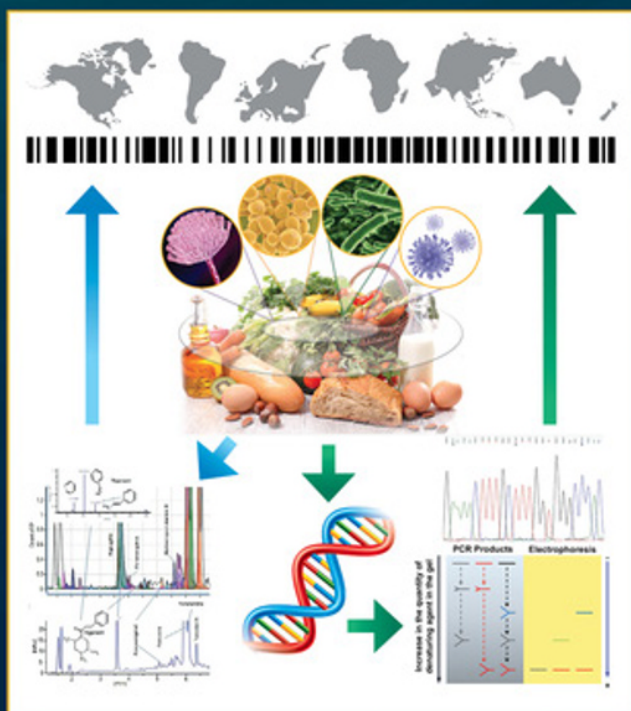


MOLECULAR TECHNIQUES IN FOOD BIOLOGY

SAFETY, BIOTECHNOLOGY,
AUTHENTICITY AND TRACEABILITY



EDITED BY
ALY FARAG EL SHEIKHA,
ROBERT LEVIN, AND JIANPING XU

WILEY

**Molecular Techniques in
Food Biology**

Molecular Techniques in Food Biology

Safety, Biotechnology,
Authenticity and Traceability

Edited by

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To my beloved wife, Omnia who has been an excellent intellectual companion and renewable source of inspiration for me

To my wonderful sons, Omar and Moaz who motivated me

Aly El Sheikha

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Preface

With increasing population size and heightened awareness of food quality, safety, and authenticity, food production and food safety have never been more important in human history. Over the decades since the introduction of molecular biology, significant improvements have been made to enhance food production, enrich food nutrition, and increase food quality and food authenticity. This book describes recent advances in food biology from the viewpoint of the development and use of molecular techniques. Our focus is the microbes associated with food and food products and the diversity of microbe-food interactions.

Molecular Techniques in Food Biology: Safety, Biotechnology, Authenticity & Traceability presents a summary of the broad microbe-food interactions, covering food microbiology, food mycology, biochemistry, microbial ecology, food biotechnology and bioprocessing, food authenticity, food origin, and food science and technology. Particular emphasis is placed on how modern molecular techniques have been and can be used to enhance food biology research, to help monitor and assess food safety and quality, and to establish effective food traceability and inspection systems.

The book comprises 19 chapters, broadly divided into six sections. The first section contains five chapters that deal with general topics to provide a global overview of safety, biotechnology, authenticity, and traceability issues related to plant- and animal-based foods. The second section includes two chapters on the molecular techniques used in studying microbes found in fruits and vegetables. The third section consists of two chapters dealing with the assessment of microbial ecology of non-fermented fish and meat products at the molecular level. The fourth section includes five chapters capturing the excitement of recent advances in molecular approaches made to decipher the microbial mechanisms in fermented foods and beverages. The fifth section comprises four chapters covering the detection of foodborne pathogens by new molecular strategies. The last chapter provides an overview of the current status and future prospects of molecular food fingerprinting.

An emerging theme among these chapters is that the detection, differentiation, and identification of microorganisms associated with food are ambiguous when they are exclusively based on morphological, physiological, and biochemical characteristics. The application of molecular tools has vastly enhanced our ability to identify these microbes and analyze their activities. In addition, there is increasing recognition that a systematic view of food products is needed in order to reveal the complexities of microbe-food interactions. These complexities include the changing trophic relationships among interacting organisms throughout the food production process. For example, beneficial

microbes can help plant and animal growth while pathogenic ones cause diseases and deter their growth. During harvesting, environmental microbes from their immediate environments are introduced which could cause spoilage. During the preparation of fine processed food, microbes and/or microbial enzymes are often needed to achieve desirable properties. Throughout these processes, microbes leave their signatures on the food that can be used for tracking and authentication purposes. For contaminated foods associated with disease outbreaks, analyses of microbial communities and populations are needed to help track the origins and spread of the specific pathogens.

We are fortunate to have experts from diverse backgrounds and agencies contributing to this book. They bring perspectives from academia, research institutes, industry, and government agencies. We believe the book will be a useful reference for research scientists, regulatory authorities, food microbiologists and technologists, epidemiologists, biotechnologists, food manufacturers, policymakers involved in food regulation, and the general public interested in food biology.

Aly Farag El Sheikha
Robert E. Levin
Jianping Xu

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The work presented here represents a collective effort from many individuals. We are indebted to all contributors who have so willingly offered their time, effort, and expertise in making this book a reality. Without their cooperation, enthusiasm, and timely submission of the chapters, this work would not have been possible.

The Editors wish to thank all the researchers whose contributions were cited in this book. We also want to thank the earlier pioneers whose work enabled the applications of the diversity of molecular techniques in food biology.

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Section I

General Topics

1

How to Determine the Geographical Origin of Food by Molecular Techniques

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1.1 Linkage Between Food and Its Geographical Origin: Historical View

Historically, food products have always been linked with a specific geographical origin. Regional product identities have a long history. In ancient Egypt, places of origin were used to identify products and to signal their quality. In the Middle Ages, European guilds gave their products certain names to ensure consumers got consistent quality, assure market exclusivity, and protect producers legally (Institut National des Appellations d'Origine [INAO] 2005). The history of some well-known cheeses can be traced back to this period, for example Parmigiano Reggiano in Italy, Stilton in the UK, and Comté in France. The process of establishing a regional reputation went parallel with the emergence of the concept of individual brands. In both cases, producers tried to enhance their products' value by associating consumers with a name: a single producer in the case of a brand, on a collective scale in the case of regional products (Boto et al. 2013).

Several regional products identified in the marketplace by geographical names date from the 19th century, including Opperdoezer Ronde potatoes (Netherlands) and Washington apples (USA). While such regional indications remained important, their significance gradually shrank with time. National and international trade evolved, and technical grades and standards developed and became more important in trade. During the 20th century, internationalization expanded rapidly. The urge for economies of scale meant that certain regions began to specialize in producing a few products. Firms marketed their products over an ever wider area. Product specialization also occurred; instead of producing a broad product assortment, companies specialized in a few standard products. This mass production resulted in the loss of many unique, specific regional products. In time, the globalization of business and markets increased further (Boto et al. 2013).

By the late 1990s, a new geographical diversity of foods had emerged. While the globalization of trade in food produce continues apace, Europe has experienced an increasing interest in foods with local and regional identities. Local food production

systems have indeed been characterized by various strategies to promote local/regional food products (Goodman 2004; Ilbery & Maye 2005; Marsden et al. 2002; Murdoch 2000).

An image of the region and regional names are often used to market products that may have a strong reputation associated with their place of production (INAO 2005). As Bérard and Marchenay (2005) point out, products do not just “come from” a region; they “are” from a region. This means that they convey values and culture, that is, an identity. In general, these products have, to a greater or lesser extent, specific qualities based on human expertise and the natural environment where they are produced. The mix of these specific qualities and the regional image creates a unique identity for the product, therefore raising its value (van de Kop & Sautier 2006).

Food quality and authentication are becoming of primary importance for both consumers and industries, at all levels of the production process, from raw materials (farm) to finished products (fork). Moreover, consumers around the world have shown an increasing interest for typical food products with reliable indicators of geographical origin. Typical food products have an important economic role at both national and international levels, as confirmed by certifications and trademarks of quality (e.g., Protected Denomination of Origin, PDO; Protected Geographical Identification, PGI; and Traditional Specialities Guaranteed, TSG), assigned to guarantee typicity and quality standards (Longobardi et al. 2015).

Figure 1.1 highlights the significant stages in determining the geographic origins of products through human history.

1.2 Scope and Approach

This chapter focuses on how to determine the geographical origin of food. Figure 1.2 illustrates the scope of the chapter and the major issues related to determining the geographical origin of foodstuffs. The demand to know the geographical origin of food has been a driving force for implementation of determining the geographic origins of food. Technological innovations, the benefits of using molecular techniques, and the drawbacks of existing approaches are reviewed below.

1.3 Definitions Related to Tracking of Food Origins

1.3.1 Geographical Area

This is the area in which the production and/or processing take place. Generally, the limits of the area are defined by natural and/or human factors which give the final product its particular characteristics. Supporting documents, such as maps, must be provided (Patent Office of the Republic of Poland [PPO] 2010).

1.3.2 Regional Products

In a general sense, van de Kop and Sautier (2006) defined a regional product as a “local product based on a territorial identity and reputation, and/or a typical product based on specific mode of production and whose quality, reputation or any other characteristics



Figure 1.1 Developments in the history of geographical origin determination.

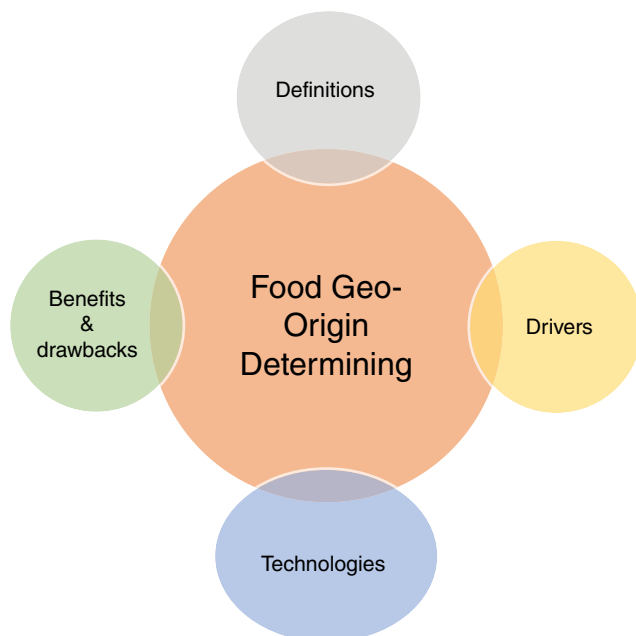


Figure 1.2 Analytic structure illustrating the scope of this chapter on determining the geo-origin of food.

are attributable essentially to its geographical origin.” The geographical origin can be a province, state, department or country, but also cross-border areas that are culturally, naturally or climatically similar.

1.3.3 Appellation of Origin (AO)

This term is defined through the World Intellectual Property Organization (WIPO 2013) as “The geographical name of a country, region, or locality, which serves to designate a product originating therein, the quality and characteristics of which are due exclusively or essentially to the geographical environment, including natural and human factors.”

Appellation of Origin was one of the earliest forms of Geographical Indication (GI) recognition and protection (WIPO 1979). Although mentioned in earlier treaties, the 26 contracting parties to the Lisbon System in 1958 first formally recognized the term “Appellation of Origin” as a form of GI, by using a single registration procedure, effective for all the signatories (Boto et al. 2013).

1.3.4 Geographical Indication (GI)

Geographical Indication is defined by the TRIPS Agreement 1994 as “Indication which identifies a good as originating in the territory of a member (country), or a region or locality in that territory, where a given quality, reputation or other characteristic of the good is essentially attributable to its geographical origin” (World Trade Organization [WTO] 2005).

1.3.5 Protected Designation of Origin (PDO)

The Protected Designation of Origin is for products closely associated with the area whose name they bear (European Commission 1992, Regulation No. 2081/92). Such a product must meet two conditions.

- Quality or characteristics of the product must be connected with the particular geographical environment of the place of origin; this environment includes inherent natural and human factors, such as climate, soil quality, and local know-how.
- Production and processing of the raw materials, up to the stage of the finished product, must take place in the defined geographical area whose name the product bears.

The PDO covers agricultural products that are produced, processed, and prepared in a given geographical area using recognized know-how. Well-known PDO products are prosciutto di Parma (ham) from Italy, Kalamata (olive oil) from Greece, and Camembert de Normandie (cheese) from France (Drivelos & Georgiou 2012).

1.3.6 Protected Geographical Indication (PGI)

Protected Geographical Indications also designate products attached to the region whose name they bear but the link is different from that between a product with a PDO

and its geographical area of origin (European Commission 1992). To be eligible to use a PGI, a product must meet two conditions.

- It must have been produced in the geographical area whose name it bears. Unlike the Protected Designation of Origin, it is sufficient that one of the stages of production has taken place in the defined area. For example, the raw materials used in production may have come from another region.
- There must also be a link between the product and the area which gives it its name. However, this feature need not be essential, as in the case of a designation of origin. It is sufficient that a specific quality, reputation or other characteristic be attributable to the geographical origin of a given product.

The PGI covers agricultural products and foodstuffs closely linked to the geographical area. At least one of the stages of production, processing or preparation takes place in the area. Typical products with recognized PGIs are Scotch beef from the UK, Calcot de Valls (onion) from Spain, and Budějovické pivo (beer) from the Czech Republic (Drivelos & Georgiou 2012).

1.3.7 Generic Name

A term or sign is considered “generic” when it is so widely used that consumers see it as designating a class or category name for all goods or services of the same type, rather than as referring to a specific geographical origin (Boto et al. 2013).

1.3.8 Food Safety

Food safety is defined as the style of preparing, handling, and storing food to prevent infection and to help ensure that food retains enough nutrients to support a healthy diet. Unsafe food means that it has been exposed to pathogens or is rotten, which can cause diseases or infections (e.g., diarrhea, meningitis, etc.) (El Sheikha 2015a; Food and Agriculture Organization of the United Nations [FAO] 2004).

1.3.9 Food Quality

Quality is a measure of the degree of excellence or degree of acceptability by the consumer. It can be defined as “a summary of features and characteristics of a product or service that bear on its ability to satisfy stated or implied needs” (FAO 2004). In simple words, the product should have attributes to “satisfy the wants/needs of the consumer or conformance with the user’s requirements.” Quality also covers safety and value for money.

Food quality can be considered as a complex characteristic of food that determines its value or acceptability to consumers. Thus it may be defined as “the composite of those characteristics which have significance in determining the degree of acceptability by the buyer. These characteristics should also have the ability to differentiate individual units of the product” (Leitzmann 1993). The important components of food quality are food safety, sensory characteristics, and nutritional value. Safety of food is a basic requirement of food quality.

1.3.10 Geo-traceability

Geo-traceability can be defined as the result of combination of geographic information and traditional data used in traceability procedures (El Sheikha 2010). In the agriculture-food sector and more particularly in the field of plant production, geo-traceability is concerned with the relationships between a production plot, its geographical location, its environment, and cultural practices. Geo-traceability requires the implementation of spatial analysis and information acquisition and processing tools that will be combined in geographic information systems (GIS) (GeoTraceAgri [GTA] 2005).

1.4 Driving Forces for Determining the Geo-origin of Food

There are many driving forces behind the development and implementation of technologies for determining the geographic origin of foodstuffs. These forces can be put into five different categories: why do people buy “origin” food products, safety and quality importance, regulatory focus, economic and social concerns.

1.4.1 Why do People Buy “Origin” Food Products?

Food consumption habits were created by the local natural resources and the social or cultural factors of the community (Delamont 1995). Such links between food and origin have disappeared over time, the main reason for this being globalization of the food industry, following the extensive growth in technology over the past century (Montanari 1994). This has led to a similarity of lifestyles across regions such that food consumption patterns within a region no longer necessarily reflect food production of that area (Ritzer 1996). However, in recent years, consumers have renewed their interest in food strongly identified with a place of origin (Drivelos & Georgiou 2012; El Sheikha 2015b). There are a number of reasons for this increasing interest, including organoleptic qualities, health, psychology (patriotism and confidence in the product), media information, and concern about animal welfare and environmentally friendly methods of production (Gilg & Battershill 1998; Mitchell 2001).

Figure 1.3 shows the interaction between consumers, food product, and origin.

1.4.2 Food Safety, Food Quality, and Consumer Protection Linked to Geographic Origin

Food scares have occurred throughout history. Atkins (2008) has discussed that, in Europe, food scares (especially zoonotic hazards) have been present in the UK for at least 150 years. Saltini and Akkerman (2012) mentioned that in Europe, foodborne illness affects about 1% of the population (approximately 7 million people) each year. In 2011, approximately 16.7% of the US population (47.8 million people) experienced food-related illness (Resende-Filho & Hurley 2012). Other types of food scare such as contamination with radioactive materials disturb the food supply chain. After the release of radioactive materials from damaged nuclear plants due to earthquake in Japan in 2011 (World Health Organization [WHO] 2011), many countries implemented intensive food control measures concerning their food trade relationship with Japan while some countries suspended food imports from Japan.



Figure 1.3 Interaction between consumers, food product, and origin. *Source:* Adapted from Vandecastelaere et al. *Linking people, places and products: a guide for promoting quality linked to geographical origin and sustainable geographical indications*, Food and Agriculture Organization of the United Nations (2010). Available at: www.fao.org/docrep/013/i1760e/i1760e.pdf. Reproduced with permission.

In addition to the public health risk, food scares lead to economic crises due to direct and indirect (damage to reputation and brand name) costs of product recall. Recent studies in Europe and North America reported that commercial frauds range from 15% to 43% of total commercial seafood products, with 75% of fraud cases related to the red snapper (*Lutjanus campechanus*) (Hellberg & Morrissey 2011; Rasmussen & Morrissey 2008). Therefore, traceability is an important component of contemporary supply chains in the production industry in general and the food sector in particular as the food sector is sensitive from a human and animal health point of view (Olsen & Aschan 2010). Figure 1.4 represents the benefits of applying an effective traceability system.

Proof of provenance has become an important topic in the context of food safety, food quality, and consumer protection in accordance with national legislation and international standards and guidelines. Provenance means being able to identify and ensure the origin of a commodity and thereby the region where it was produced. Incidents such as the outbreak of *Salmonella* food poisoning via contaminated peppers from Mexico, which occurred in the USA in 2008, have demonstrated the need for effective traceability systems and the deficiencies in current paper-based systems. The failure to trace the contaminated batch of peppers to their origin resulted in a wide-scale, costly, and lengthy recall procedure involving many producers in Mexico and retail outlets in the USA (International Atomic Energy Agency [IAEA] 2011).

Therefore, origin of food is one of the most important criteria for ensuring food safety and quality. At the same time, origin is an indispensable basis in the concept of quality from farm to fork. In this context, origin detection tools are regarded as important

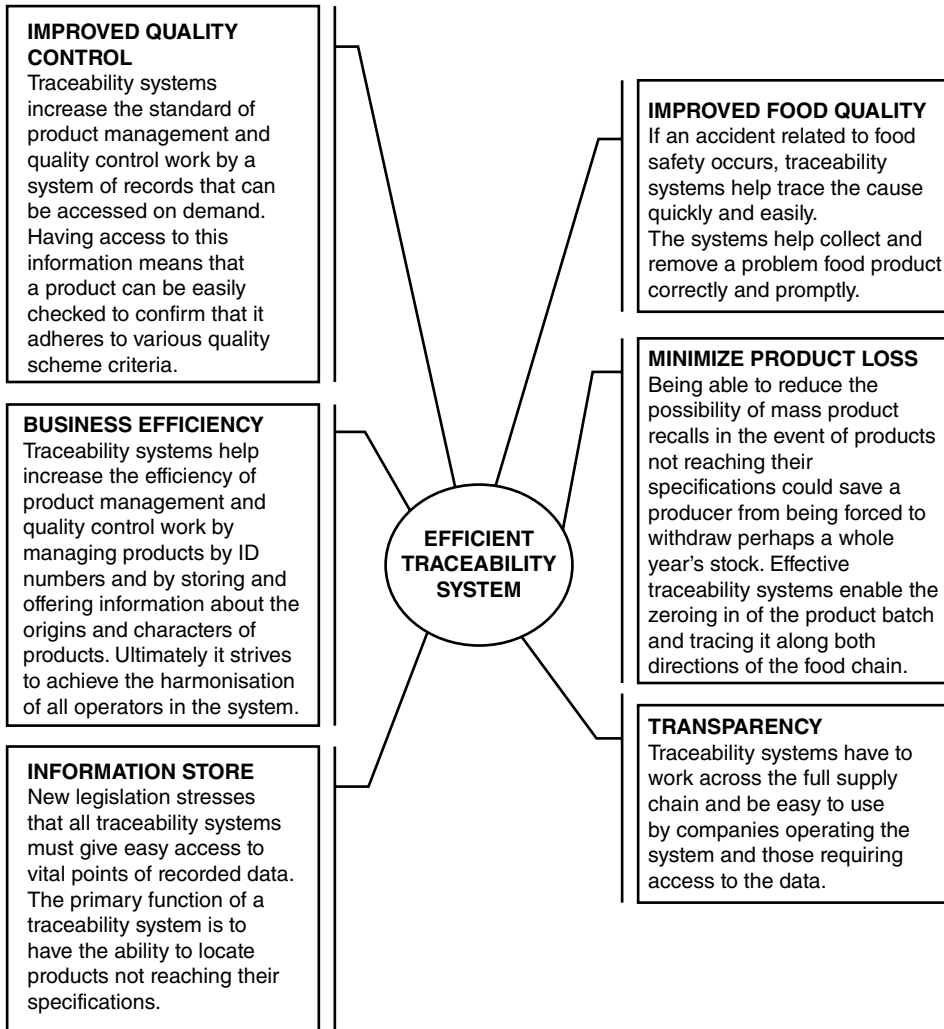


Figure 1.4 What are the benefits of applying an effective traceability system on food chain components? *Source:* El Sheikha & Montet (2016). Reproduced with permission of Taylor and Francis.

aspects of food traceability. Providing early warning systems for avoiding safety and quality problems and recalling effectively are some of the benefits of determining the geographic origin of food products.

1.4.3 Regulatory Focus

The 1883 Paris Convention was the first international multilateral treaty to include provisions relating to indications of geographical origin. Article 1(2) of the Convention recognizes “indications of source” and “appellations of origin” as subject matter for industrial property (WIPO 1979). Indications of source offer a measure of protection

for origin-based product names and icons without the burdens associated with demonstrating specificity and/or reputation, developing a binding product specification and instituting a system of certification control. Indications of source are particularly applicable for marketing through nation branding (Boto et al. 2013).

European consumers are paying increased attention to the quality and authenticity of foodstuffs. European Union regulations allow food products to be distinguished by their specific characteristics and their geographical origin. In 1992, EU regulations 2081/92 and 2082/92 introduced an integrated framework for the protection of geographical indications and designations of origin for agricultural products and foodstuffs. Furthermore, laws enforce labeling of the geographical origin of agricultural products in many countries due to consumer demands for more information on foods. EU regulations 2081/92 and 2082/92 have been replaced by regulations 510/2006 and 509/2006, respectively, and EU regulation 1898/2006 was added (Koç et al. 2012). The EU regulations allow the application of the following geographical indications to a food product: PDO, PGI, and (TSG) (Mout 2004).

The EU has established regulations for every product available. Regulation CE 1760 17/07/2000 made the indication of origin on meat carcasses mandatory, Commission Directive 2001/110/CE posed the same condition for honey, Commission Regulation No 2065/2001 established rules for the application of Council Regulation 104/2000 to inform consumers about fishery and aquaculture products (Ghidini et al. 2006). EC Regulation 182/2009 has detailed rules regarding the geographical origin labeling of virgin and extra virgin olive oil in the European Community. As of December 2014, EU Council Regulation 1169/2011 EU made it obligatory that all fresh and frozen meat, as well as fish produce, be clearly labeled with the point of origin (Wilkes et al. 2016). This is also demonstrated by the existence of a European Wine Databank on authentic European wines (as foreseen in EC Regulation 2729/2000) (Luykx & van Ruth 2008). Figure 1.5 shows products with protected signature names in Europe.

In addition to regulations enabling producers to legally protect regional food specialties from counterfeit copycat products and name abuse, regulations also seek to achieve wider social and environmental objectives with respect to the rural economy.

1.4.4 Economic Concern

The market for GI food products is considerable, especially in the United States, Europe, and the more affluent countries. The estimated value for sales of GI products worldwide is well over USD 50 billion. A number of countries, ranging from Scotland to Australia and China to Chile, have GI exports in excess of USD 1 billion. In France, the market value of GI products is almost USD 24 billion, or close to 10% of the national food market's total value. Products registered under Italy's 430 GIs generate a value of some USD 13 billion and employ about 300 000 persons, while Spain's 133 GI-designated products generate approximately USD 4 billion (Rondot et al. 2004).

Chever et al. (2012) published a study analyzing the value of the EU name protection scheme for all GIs (agri-food products, wines, and spirits). The products with GIs in EU countries are worth about USD 54 billion worldwide. The study also analyzes the value premium of products bearing a GI, that is, the premium that a GI can expect on the market, compared to similar non-GI products. On average, GI products were estimated to achieve a price 2.23 times higher than their non-GI counterparts.

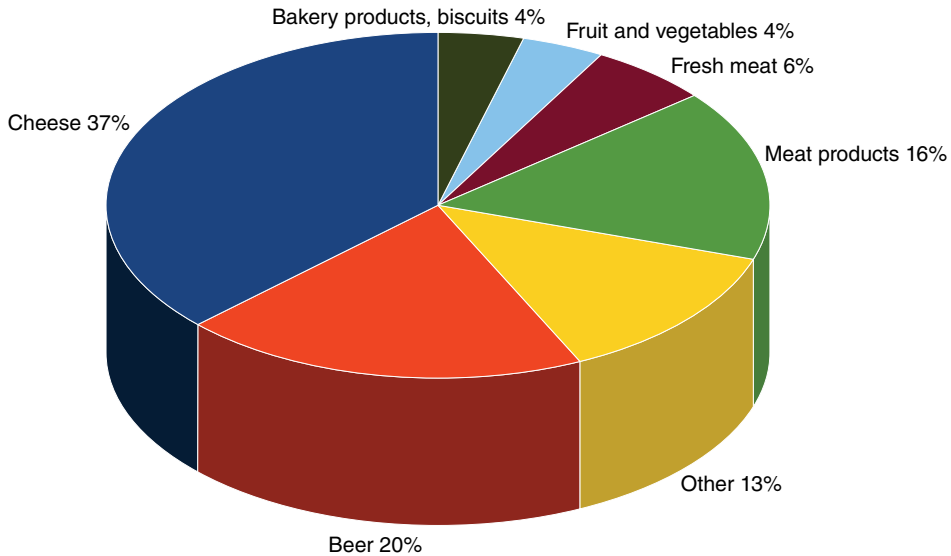


Figure 1.5 European food products with protected signature names. *Source:* Drivelos & Georgiou (2012). Reproduced with permission of Elsevier.

1.4.5 Social Concern

Increasing confidence of consumers in their food, changing lifestyles, increasing consumer income, and increasing societal awareness about health are some of the social issues that motivate food companies to implement GI systems (El Sheikha 2017). The improvement in food crisis management enables concerned agencies to build capacity to safeguard food safety and security which in turn strengthens the social and political security of a nation. In contemporary food traceability systems, companies should not only attempt to comply with government rules but should also adequately provide information that consumers need to know such as a variety of food attributes, country of origin, animal welfare, and genetic engineering-related issues (Golan et al. 2004).

Some origin-linked products have been produced for a long period in the same social and cultural environment. They incorporate producer know-how regarding how to manage a sound production process and attain high specific quality within a particular local environment. The link between product, people, and place often makes the origin-linked product an element of identity for local populations, transcending even its economic impact. As a consequence, the social dimension of certain products has many aspects (Boto et al. 2013).

- The origin-linked product is related to the preservation of the natural and cultural heritage, traditions, know-how, and lifestyle in marginal areas.
- The collective dimension of the origin-linked product strengthens social linkages between local actors, not only through local organizations and greater equity in the production sector, but also externally, as all local stakeholders are involved (for example, public actors, stakeholders of the tourism industry, schools, etc.).
- The sustainable management of various local resources used for food and agriculture contributes to food and livelihood security, while the preservation of typical products offers consumers broader food diversity.

1.5 Geo-origin Determination ... Evolution of Molecular Techniques

1.5.1 New and Sophisticated Techniques are Increasingly Needed ... Why?

Reports on analytical methods for determining the geographical origin of agricultural products have been increasing since the 1980s. The initial focus was on processed agricultural products such as wine (Etiévant et al. 1988; Frías et al. 2003; Latorre et al. 1994; Martin et al. 1999), honey (Sanz et al. 1995), teas (Fernández-Cáceres et al. 2001; Marcos et al. 1998), olive oil (Angerosa et al. 1999), and orange juice (Mouly et al., 1999), while later studies examined fresh products such as potatoes (Anderson et al. 1999; Chung et al. 2016), Welsh onions (Ariyama et al. 2004a,b; Ariyama & Yasui 2006), pistachios (Anderson & Smith 2005), and garlic (Smith 2005), chiefly because worldwide trade in fresh agricultural products has increased year by year and the law now enforces labeling of their geographical origin.

The use of GIs allows producers to obtain market recognition and often a premium price. False use of GIs by unauthorized parties is detrimental to consumers and legitimate producers. From this point of view, the development of new and increasingly sophisticated techniques for determining the geographical origin of agricultural products is highly desirable for consumers, agricultural farmers, retailers, and administrative authorities. It is an analytically challenging problem that is currently the focus of much attention within Europe and the USA (Luykx & van Ruth 2008).

1.5.2 Overview of Molecular Techniques Used for Geo-origin Determination of Foods

Various techniques have been studied based on organic constituents, mineral contents or composition, light- or heavy-element isotope ratios, or combinations thereof. If the components have sufficient discriminatory power, the set of their concentrations will form a characteristic pattern or “fingerprint” relating to the geographical origin of the sample.

Molecular approaches that have been developed so far for determining geographical origin are outlined and evaluated below. For this overview, the molecular approaches have been subdivided into five groups: mass spectrometry techniques, spectroscopic techniques, separation techniques, molecular biology techniques, and other techniques. All techniques and abbreviations are summarized in Figure 1.6.

1.5.2.1 Mass Spectrometry Techniques (MS)

Isotope ratio mass spectrometry (IRMS) is a method that can be used to differentiate chemically identical compounds based on their isotopes (Brenna et al. 1997). This technique is applicable for the determination of the geographical origin of numerous food products. Geographical origin of food is determined also by ICP-MS which analyzes inorganic elements. Furthermore, ICP-AES (atomic emission spectroscopy) and AAS (atomic absorption spectroscopy) have also been successfully combined with ICP-MS to classify onions (Ariyama et al. 2004a, 2007) and tea (Moreda-Piñeiro et al. 2003) according to their regions of origin. On the other hand, qualitative and quantitative analysis and geographical origin determination can be administered by GC-MS technique (Luykx & van Ruth 2008).

Mass Spectrometry Techniques	Spectroscopy Techniques	Separation Techniques	Molecular Biology Techniques	Others
<ul style="list-style-type: none"> • IRMS • ICP-MS • PTR-MS • GC-MS 	<ul style="list-style-type: none"> • NMR • IR • Fluorescence Spectroscopy • Atomic Spectroscopy 	<ul style="list-style-type: none"> • HPLC • GC • CE 	<ul style="list-style-type: none"> • ELISA • DNA Technology 	<ul style="list-style-type: none"> • Sensor Technology • Sensory Analysis

Figure 1.6 Molecular techniques used for determining the geographical origin products. CE, capillary electrophoresis; ELISA, enzyme linked immunosorbent assay; GC-MS, gas chromatography mass spectrometry; HPLC, high-performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; IR, infrared spectroscopy; IRMS, isotope ratio mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PTR-MS, proton transfer reaction mass spectrometry.

Generally, MS, ICP-AES, ICP-MS, and GC-MS serve as traceable tools in combination with each other. Antimicrobial, antibiotic, and pesticide residues in food are detected by MS-based methods (Herrero et al. 2012).

1.5.2.2 Spectroscopy Techniques

Spectroscopy-based traceability tools are used for the analysis of semi-solid and liquid food. By these techniques, finding the specific fingerprint of each food sample is considered to be an easy method to trace its origin (Aarnisalo et al. 2007; Luykx & van Ruth 2008).

Site-specific natural isotope fractionation (SNIF)-NMR is often used in food analysis and allows determination of the geographical origin of foods based on the isotopic ratio of a given nucleus found in a constituent of the analyzed food (Reid et al. 2006). SNIF-NMR has particularly been used for the geographical authentication of various wines (Martin et al. 1999; Ogrinc et al. 2001). Furthermore, this technique was successfully applied to identify the geographical origin of natural mustard oils (Remaud et al. 1997). The main drawback of SNIF-NMR is that it requires laborious sample preparation involving many purification and concentration steps (Ibañez & Cifuentes 2001).

Infrared spectroscopy is the measurement of the wavelength and intensity of the absorption of infrared light by a sample (Putzig et al. 1994). With respect to mid-infrared (MIR), various wines (Picque et al. 2005), cheeses (Karoui et al. 2004a), olive oils (Tapp et al. 2003), and honey (Ruoff et al. 2006) have been differentiated on the basis of geographical origin. With near-infrared (NIR) spectroscopy, the geographical classifications of grapes (Arana et al. 2005), wines (Liu et al. 2006), rice (Kim et al. 2003), soy sauce (Iizuka & Aishima 1997), and olive oils (Downey et al. 2003) have been accomplished.

Fluorescence spectroscopy provides information on the presence of aromatic amino acids and their environment in biological samples (Luykx et al. 2004). In this way, fluorescence spectroscopy allows determination of the geographical origin of various cheeses (Karoui et al. 2004a, b, 2005a), milks (Karoui et al. 2005b), and olive oils (Dupuy et al. 2005).

Via AAS, it was possible to relate the selenium content of beef to a geographical region (Hintze et al. 2001) and to geographically discriminate honeys (González Paramás et al. 2000) and wines (Frías et al. 2001) by measuring various mineral elements. A combination of AAS and AES allowed determination of the geographical origin of orange juice, nuts (Schwartz & Hecking 1991), and potatoes (Galdón et al. 2012; Rivero et al. 2003).

1.5.2.3 Separation Techniques

By HPLC, GC and CE methods, sample molecules can be partitioned to mobile and stationary phases (Aarnisalo et al. 2007).

High-performance liquid chromatography is a chromatographic method used for determining the amount of soluble and insoluble contents in the solution such as carbohydrate, fat, protein, vitamins, mycotoxins, and proteins (Luykx & van Ruth 2008). HPLC is not only an accurate and quick analysis but is also considered as an ideal method for determining phenolic compounds and organic acids (Aarnisalo et al. 2007). European wines from different geographical origins have been correctly classified on the basis of their chromatography profiles obtained with HPLC in combination with a UV-vis and/or fluorescence detector (Luykx & van Ruth 2008). HPLC has also been used to geographically discriminate honey (Tomás-Barberán et al. 1993), nuts (Gómez-Ariza et al. 2006), olive oil (Stefanoudaki et al. 1997), and cheese (di Cagno et al. 2003) based on the HPLC profiles of flavonoids, metal-binding proteins, triglycerides, and peptides, respectively.

Volatile and semi-volatile structures, flavors, and pesticides have been analyzed by GC (Luykx & van Ruth 2008). The contamination of sample or column is one possible limitation. However, a rapid and reproducible operation and a high sensitivity on a small amount of sample are considered as GC advantages (Aarnisalo et al. 2007). By analyzing the GC profiles of various compounds (e.g., alkanes, aldehydes, alcohols, acids) present in wine, it is possible to classify wines according to their geographical origin (Etiévant et al. 1989; Shimoda et al. 1993). Determination of the fatty acid composition and corresponding concentrations by GC allowed the geographical discrimination of milk samples (Collomb et al. 2002) and olive oils (Olliver et al. 2003). Furthermore, determination of the geographical origin of cocoa masses (Hernández & Rutledge 1994) and orange juices (Ruiz del Castillo et al. 2003) was accomplished via GC analysis.

Capillary electrophoresis is an electrokinetic separation technique that separates components based on their different electrokinetic mobility. This method can be used in various analyses ranging from simple inorganic ions, small organic molecules, and peptides to viruses and microorganisms (Kvasnička 2005). Delgado et al. (1994) were the first to propose that CE be used for studying the geographical origin of a food product. Their study concerned the determination of flavonoids which accumulate in different proportions in honey depending on its geographical origin. In a similar way, CE has been applied to geographically discriminate Chinese fruit extracts (Peng et al. 2006). Furthermore, CE profiles were able to differentiate herb samples based on their geographical origins (Wang et al. 2005).

1.5.2.4 Molecular Biology Techniques

Enzyme linked immunosorbent assay is the most commonly used enzyme-based method with high sensitivity. It is economical and efficient (Ahmed 2002). Enzyme-based traceability tools are used in various implementations such as verifying suitability of meat and dairy products (Aarnisalo et al. 2007; El Sheikha et al. 2017), determining authenticity in fish, fish products and fruit juice and detection of GM products or allergens (Ahmed 2002; Asensio et al. 2008; Sass-Kiss & Sass 2000, 2002; Valdes et al. 2003; Williams et al 2004).

The characteristics of DNA make it a useful geo-origin marker for food. DNA-based techniques are more effective, and can also be applied to different food matrices

(Lockley & Bardsley 2000; Mafra et al. 2008). Furthermore, DNA is more informative than proteins, and can be easily extracted in the presence of small traces of organic material (Hellberg & Morrisey 2011). PCR-based methods are extremely sensitive, often faster than other technologies, and are widely used in agriculture and zootechnology (Doulaty Baneh et al. 2007; Grassi et al. 2006; Labra et al. 2004; Mane et al. 2006; Teletchea et al. 2005). In recent years, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) has been largely used in the field of food traceability and safety in order to characterize bacteria, yeasts, and molds in food products (Dalmacio et al. 2011; El Sheikha & Xu 2017; El Sheikha et al. 2009; Rychlik et al. 2017; Zheng et al. 2012).

More details regarding DNA-based approaches and other recent techniques which use innovative fingerprinting of food will be discussed in Chapter 19.

1.5.2.5 Other Techniques

Sensor technology, sometimes referred to as “electronic nose technology,” is based on detection by an array of semi-selective gas sensors of the volatile compounds present in the headspace of a food sample (Strike et al. 1999). The electronic nose has been successfully applied to differentiate geographical origins of olive oils (Guadarrama et al. 2001), wines (Penza & Cassano 2004), orange juices (Steine et al. 2001), and cheeses (Pillonel et al. 2003).

Sensory evaluation is considered as an important technique to determine product quality. It comprises a set of techniques for accurate measurement of human responses to foods (Pérez Elortondo et al. 2007). Appearance, odor, flavor, and texture properties are important characteristics determining the quality of food products. Sensory analysis requires panels of human assessors on whom the products are tested, and recording of their responses. By applying statistical techniques to the results, it is possible to make inferences about the products under test (Luykx & van Ruth 2008). Sensory analyses have also been applied to geographically discriminate a spirit drink (Lachenmeier 2007), cheeses (Pillonel et al. 2002), and olive oils (Stefanoudaki et al. 2000).

1.6 Pros and Cons of Molecular Techniques Used as Geo-Discriminative Tools of Food

Although conventional analytical approaches such as IRMS and ICP-MS can provide a good indication as to the likely geographical origin of a sample, instrumentation and running costs, plus the requirement for highly trained analysts, and a protracted workflow, make their use for routine sample analysis difficult. With lower cost implications and an increasing number of nucleic acid-based assays becoming available for food authenticity testing, DNA-based molecular methods have experienced a rapid adoption by many enforcement agencies. Recent technical advances with nucleic acid-based marker systems have made possible the exploitation of genetic variation, where present, which can be used to provide an indication as to where a product may have originated (Chauhan & Rajiv 2010; El Sheikha & Montet 2016; Leal et al. 2015; Lockley & Bardsley 2000; Wilkes et al. 2016; Woolfe & Primrose 2004). A number of techniques have been, or are currently in the process of being evaluated for this purpose. Table 1.1 presents an overview of these molecular methods with their pros and cons.

Table 1.1 Pros and cons of the molecular techniques used for determination of the geo-origin of food products.

Techniques	Sensitivity	Simplicity	Time analysis	Costs	Reported application	Identification/ profiling
<i>Mass spectrometry</i>						
IRMS ^a	+ ^b	+/- ^c	+/-	- ^d	+	I* + P**
ICP-MS	+	+/-	+	-	+	I + P
PTR-MS	+	+	+	-	-	P
GC-MS	+	+	+/-	-	+	I + P
<i>Spectroscopy</i>						
NMR	-	+/-	+/-	-	-	I + P
IR	+/-	+	+	+	+	P
Fluorescence	+	+	+	+	-	P
Atomic	+/-	+/-	+/-	+/-	+	I + P
<i>Separation</i>						
HPLC	+/-	+	+/-	+	+	P
GC	+	+	+/-	+	+	P
CE	-	+	+/-	+	-	P
<i>Molecular biology</i>						
ELISA	+	+	+	+	+	I + P
DNA technology	+	+/-	+/-	+	+	I + P
<i>Other</i>						
Sensor technology	-	+	+	+/-	+/-	P
Sensory analysis	+/-	+/-	-	-	-	P

^a For abbreviations of the techniques, see Figure 1.6.

^b (+) Favorable;

^c (+/-) moderate;

^d (-) unfavorable.

* Possibility of identifying;

** possibility of profiling.

Source: Adapted from Luykx and van Ruth (2008). Reproduced with permission of Elsevier.

1.7 Conclusions

Consumer awareness is increasing day by day and the interest of consumers in high-quality foods with a clear geographical identity has grown rapidly. The concept of food traceability must be evaluated with total quality from farm to fork. In this context, food origin is the base point for ensuring the quality of the whole process. Therefore, participation in protected food name systems (PDO, PGI, TSG) is encouraged in the EU. This means that suitable techniques for determining the geographical origin of food products are highly desirable.

Unfortunately, it is difficult to develop a 100% accurate method for determining geographic origin, and the techniques which have been developed usually cannot avoid a certain number of mistakes. In the last 25 years, molecular tools for studying food have become more sensitive, reliable, and faster. These methods are capable of analyzing specific characteristics of a product which are influenced by geographically specific factors. Current molecular methods are quick, precise, and reliable, and as a result analysis of genetic variation has rapidly become the method of choice for a number of applications, including that of food authenticity. Consequently, the development of food authenticity is beneficial both for raising the awareness of consumers and for ensuring food safety.

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2

Unraveling Pathogenic Behavior of Phytopathogens through Advanced Molecular Techniques

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2.1 Introduction

Plants are prone to infections by different pathogenic microorganisms from the emergence of seedlings all the way to maturity. Pathogens are ubiquitously distributed in many ecological niches and can gain entry into plants via stomata, roots, flowers, fruits, and other plant parts. Globally, huge crop losses are incurred due to infestations by microbial pathogens and such infectious diseases have been and remain an important constraint on efforts to increase crop production around the world (Strange & Scott 2005). It has been estimated that crop losses due to plant pathogens in the United States alone cost about 33 billion dollars each year, out of which about 65% (21 billion dollars) could be attributed to foreign plant pathogens (Pimentel et al. 2005). Therefore, monitoring of plant health and early diagnosis of plant disease are crucial for limiting the extent of plant damage by pathogens. Early information on disease detection can be helpful for facilitating the control of diseases through appropriate and accurate management strategies such as vector control through application of pesticides and fungicides, biocontrol agents, and disease-specific chemicals (Narayanasamy 2001, 2005).

So far, various characteristics related to pathogen morphology, biology, and physiology have been used for the differentiation and detection of pathogens down to species level. Apart from the routinely used conventional methods for detecting causal organisms of diseases, current developments in molecular biology techniques, particularly the introduction of a range of DNA markers, have greatly enhanced our capacity to safeguard the health of plants. For pathogen detection, plant pathologists have mostly relied upon basic techniques of isolating microorganisms and observing symptoms they induce on susceptible hosts. Since the dawn of molecular biology techniques, there has been a radical shift in the types of approaches used to differentiate and identify plant pathogens and to develop disease management plans.

However, in order to achieve accurate identification of the pathogen which is necessary to devise methods for crop disease management, some important criteria need to be studied. These include the quantity of pathogen in the crop, a guesstimate of the

effectiveness of available practices for controlling the pathogens, using the right quality seeds of the particular crop to be sown, and analysis of the reciprocity between the plant and the pathogen in order to decode the process of pathogenesis (Narayanasamy 2011). Determinations of disease potential can help guide optimal crop rotation regimes, varietal selections, targeted control measures, harvest timings, and crop postharvest handling. Pathogen detection prior to infection can also reduce the incidence of disease epidemics (Wakeham & Pettitt 2016).

In this chapter, we provide a detailed summary of various molecular methods used for disease detection and future prospects for plant disease diagnostics and pathogen detection worldwide.

2.2 Plant Pathogens: A Menace to Agricultural Productivity

Plants live in a world surrounded by microorganisms, which are capable of growing on the plants throughout their life cycles. These plants are constantly exposed to microorganisms such as bacteria, fungi, and viruses, which may be beneficial, neutral or harmful. Plant pathogens have developed adaptations to invade and overcome the innate defense mechanisms of plants and cause diseases. Such diseases can lead to significant losses, up to complete crop failure. Given the significance of this destruction, there is an urgent need for early and effective diagnosis of the pathogens.

The biotic agents of disease are generally classified according to their effects on their hosts. Plant pathogens have been broadly divided into necrotrophs – those that kill the hosts to obtain nutrients for their survival and reproduction – and biotrophs – those that need a living host to complete their life cycle (Dangl & Jones 2001). Among microbial pathogens, viruses are typical examples of biotrophs, while bacteria and fungi adopt either a biotroph or a necrotroph lifestyle. These biotic agents can, at times, fatally compromise food security (Strange & Scott 2005). The Irish famine, caused by *Phytophthora infestans* (Large 1940; Strange 2003), the Great Bengal Famine of 1943 (Padmanabhan 1973) and the southern corn leaf blight epidemic in 1970–1971 in the USA (Ullstrup 1972) are a few examples.

2.2.1 Plant Disease Diagnostics

Endemic, emerging, and reemerging plant pathogens constantly challenge our ability to look after plant health globally. In addition, globalization, climate change, increased human movement, pathogen and vector evolution and adaptation have collectively increased the spread of invasive plant pathogens (Anderson et al. 2004; Garrett et al. 2006; Miller et al. 2006). Precise and early diagnosis as well as frequent pathogen inspections will be important for predicting and preventing outbreaks at local, regional, and global levels (Miller et al. 2009).

Relying on disease symptoms alone is often not sufficient for accurate diagnosis. In addition, the disease may have progressed significantly when visible symptoms first appear. Furthermore, the appearance of symptoms can be highly variable. Biological techniques for disease diagnosis and pathogen detection are most often accurate but too slow and not feasible for large-scale use. In this regard, recent advances in

molecular biology and biotechnology offer a good alternative for the development of fast, unambiguous, and sensitive tools for the detection of plant pathogens. Besides this, the development of molecular techniques has made field-based community ecology of arbuscular mycorrhizal fungi (AMF) also truly feasible (Hart et al. 2015). In the future, this could be tapped to aid in biocontrol through AMF.

The direct molecular approach was used to create the first comprehensive census of soil which revealed both hyperdiversity and fine-scale niche partitioning (Taylor et al. 2014). In another study, molecular techniques were used to identify fungi isolated from stored apples in Riyadh in order to aid in postharvest management (Alwakeel 2013). Apart from the diagnostic methods, plant disease diagnostic networks have been developed worldwide to tackle the problems of effective disease diagnosis by developing collaboration and assistance among institutions and experts within countries and across national boundaries.

2.2.2 Need for Disease Diagnosis

Even though the monetary, societal, and environmental consequences of plant diseases have taken a back seat with respect to the diseases of humans and animals, scientists and people outside the plant pathology community have started giving this area some attention (Brownlie et al. 2006; King et al. 2006; Lemon et al. 2007); there has been quite a bit of development since 2008, for example in fungal phytopathogen barcoding. Food security is endangered in resource-poor countries, with more than 800 million people without access to sufficient food while 1.3 billion live on less than \$1 a day and at least 10% of worldwide food production is destroyed by plant diseases (Christou & Twyman 2004; FAO 2000; Strange & Scott 2005). The statistics and the seriousness of the issue cannot be ignored.

As a direct effect, the health of humans and domestic animals is affected by the toxins produced by pathogenic fungi such as *Aspergillus* and *Fusarium*, which taint food, resulting in a gamut of diseases and disorders along with the problem of undernourishment (Fink-Gremmels 2008; Gong et al. 2008; Jolly et al. 2008; Jurick et al. 2007; Verstraete 2008). On the other hand, losses due to crop failures indirectly spread human infections, diseases, and environmental damage as a result of population movement from the countryside (Anderson et al. 2004). Also, reduction in pathogen recognition and disease diagnosis leads directly to insufficient command over disease control and a fall in crop production and quality. The main reasons for disease diagnosis are highlighted in Figure 2.1.

2.2.3 Merits of Modern Diagnostic Methods

The capacity for traditional pathogen identification is generally insufficient to meet the needs in both developed and developing countries and therefore with the arrival of molecular biology, a considerable shift has been observed in the types of approaches used to distinguish and categorize plant pathogens and to invent disease management strategies (Boonham et al. 2008; Schaad et al. 2003; Tinivella et al. 2008). The sensitivity of molecular techniques generally refers to the smallest amount of microorganism that

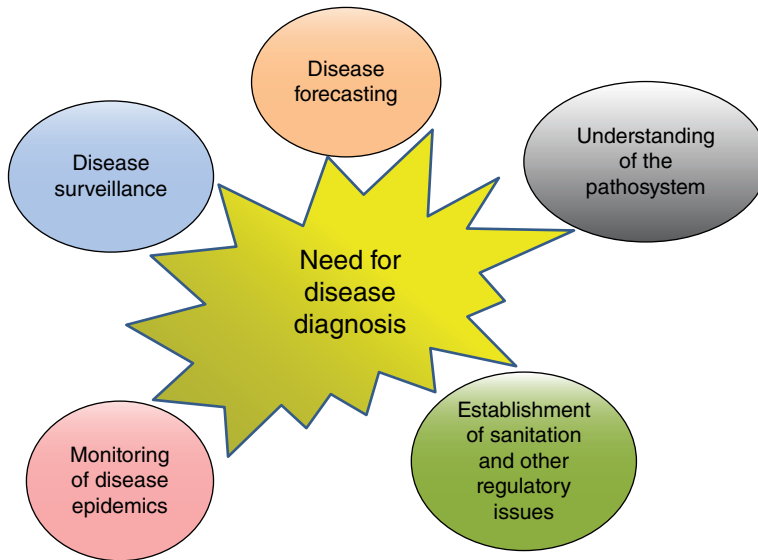


Figure 2.1 Representation of the need for disease diagnosis.

can be identified in the sample. The other indicator is specificity. Overall, the superiority of modern diagnostic methods over conventional ones can be summarized as follows.

- Rapid identification of disease-causing agent.
- Capable of distinguishing closely related species.
- A high sensitivity for detecting a small number of cells in a small amount of sample.
- Kits made for commercial purposes are cost-effective and user friendly with better adaptability for scanning very large numbers of samples.
- Low labour costs as established protocol reduces the dependence on trained personnel.
- One-time investment pays off over a long and extended run.

2.2.4 Diagnosis Via Direct and Indirect Methods

Early recognition is crucial in the control of emerging and new infectious diseases, whether naturally occurring or deliberately introduced. Limiting the spread of such diseases in today's closely knit globe requires constant monitoring, accurate recognition, and early diagnosis of the microbial source.

After the commencement of symptoms, the presence of disease in plants is confirmed using disease detection methods. Currently, the most common and routinely used plant disease detection procedures available are enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) (El Sheikha & Ray 2014; El Sheikha et al. 2013; Prithiviraj et al. 2004; Ruiz-Ruiz et al. 2009; Saponari et al. 2008; Yvon et al. 2009). In spite of the accessibility of these techniques, there is still a requirement for a quick and discriminatory technique for speedy detection of plant diseases.

Direct and indirect methods are the two broad areas into which the disease detection techniques can be broadly categorized (Figure 2.2). A highly developed detection technique can offer swift, precise, and dependable detection of plant diseases in the early

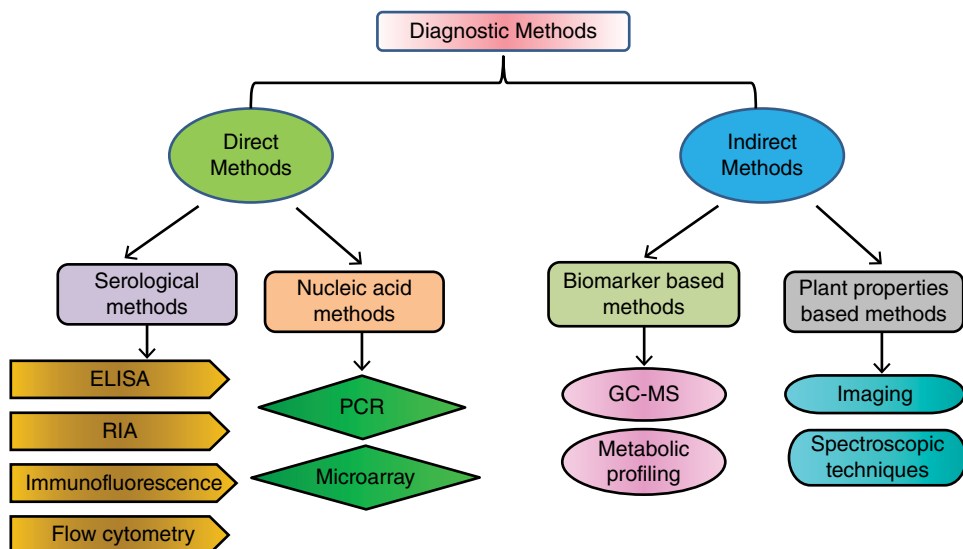


Figure 2.2 Various methods of disease diagnosis. ELISA, enzyme-linked immunosorbent assay; GC-MS, gas chromatography mass spectrometry; PCR, polymerase chain reaction; RIA, radioimmunoassay.

stages for monetary, production, and agricultural profits. Fatty acid profiles, protein methods, and serological methods are frequently used for pathogen identification (Pandey et al. 2015). Amongst the direct methods, serological methods including ELISA, flow cytometry, and immunofluorescence are used along with molecular methods such as fluorescent *in situ* hybridization, PCR, and DNA arrays. The indirect methods generally use spectroscopic and imaging techniques along with volatile organic metabolites as possible biomarkers for disease detection. These methods with examples of diseases diagnosed in past are discussed in detail in the following sections.

2.2.4.1 Direct Methods

Serological Methods

Serological tests were developed to identify viruses as they could not be cultured on artificial media. Immunofluorescence (IF) colony staining (van Vuurde 1990), ELISA, and immuno-strip tests (Hampton et al. 1990) constitute immunodiagnostic techniques. ELISA is by far the most universal immunodiagnostic technique which has been used by scientists around the world for virus and bacteria detection since the 1970s (Clark & Adams 1977), long before DNA-based procedures were available. There are over 800 diverse antisera accessible in the American Type Culture Collection (www.lgcstandards-atcc.org) for plant viruses. Immunological techniques concerning the precise and definite reactions between the antigen and the antibody have progressed as significant diagnostic tools in medicine and recently in agriculture as well. By specifically forecasting the pathogens these techniques can be helpful for enabling proper application of fungicides at suitable times (Narayanasamy 2001, 2005).

All immunodiagnostic assays are based on the principle of detection and quantification of the binding of the specific antigen to the raised antibody. Some of the major immunodiagnostic assays are discussed below.

ELISA Similar to PCR in nucleic acid-based diagnostic techniques, ELISA was a landmark in the immunodiagnosis of diseases caused by microbial plant pathogens, particularly viruses (Clark & Adams 1977). In the ELISA-based disease detection methodology, the microbial protein (antigen) linked with a plant disease is infused into an animal that generates antibodies against the antigen. The antibodies thus produced are removed from the animal's body and used for antigen recognition with a fluorescent dye and enzymes. The sample fluoresces in the presence of the disease-causing micro-organism (antigen), thereby confirming the presence of a particular plant disease.

Different adaptations have been developed suited to the needs of the research and researcher. One of the simplest adaptations is the plate trapped antigen (PTA-ELISA) method, which helps in detection of the pathogen through development of color produced when an antigen–antibody complex is formed on the ELISA plate. The procedure uses a specific antibody intended for a specific antigen. The difference between the composition of the stage-specific secretion and development of the extracellular matrices secreted by *Stagonospora nodorum* was detected using this technique.

Similarly, double antibody sandwich (DAS) ELISA has been used for detection and measurement of *Macrophomina phaseolinain* plant tissues (Afouda et al. 2009). For detection of various stages in the life cycle of *Puccinia graminis* in natural and inoculated plants, direct antigen coating (DAC) ELISA has been used by a group of researchers (Delfosse et al. 2000).

In viral disease diagnosis, among the major developments that have taken place over the past 25 years, ELISA is the most noteworthy (Clark & Adams 1977). Commercial monoclonal antibodies for detection of plant pathogenic viruses are now available from several companies such as Agdia (Elkhart, USA), Adgen (Ayr, UK), and Agritest (Valenzano, Italy). In an experiment, tobacco mosaic viruses were detected by means of surface imprinting methods on polymer-coated quartz crystal microbalances in aqueous media, with a detection range varying from 100 ng/mL to 1 mg/mL within minutes (Dickert et al. 2004). Similarly, viruses responsible for causing the maize stripe and maize chlorotic stripe syndromes in Mauritius were purified and categorized using an antisera and ELISA diagnostic method (Roca de Doyle et al. 2007).

Radioimmunoassay Radioimmunoassay (RIA) is used customarily in clinical microbiology laboratories but seldom for the detection of plant pathogens (Ghabrial & Shepherd 1980; Savage & Sall 1981). The RIA is also a microplate technique based on the double antibody sandwich principle which essentially follows the protocol of the ELISA procedure (Clark & Adams 1977), apart from the substitution of radioisotope ¹²⁵I-labelled T-globulin in place of v-globulin enzyme conjugate. However, the RIA has found limited application in plant pathology, chiefly because the appropriate equipment is quite expensive along with the short half-life of some isotopic reagents which have associated hazards connected with handling and disposing of radioactive material.

Immunofluorescence Serodiagnosis is helpful in identifying viruses and bacteria in plants. Immunofluorescence analysis is a new approach in serodiagnosis that helps to detect the contributory agents directly in plant cells and tissues. In direct immunofluorescence antibody assay (IFA), antibodies specific to pathogens are conjugated with fluorescent dye molecules, normally fluorescing in isothiocyanate (FITC) or rhodamine isothiocyanate (RITC). Antigens present in samples attached to the microscope slides are observed by

means of a fluorescence microscope. Indirect IFA, similar to indirect ELISA, uses a second, tagged antibody to identify specific antibody–antigen binding. Immunofluorescence assays have been particularly useful in detecting and localizing fungi in plant material and soil (Choo & Holland 1970; Dewey et al. 1984; Gabor et al. 1993; Salinas & Schots 1994; White 1976) and diagnosing diseases caused by plant pathogenic bacteria (de Boer & McNaughton 1986; Miller 1984). An efficient IF method has been developed for the detection of host tissue infected with *Fusarium oxysporum* (Arie et al. 1995). This technique has been well exploited by combining microscopy with fluorescent immunoassays to localize and quantify mycelia colonization by *Botrytis* spp. in infected leaves of *Cyclamen persicum* (Kessel 1999).

Similarly, an indirect immunofluorescence spore assay (IFSA) was developed for identifying urediniospores of the rust pathogen *Phakopsora pachyrhizi* by confining them on standard glass slides set with double-sided tape or a thin coating of petroleum jelly. Quantitative results can be attained by counting the number of fluorescing units (i.e., bacterial cells or fungal spores), but this is likely to be very tiresome, thereby limiting the number of samples that can be assessed. To overcome this problem, automated computer-driven microscope systems are being developed to offer quantitative estimation of cell populations, thereby allowing large-scale use.

Flow Cytometry Flow cytometry (FCM) is a method for speedy recognition of cells or other particles as they pass independently through a sensor in a liquid stream. Bacterial cells are recognized by fluorescent dyes bound to specific antibodies and distinguished electronically using a fluorescence-activated cell sorter, which measures several cellular factors based on light scatter and fluorescence. It has been used in seed health testing and other fields. With this advanced technique, tests such as total particle count, distinction between living and dead cells, and discrimination of target and non-target bacterial populations associated with seeds or other plant material can be carried out simultaneously. This technique has also been successfully adapted for the analysis of viability, metabolic condition, and antigenic markers of bacteria (Davey & Kell 1996). In the detection of plant pathogens, the technique has been applied for diagnosis of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seed extracts (Alvarez & Adams 1999), identification of *Xanthomonas axonopodis* pv. *dieffenbachiae*, causal agent of anthurium blight (Alvarez & Adams 1999), detection of *X. campestris* pv. *campestris* in seed extracts of *Brassica* sp. (Chitarra et al. 2002), and for determining the viability of *Ralstonia solanacearum* in seed potatoes (van der Wolf et al. 2004).

Fluorescent *In Situ* Hybridization Fluorescent *in situ* hybridization (FISH) is a technique for bacterial identification that combines the simplicity of microscopic observation and the particularity of the process of hybridization (Volkhard et al. 2000). Its use in detection of plant pathogenic bacteria is comparatively recent (Wullings et al. 1998). The sensitivity of the FISH method is comparable to that of amplification technologies which are the result of the high selectivity of DNA probes. Fluorescent probes targeting the 23S rRNA gene have been useful in detecting *R. solanacearum* race 3 biovar 2 from potato peels (van der Wolf & Schoen 2004). Similarly, bacterial ring rot disease in potatoes caused by *Clavibacter michiganensis* subsp. *sepedonicus* was improved by introduction of FISH targeting the 16S rRNA gene (Li et al. 1997).

2.2.4.2 Nucleic Acid-based Detection Methods

Nucleic acid hybridization, which relies on the high degree of specificity inbuilt in the pairing of nucleotide base sequences, is a well-established and commonly used tool in molecular biology.

Traditional techniques for detection of seedborne fungi are based on incubation and grow-out methods. Recently, new identification techniques, based on DNA analysis, have been applied and are very efficient due to their high sensitivity and specificity. The most common technique is conventional PCR, while other recent techniques include nested PCR, to obviate low levels of target pathogens, multiplex PCR, to detect several pathogens simultaneously, real-time PCR, to quantify fungi on seeds, and magnetic-capture hybridization PCR. The main drawbacks of molecular methods are the inability to distinguish between vital and non-vital inocula, and the difficulty in obtaining a quality DNA template, due to PCR inhibitors in seeds (Mancini et al. 2016).

Polymerase Chain Reaction Based

The invention of PCR in 1984 by Kary Mullis has been among the greatest innovations in science. PCR has revolutionized basic biology and has greatly influenced applied sciences such as diagnostics and forensics. Owing to its efficiency and sensitivity, the PCR (the exponential amplification of a target DNA strand catalyzed by a thermostable DNA polymerase) has become the foundation of nucleic acid-based pathogen studies (Vincelli & Tisserat 2008). Based on the desired molecular targets, PCR has evolved technologically, enhancing the feasibility and accuracy of nucleic acid-based analysis.

Due to its precision, the method is applied for diagnostic purposes, including the detection of plant pathogens. Some of the routinely used methods are as follows.

Standard PCR Standard PCR technique using Taq polymerase has been used for identification of pathogens that are difficult to identify morphologically, such as *Pythium* (André Levesque et al. 1994), *Sclerotium rolfsii* (Jeeva et al. 2010), and *Colletotrichum capsici* (Torres-Calzada et al. 2011), by developing specific sequences from the conserved ITS regions.

RT-PCR Reverse transcription PCR (RT-PCR) is one of many variants of the PCR. In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase and the resulting cDNA is then used as a template for exponential amplification using PCR. RT-PCR is currently the most sensitive method of RNA detection available. RT-PCR uses the mRNA of the structures, thereby reducing the error due to dead tissue contamination as mRNA degrades rapidly in dead cells (Sheridan et al. 1998). Study of viable populations of *Mycosphaerella graminicola* in wheat by Guo et al. (2005) and analysis of plant and fungal gene expression during disease development by other workers have also been conducted using this technique.

Nested/TAIL-PCR A modification of standard PCR, nested PCR is aimed at reducing product contamination due to the amplification of unintended primer binding sites (mispriming). Primer design for nested PCR thus involves designing two primer pairs, one for the outer fragment and one for the inner (Porter-Jordan et al. 1990). This technique has been used for study of pathogenic behavior of phytoplasma in sugarcane

(Wang et al. 2014), maize associated *Curvularia lunata* (Hou et al. 2013), and quarantine *Fusarium* species (Hong et al. 2010).

Thermal asymmetric interlaced (TAIL)-PCR is a powerful tool for the recovery of DNA fragments adjacent to known sequences and utilizes three nested primers in consecutive reactions together with an arbitrary degenerate (AD) primer having a lower melting temperature (T_m) so that the relative amplification frequencies of specific and non-specific products can be thermally controlled. TAIL-PCR has recently been employed to study the pathogenic nature of vascular wilt pathogens in Brassicaceae (Yadeta et al. 2014) and pathogenic nematodes (Peng et al. 2013).

Real-time PCR/multiplex PCR Polymerase chain reaction conditions can interfere with exponential amplification and affect product concentration, in contrast to the exponential nature of PCR in theory which estimates the amount of starting material to be calculated from the amount of product at any point in the reaction. Real-time PCR hence allows for DNA amplification via PCR as well as detection of the amplified DNA fragment (the amplicon) within the same, sealed reaction vessel (Overbergh et al. 2003). The amplicon is detected through the use of oligonucleotide probes which emit fluorescence of defined wavelengths in proportion to the amount of amplicon present after each thermocycle. The most extensively used real-time PCR assays use TaqMan probes.

Identification and diagnostic studies of *Phytophthora* spp. (Bilodeau et al. 2009), along with many other pathogens (Barnes & Szabo 2007; Børja et al. 2006), have been developed using TaqMan probes. Simultaneous and sensitive detection of different DNA or RNA targets in a single reaction can be obtained through multiplex PCR. The presence of more than one pathogen in plant material can be studied through modified standard protocols, by looking for common specific sequences in two or more of them, or detecting related viruses or bacteria on multiple hosts. Multiplex PCR is useful in plant pathology because different bacteria and/or RNA viruses frequently infect a single host and consequently sensitive detection is needed for the propagation of pathogen-free plant material.

There are several examples in plant pathology of simultaneous detection of several targets and the amplification by multiplex PCR of two or three plant viruses has been reported by Grieco and Gallitelli (1999) and Jacobi et al. (1998). Establishing the pathogenic behavior of phytoplasmas (Kazikawa & Kamagata 2014) and *Cylindrocarpon* spp. (Alaniz et al. 2009) are recent examples of the utility of multiplex PCR in phytopathology. Real-time PCR is advantageous over conventional PCR as it provides the data in real time, has a much greater quantitation range and sensitivity, a reduced risk of sample contamination during PCR set-up due to laboratory contamination with amplicon, and a greater amenity for multiplexing (simultaneous testing for multiple pathogens) (Vincelli & Tisserat 2008).

Given the various biases introduced by primer choice and PCR amplification process, PCR-free third-generation sequencing technologies offer great promise (Tedersoo & Nilsson 2016).

Another new technique is the application of high-resolution melting (HRM) analysis for pathogen detection. HRM analysis is a rapid, accurate and powerful tool, capable of differentiating even closely related fungal isolates. The HRM technique is based on monitoring the melting of PCR amplicons, using saturating concentrations of a fluorescent intercalating dye that binds to double-stranded DNA (Zambounis et al. 2015).

Microarray/biochips Based

Microarray has evolved as one of the most efficient systems for simultaneous analysis of large-scale gene expression patterns and has thus occupied center stage in studying plant–pathogen interactions at diagnostic and genomic scales. The underlying principle of DNA microarray is base-pairing of complementary sequences by hybridization and this specific binding of DNA allows a target DNA or RNA to hybridize to a specific complementary DNA probe on the array (Hadidi et al. 2004). This technology is constantly evolving and presently can be grouped into two basic types: cDNA microarrays and oligonucleotide-based arrays (Mah et al. 2004). In a study carried out by Lee et al. (2003), four species of selected cucurbit-infecting tobamoviruses were detected by designing a plant virus cDNA chip with a manual spotting system by using viral microarray technology.

Nanopore Biosensors Another recent advancement is the application of nanotechnology-based techniques. In this regard, nanopores are used as biosensors. Nanopores are electrochemical sensors consisting of an electrically insulated material permeated by one or more pores, of 10–150 nM diameter. For nucleic acid-based nanopore detection, specific capture oligonucleotides are immobilized on the surface of the nanopores and used to detect complementary single-stranded sequences as they pass through the opening. Electric current is used to electrophoretically move the charged nucleic acid molecules through the pores. The observation that different strands of DNA or RNA can have a different effect on the ionic current has also led directly to the field of nanopore sequencing which will be another future generation of sequencing technology (Khan 2014).

2.2.4.3 Indirect Methods

Gaseous and Non-volatile Metabolite Profiling

The gaseous organic compounds released by plants contribute approximately 33% of the total gaseous organic compounds released in the atmosphere (Guenther 1997). However, various physicochemical factors including humidity, temperature, light, soil condition, and fertilization, as well as biological factors such as growth and developmental stage of the plant, insects, and presence of other herbs (Vuorinen et al. 2007), are known to affect the amounts released by plants. These gaseous organic compounds directly or indirectly influence the relationship between plants and pathogens (Vuorinen et al. 2007). Compounds isolated from leaves of citrus plants such as quercetin, hesperidin, and naringenin can be used as biomarkers to identify huanglong-bing disease of these plants (Cevallos-Cevallos et al. 2009).

Gas chromatography (GC)-based and electronic nose system-based techniques are used for assessing the profile of volatile metabolites released by plants. An electronic nose system consists of a series of gas sensors that are sensitive to a range of gaseous organic compounds. Electronic nose systems are used to determine food quality, identify diseases in humans, and detect microorganisms in food products, among others (Sankaran et al. 2010). The potential of using plant volatile signatures for pest and disease monitoring in cucumber, pepper, and tomato plants was examined by Laothawornkitkul et al. (2008). Similarly, electronic nose and GC-MS systems were used to identify and differentiate the volatiles released by plants under normal and disease conditions (Li et al. 2009). The berries infected with *Colletotrichum gloeosporioides* could be distinctively differentiated from the other groups. However, there was some overlap in the gaseous organic compound profiles of the berries infected with *Botrytis*

cinerea and *Alternaria* spp. A PEN2 electronic nose system was used, consisting of an array of 10 metal oxide-based sensors for determining the gaseous organic compounds profile in wheat plants damaged by age and insects (Zhang & Wang 2007). The use of electronic nose systems for identifying plant diseases is a relatively new domain for this technology.

The variability in the volatiles released from onion bulbs infected with bacterial *Erwinia carotovora* causing soft rot and fungal species *Fusarium oxysporum* and *Botrytis allii* causing basal and neck rots, respectively, was determined by GC-MS analysis (Prithiviraj et al. 2004). This study suggested that 25 volatile compounds (among the 59 consistently detected compounds) released from onion can be used to identify the disease based on gaseous organic compound profiling. Similar investigations on potato tubers infected by *Erwinia carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, *Pythium ultimum*, *Phytophthora infestans*, or *Fusariums ambucinum* using solid phase microextraction (SPME) fiber along with GC-flame ionization detector (FID) indicated the potential of gaseous organic compound profiling for disease detection (Kushalappa et al. 2002). The production of volatiles increased with an increase in disease severity. The gaseous organic compound release profiles of damaged and undamaged leaves of oak trees infested by gypsy moth larvae has been studied. The authors reported that the leaves released linalool, homoterpene (E)-4, 8-dimethyl-1,3,7-nonatriene, germacrene D, α -caryophyllene, and several other sesquiterpenes upon days of caterpillar growth on the leaves. These gases were not present in the gaseous organic compound released by the control plants (Staudt & Lhoutellier 2007).

Spectroscopic and Imaging Techniques for Diagnosis of Plant Diseases

Numerous spectroscopic and imaging techniques have been applied for the detection of symptomatic and asymptomatic plant diseases. These include fluorescence imaging, multispectral or hyperspectral imaging, infrared spectroscopy, fluorescence spectroscopy, visible/multiband spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy. Hahn (2009) summarized multiple methods (sensors and algorithms) for pathogen detection, with special emphasis on postharvest diseases. These techniques could be integrated with an autonomous agricultural vehicle that could give information on disease detection at early stages to control the spread of plant diseases. This approach can also be used to identify stress levels and nutrient deficiencies in plants.

Application of fluorescence spectroscopy to detect stress caused by citrus canker (bacterial disease caused by *Xanthomonas citri* (*Xanthomonas axonopodis* pv. *citri*)) and mechanical injury has been employed (Belasque et al. 2008). This study provided the possibility of fluorescence spectroscopy for disease detection and discrimination between mechanical and disease stress. A similar technique was applied to detect water stress and differentiate citrus canker leaves from variegated chlorosis leaves (Marcassa et al. 2006). The above investigations were able to classify healthy from citrus canker-affected leaves, but were unable to identify water stress or distinguish between variegated chlorosis and citrus canker-infected leaves.

Visible and infrared spectroscopies have been used as a rapid, non-destructive, and cost-effective method for the diagnosis of plant diseases. These approaches have also been conducted on the detection of stress, injury, and diseases in plants. The near infrared (NIR)-based method was employed for detecting fire blight disease in asymptomatic pear plants under greenhouse conditions (Spinelli et al. 2006). Purcell et al. (2009) assessed the application of NIR spectroscopy for the examination and rating of

sugarcane resistance against Australian sugarcane disease and Fiji leaf gall. The application of hyperspectral reflectance to identify cotton canopy infected with *Verticillium* wilt was studied by Chen et al. (2008). Delalieux et al. (2007) used hyperspectral reflectance data (350–2500 nm) to detect apple scab caused by *Venturia inaequalis*.

Fluorescence imaging is an advanced version of fluorescence spectroscopy, where fluorescence images (rather than single spectra) are achieved using a camera. A xenon or halogen lamp is used as a UV light source for fluorescence excitation, and the fluorescence at specific wavelengths is recorded using a charge coupled device-based camera system. Chlorophyll fluorescence imaging could be an effective device in monitoring leaf diseases (Lenk et al. 2007). The blue-green fluorescence is applied to evaluate the effectiveness of this technique in observing the development of tobacco mosaic virus infection in tobacco plants. Bravo et al. (2004) employed fluorescence imaging for detecting yellow rust in winter wheat. Application of hyperspectral reflectance imaging in combination with multispectral fluorescence imaging through sensor fusion was used to detect yellow rust (*Puccinia striiformis*) disease of winter wheat (Moshou et al. 2005). The imaging techniques are an improvement over spectroscopic techniques as these methods acquire spectral information over a larger area and offer three-dimensional spectral evidence in the form of images.

2.3 Future Directions

The likelihood that a particular pathogen will cause severe economic and social problems cannot be ignored and therefore the motto “Predict and Prevent” should be adopted by plant pathologists around the globe, as it is by people working on human and animal diseases. However, such a plan would involve a global effort in early recognition of budding diseases, keeping an eye on pathogen movement, and preparing alleviating reactions well in advance. Further, the number of institutions developing investigative technology for plant-based agriculture is small and therefore, with the combined efforts of government and private sector agencies, this gap should be minimized. The development of reasonably priced field tests which can accurately detect new disease epidemics should be encouraged in order to rapidly promote decision-making processes for disease management. Also, technologies such as microarray, which is currently too expensive for broad exploitation for plant pathogen identification, should be made more affordable through efforts to bring down the price of diagnostic chips. It is therefore hoped that more sensitive and specific devices will be developed for a variety of pathogens that can be easily used in the field.

Recent developments have propelled the need for improved use of molecular diagnostic techniques resulting in more suitable, efficient, and precise assays. It is expected that this drift will increase over the years to come. Further, advanced assays reduce the dependence of growers, crop experts, and plant health specialists on symptomatology and/or lengthy diagnostic procedures, thereby permitting early identification of pathogens. Also the disciplines involving molecular and traditional diagnostics should preferably work in groups for any structure of diagnostics to remain efficient and of practical significance. However, diagnostic tools will no doubt continue to progress and the degree of their applicability will finally be determined by simplicity of method, price, and the consequences of the results they deliver.

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3

Molecular Characterization of Ochratoxigenic Fungal Flora as an Innovative Tool to Certify Coffee Origin

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3.1 Introduction: Coffee Factsheet

Coffee belongs to the Rubiaceae family, which includes more than 500 genera and 800 species. The shrub (called coffee) is a perennial plant of the *Coffea* genus whose height can reach 8 m (Bridson & Verdcourt 1988). There are about 100 botanical species in the *Coffea* genus; the best-known ones are *Coffea arabica* Linné and *Coffea canephora* Pierre Ex Froehner where *Coffea robusta* Lindon is a variety (Charrier & Berthaud 1985).

For good growth, coffee requires a hot and humid climate in tropical or subtropical regions. The cultivation of coffee is favorable in the equatorial belt bounded by the 30° parallel North and South (Figure 3.1). At flowering, the flowers come in groups and give rise to fruit commonly called cherries (Figures 3.2, 3.3). The cherries are up to 10–18 mm long and 10–25 mm in diameter (Nganou 2012).

Currently, there are about 25 types of coffee trees distributed in a diversity of ecological niches that vary significantly in soil, weather, and altitude. Among these, only the following three main species are cultivated for commercial coffee consumption (Davis et al. 2006).

- *Coffea arabica* is originally grown in the mountains of Yemen in the Arabian Peninsula (hence its name – *arabica*) as well as the south-western highlands of Ethiopia and south-eastern Sudan. This species produces about 70% of the world's coffee beans.
- *Coffea canephora* (referred to as *robusta*) takes its origins from central and western sub-Saharan Africa. It is also grown in Brazil, Africa, and South-East Asia – India, Indonesia, and Vietnam have now become the world's largest *robusta*-producing region. The species makes up about 27% of the world coffee bean market. Coffee beans from this species are often used in instant coffee.

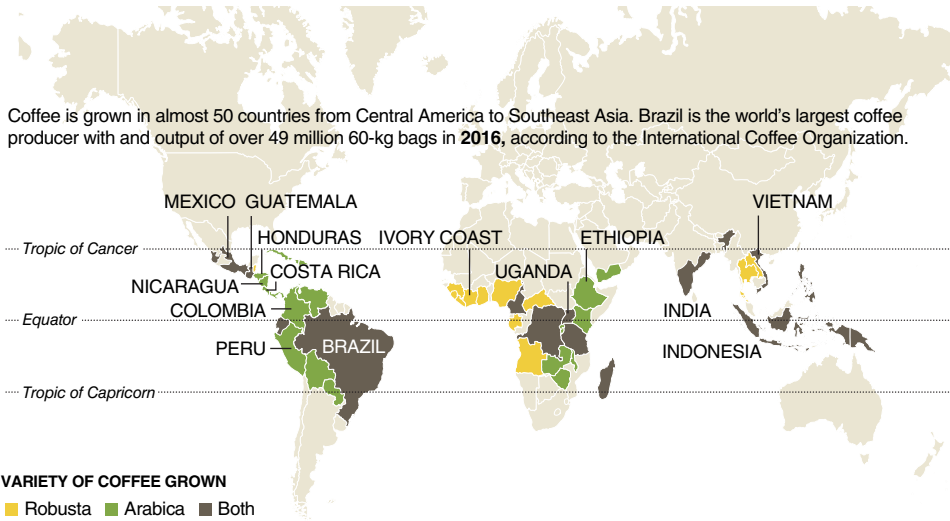


Figure 3.1 Map of coffee-producing countries. Adapted from International Coffee Organization (2016).



Figure 3.2 Photo of coffee flowers (*Coffea arabica*). Source: Marcelo Corrêa (2002). https://en.wikipedia.org/wiki/File:Coffee_Flowers.JPG. Licensed under CC BY-SA 3.0.

- *Coffea liberica* Bull. ex Hiern. was first discovered in Liberia, West Africa. It can grow up to 9 m tall and has cherries larger than those produced by arabica plants. Figure 3.4 shows the differences between the coffee beans of the three species (*arabica*, *robusta*, and *liberica*). This species is mainly grown in Malaysia and the Ivory Coast and makes up about 3% of the world's coffee bean market. *C. liberica* is also known as *C. arnoldiana* De Wild or more commonly as Liberian coffee.



Figure 3.3 Photo of coffee fruit (*Coffea arabica*). Source: Forest & Kim Starr (2007). https://en.wikipedia.org/wiki/File:Starr_070308-5472_Coffea_arabica.jpg. Licensed under CC BY 3.0.

Figure 3.4 *Liberica*, *robusta*, and *arabica*: coffee bean differentiation.



However, via a process of grafting, many cultivars have been developed from these species in an effort to boost production volumes, increase disease resistance or improve cup quality.

The *robusta* strain produces less expensive beans, largely because it can be grown under less ideal conditions than the *arabica* strain. Coffee made from *arabica* beans has a deep reddish cast, whereas *robusta* brews tend to be dark brown or black in appearance.

The coffees made from the two commonly used beans differ significantly. *Robusta* beans are generally grown on large plantations where the berries ripen and are harvested at one time, thereby increasing the percentage of under- and overripe beans. *Arabica* beans, on the other hand, comprise the bulk of the premium coffees that are typically sold in whole bean form so purchasers can grind their own coffee. Whether served in a coffee house or prepared at home, coffee made from such beans offers a more delicate and less acidic flavor (Clarke & Macrae 1987). Figure 3.5 illustrates the principal stages of coffee production from harvesting to final products (ground or instant coffees).

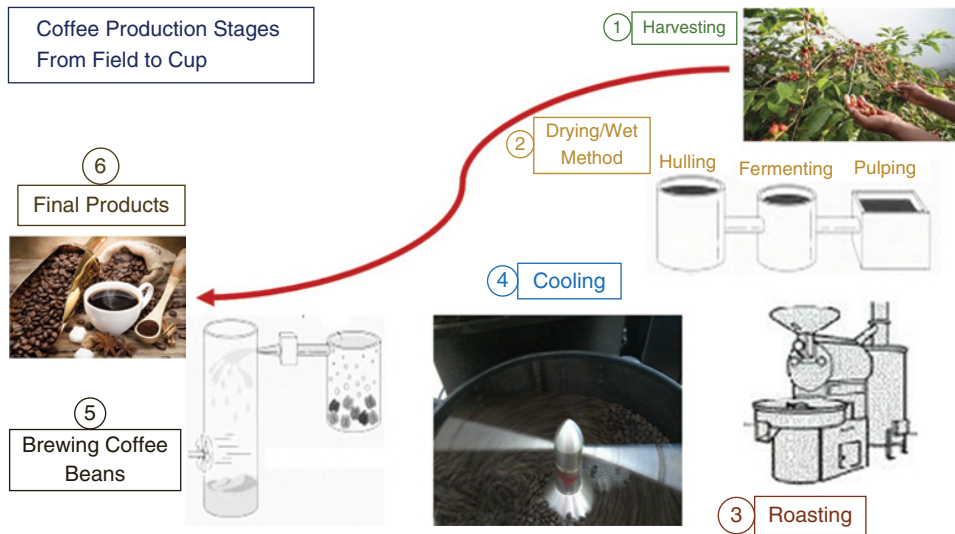


Figure 3.5 Diagram showing stages of coffee production.

The beverage prepared by moving hot water through ground coffee gives a liquid that has lost its carbohydrates, fats, and proteins, and therefore has a low energy content, but which has retained its polyphenols and caffeine. A cup of coffee (200 mL) provides about 175 mg of chlorogenic acid. The coffee drink has a total polyphenol content of 323 mg/g, like red wine. Coffee in one of the drinks with the highest antioxidant activity, with a total oxygen radical absorbance capacity (ORAC) value of 2541 μmol Trolox equivalents (TE)/g, greater than that of green tea with a value of about 1500 μmol TE/g (Carlsen et al. 2010).

Coffee is one of the most important commodities in the world's economy (Durand et al. 2013). The coffee sector provides employment to more than 100 million people, 25 million of whom are involved in cultivation and harvesting (Nganou 2012). On average, world harvest is about 8.9×10^6 tonnes (2015–2016). South America provides almost half of this amount (ICO 2016). Table 3.1 illustrates the global production of coffee and top producers' statistics from 2012–2013 to 2015–2016.

The industrial countries consume about 75% of world coffee production and their consumption per capita has been nearly constant for two decades (ICO 2016). Europe is the largest consumer. Brazil has the highest consumption of exporting countries while European Union members have the highest consumption among the importing countries (Table 3.2).

3.2 The Microflora of Coffee

The constant consumer demand for high-quality coffees has led to higher safety standards, including the need to understand the whole microbiotic environment of natural and processed coffee, and to determine the role of these microorganisms in the characteristics (safety and quality) of the final beverage.

Table 3.1 The global production of coffee and top producers' statistics.

Crop year	2012–13	2013–14	2014–15	2015–16
Coffee variety				
<i>Arabica</i>	91 511*	90 540	85 239	85 814
<i>Robusta</i>	59 346	61 564	61 410	62 179
<i>World total</i>	<i>150 858</i>	<i>152 105</i>	<i>146 648</i>	<i>147 994</i>
Africa				
Ethiopia	6 233	6 527	6 625	6 700
Uganda	3 914	3 633	3 744	4 000
Ivory Coast	2 072	2 107	1 750	1 900
Tanzania	1 109	809	728	875
Kenya	875	838	765	760
Cameroon	371	404	483	570
Madagascar	500	584	500	449
Congo, DR	334	347	335	335
Rwanda	259	258	238	278
Burundi	406	163	248	274
Togo	78	135	185	200
Guinea	234	101	147	177
Others	289	299	255	312
<i>Total</i>	<i>16 673</i>	<i>16 205</i>	<i>16 005</i>	<i>16 831</i>
Asia & Oceania				
Vietnam	23 402	27 610	26 500	27 500
Indonesia	10 726	10 300	9 935	11 525
India	5 303	5 075	5 450	5 833
Papua New Guinea	716	835	798	712
Philippines	599	593	603	605
Laos	541	550	506	520
Thailand	608	638	497	500
Yemen	188	185	150	130
Others	97	117	154	102
<i>Total</i>	<i>42 181</i>	<i>45 903</i>	<i>44 592</i>	<i>47 428</i>
Mexico & Central America				
Honduras	4 537	4 568	5 400	5 400
Guatemala	3 963	3 439	3 310	3 400
Mexico	4 327	3 916	3 591	2 800
Nicaragua	1 991	1 941	1 898	2 100

(Continued)

Table 3.1 (Continued)

Crop year	2012–13	2013–14	2014–15	2015–16
Costa Rica	1658	1444	1408	1492
El Salvador	1255	525	698	565
Dominican Republic	488	425	397	400
Haiti	350	345	343	342
Panama	80	113	106	107
Cuba	88	107	101	100
Others	36	32	34	33
Total	18 773	16 856	17 287	16 739
South America				
Brazil	56 925	55 050	51 116	48 423
Colombia	9 927	12 124	13 333	14 009
Peru	4 453	4 338	2 883	3 301
Ecuador	828	666	644	644
Venezuela	952	805	651	500
Bolivia	115	128	106	89
Others	30	30	31	30
Total	73 230	73 141	68 764	66 997

* In thousand 60 kg bags.

Source: Adapted from ICO (2016).

Table 3.2 The global consumption of coffee.

Coffee year (October–September)	2012–13	2013–14	2014–15	2015–16	Consumption per capita (cup/day)*
Countries level					
Top exporting countries					
Brazil	20 330**	20 085	20 333	20 500	0.484
Philippines	3 590	3 210	3 563	4 155	0.608
Ethiopia	3 400	3 650	3 675	3 700	0.100
Indonesia	2 418	2 550	2 770	2 650	0.041
Mexico	2 354	2 354	2 354	2 354	0.212
Vietnam	1 825	2 000	2 200	2 300	0.034
Colombia	1 441	1 469	1 494	1 600	0.479
Thailand	1 230	1 300	1 350	1 400	0.149
India	1 208	1 248	1 310	1 355	0.024
Venezuela	1 195	1 135	1 126	1 126	0.321
Total	44 350	44 209	45 374	46 369	

Table 3.2 (Continued)

Coffee year (October–September)	2012–13	2013–14	2014–15	2015–16	Consumption per capita (cup/day)*
Top importing countries					
European Union	41 661	42 130	42 039	42 295	–
USA	23 120	23 679	24 272	24 500	0.931
Japan	7359	7479	7609	7734	1.590
Russia	3660	3928	3898	3900	0.650
Canada	3510	3813	3629	3535	1.009
Algeria	2123	2147	2154	2154	0.765
South Korea	1748	1873	1963	1980	0.329
Australia	1564	1543	1713	1770	0.365
Saudi Arabia	1256	1317	1 30	1330	0.277
Ukraine	1313	1246	1106	1069	0.309
Switzerland	1 104	1 051	1 079	1 108	0.971
Total	101 018	102 808	104 021	104 933	
Regional level					
Europe	50 239	50 845	50 608	50 870	–
Asia & Oceania	28 329	28 745	30 516	31 609	–
North America	26 631	27 492	27 901	28 035	–
South America	24 587	24 167	24 426	24 717	–
Africa	10 447	10 571	10 704	10 815	–
Mexico & Central America	5135	5198	5239	5257	–
World total	145 367	147 017	149 395	1513	

* Source: Euromonitor International (2014).

** In thousand 60 kg bags.

Source: Adapted from ICO (2016).

Many microbial species have been isolated from coffee cherries during natural or dry fermentation. This microflora consists of spoilage microorganisms, generally found on the surface of fruits and soil surrounding the coffee. Silva et al. (2000, 2008) have shown that among the 940 microbial isolates they analyzed, 375 isolates were bacteria, with the majority of the bacteria (80.4%) in the Gram-positive *Bacillus* genus. This genus was found in the coffee beans throughout the fermentation, drying, and storage stages. Gram-negative bacteria represented 19% of the total bacterial isolates. Gram-negative bacteria belonged predominantly to the Enterobacteriaceae family, and the most frequent genera were *Enterobacter* and *Serratia*. The following species were identified: *Enterobacter aerogenes*, *Klebsiella oxytoca*, *Shewanella putrefaciens*, *Shigella dysenteriae*, and *Yersinia* spp. The second microbial group in the succession isolated and identified from coffee cherries and beans was yeasts. *Debaryomyces hansenii* and

Pichia guilliermondii were the most frequently identified among the 202 yeast isolates. A total of 363 filamentous fungal isolates were identified, including 132 isolates of *Aspergillus*, 101 *Penicillium*, 58 *Cladosporium*, 44 *Fusarium*, 15 *Pestalotia*, and 13 *Paecilomyces*. *Cladosporium cladosporioides* was the fungal species most commonly found in coffee cherries and during the fermentation process.

3.2.1 Occurrence of Ochratoxigenic Fungi in Coffee

Ochratoxin A (OTA) contamination is commonly associated with cereals, fresh grapes, dried fruits, wine, beer, cocoa, and coffee. In a European assessment of the contribution of each food commodity to the mean total dietary intake of OTA (Miraglia & Brera 2002), cereals and their derived products were considered the major source of human OTA exposure, accounting for half of all contributions (50%). In this study, wine and coffee took second and third place, contributing about 13% and 10%, respectively. The other contributing food commodities were spices (8%), beer (5%), cocoa (4%), dried fruits (3%), and meat (1%).

The presence of OTA in coffee was first reported by Levi et al. (1974) and has since been found in green coffee beans (Nakajima et al. 1997; Tsubouchi et al. 1985), roasted coffee (Studer-Rohr et al. 1995), and instant coffee (Lombaert et al. 2002; Patel et al. 1997). OTA has also been detected in brewed coffee (Pérez de Obanos et al. 2005; Stegen et al. 1997). Many species of *Aspergillus* and *Penicillium* can produce OTA in coffee, but the most important sources of this toxin in coffee have been attributed to three species: *Aspergillus niger*, *A. carbonarius*, and *A. ochraceus* (Frank 2001; Noonim et al. 2008; Taniwaki et al. 2003). These three species have been reported in 408 coffee samples from four regions of Brazil (Taniwaki et al. 2003). In this study, coffee samples (*Coffea arabica*) were collected from selected farms that cover different stages of coffee bean maturation and coffee processing: cherries, including immature and overripe fruits from trees, overripe cherries from the soil, all types of cherries from the drying yard and storage in barns. The presence of these species was low in fruits obtained from trees, but higher in samples from the ground (overripe fruits), from the drying yard and storage. From all samples analyzed, 872 isolates of *A. ochraceus*, *A. niger*, and *A. carbonarius* were obtained. *A. niger* was the most common species found (549 isolates, 63% of the three species), but only 3% of them produced OTA. *A. ochraceus* was also common (269 isolates, 31%), and 75% of those studied were capable of OTA production, a much higher percentage than reported elsewhere. *A. carbonarius* was much less common (54 isolates, 6%), and 77% of the *A. carbonarius* isolates were capable of producing OTA. *A. carbonarius* was found in the hottest region studied, and only from beans in the drying yard or storage (Taniwaki et al. 2003).

Several other studies have been carried out to analyze the presence of ochratoxigenic fungi in coffee (Batista et al. 2009; Noonim et al. 2008; Silva et al. 2008; Vilela et al. 2010). The main OTA-producing species in coffee beans belong to the genera *Aspergillus* Section *Circumdati* and Section *Nigri* (Batista et al. 2003, 2009; Gil-Serna et al. 2011). The OTA-producing species of the genus *Aspergillus* are *A. ochraceus* (Batista et al. 2009; Frisvad et al. 2004; Gil-Serna et al. 2011; Perrone et al. 2007; Taniwaki et al. 2003), *A. niger* (Perrone et al. 2007; Samson et al., 2004; Taniwaki et al., 2003), *A. carbonarius* (Perrone et al. 2007; Samson et al. 2004; Taniwaki et al. 2003), *A. sulphureus* (Batista et al. 2009), *A. sclerotiorum* (Batista et al. 2009), and *A. westerdijkiae* (Frisvad et al. 2004; Gil-Serna et al. 2011). Among these, *A. ochraceus* is the most commonly found in coffee

in Brazil and is an important OTA producer (Batista et al. 2009; Suárez-Quiroz et al. 2004b; Vilela et al. 2010). *A. carbonarius* is also common in grape and *robusta* coffee. However, its occurrence in coffee beans is not frequent in Brazil, unlike in Thailand where this species is commonly isolated (Noonim et al. 2008; Taniwaki et al. 2003). In Philippine coffee beans, the dominant species was *A. niger* which was isolated from 55% of the beans analyzed. In contrast, the occurrence of *A. ochraceus* was less than 1%. The genus *Penicillium* had a 16% share in the total mycobiota of coffee beans (Alvinda & de Guzman 2016).

3.2.2 The Risks of OTA-producing Fungal Presence in Coffee

The presence of fungi in coffee beans does not only affect quality in terms of flavor and aroma of the beverage but also presents a safety risk for the final product, due to the production of toxic secondary metabolites, the mycotoxins, which can be harmful to consumers at certain concentrations (Bennett & Klich 2003; Vilela et al. 2010). Ochratoxin has nephrotoxic effects and is a potential carcinogen in humans (Holzhäuser et al. 2003; Schlatter et al. 1996). According to Petzinger and Weidenbach (2002), these additional effects have gained increased attention since they have been found associated with low concentrations of OTA.

The Joint Committee FAO/WHO of Experts on Food Additives (JECFA) has set a tolerable weekly intake limit of OTA for humans at 100 ng/kg body weight (JECFA 2001). The European Union set a maximum level for OTA content at 5 µg/kg in roasted and ground coffee and 10 µg/kg in instant coffee (European Commission 2006b). However, OTA content in green coffee is not regulated yet and the complete degradation of OTA is not always achieved during normal commercial roasting (Castellanos-Onorio et al. 2011).

3.3 Detection of Ochratoxigenic Fungi in Coffee by Molecular Techniques

3.3.1 Why Do We Need to Use Molecular Approaches?

The severe consequences of OTA contamination demand efficient and cost-effective methodologies for detecting OTA producers in coffee. Castellanos-Onorio et al. (2011) reported that the reduction of OTA level greatly depended on the initial OTA contamination in green beans. In some cases, the legal OTA level in roasted coffee in Europe could not be reached. As a result, the OTA content in green coffee must be managed upstream by controlling the fungal flora.

3.3.2 Molecular Methods Used for the Detection of Ochratoxigenic Fungi in Coffee

Polymerase chain reaction (PCR) was developed in 1985 for the *in vitro* amplification of specific segments of DNA (Mullis & Faloona 1987; Saiki et al. 1985). This technique has allowed the precise identification and fast detection of ochratoxigenic species without the need for isolating pure cultures.

A specific PCR assay for the detection of *A. carbonarius* was developed by Patiño et al. (2005) based on internal transcribed spacer (ITS) sequences. Schmidt et al. (2004b)

used amplified fragment length polymorphism (AFLP) to detect specific markers for *A. carbonarius*. A certain number of amplified fragments were found to be specific to this species. The marker fragments were cloned, sequenced, and used to design a specific primer pair to detect this species. Several strains representing closely related black aspergilli, i.e., *A. carbonarius*, *A. niger*, and *A. tubingensis*, were analyzed by random amplification of polymorphic DNA (RAPD) with the aim of developing species-specific primers for the detection of *A. carbonarius* in coffee beans (Fungaro et al. 2004). Based on RAPD markers, Sartori et al. (2006) developed specific primers to detect *A. niger* in coffee beans.

Studies concerning fungi with the potential for colonizing Brazilian coffee beans and producing OTA showed that *A. ochraceus* (now *A. westerdijkiae*), *A. carbonarius*, and *A. niger* are the major species in Brazilian coffee beans. Based on this observation, Sartori et al. (2006) developed a multiplex PCR assay that can detect these three target fungi species directly from coffee bean samples. Multiplex PCR (m-PCR) is a procedure that allows the simultaneous amplification of more than one target sequence in a single PCR reaction, decreasing the number of reactions that must be performed to assess the possible presence of different species in a food sample. Sartori et al. (2006) first analyzed the value of the m-PCR assay with DNA obtained from coffee beans inoculated with these three species. Based on microsatellite-primed PCR (MP-PCR) profiles, using three microsatellite primers, three main groups were obtained by cluster analysis: *A. niger*, *A. ochraceus*, and *A. carbonarius* species isolated from coffee beans in Saudi Arabia. A clear-cut association was found between the MP-PCR genotypes in these fungi and their abilities to produce OTA (Moslem et al. 2010).

Although conventional PCR is a valuable tool for detecting and monitoring mycotoxigenic fungi, it is not appropriate to quantify a given fungus species in a food sample. Small differences in reaction efficiency per cycle can result in a substantial difference in the final product quantity, and so it is very difficult to extrapolate the initial concentration of the template in the sample from the final product (Hill & Wachsmuth 1996). Fortunately, the introduction of real-time PCR technology has increased the reliability of PCR results compared to those obtained by conventional methods, thus opening new avenues for quantifying ochratoxigenic fungi in food. Real-time PCR is more sensitive than classic PCR and does not require gel electrophoresis. The analysis can be concluded in less than 5 h. Real-time PCR also significantly reduces the time and labor required during their assay, making it appropriate for large-scale analyses.

Because *A. westerdijkiae* consistently produces large amounts of OTA, Morello et al. (2007) evaluated the potential of real-time PCR for quantification of this species in coffee beans. The authors also assessed the sensitivity of this method in order to detect *A. westerdijkiae* in coffee beans. Serial dilutions (10^{-1} – 10^{-9}) of DNA extracted from infected coffee beans after 48 h of incubation generated a positive signal at up to 10^{-5} dilution, showing that less than 10 and more than one single copy of the *A. westerdijkiae* haploid genome can be detected by this methodology. This value also indicated that fewer than 10 haploid genomes could be detected per 0.1 g of coffee beans. Thus, the real-time PCR assay was more than 100 times more sensitive than the CFU technique.

The sensitivity of the PCR method is crucial for the detection of foodborne microorganisms. Unfortunately, there is no standard for reporting sensitivity. Some authors refer to sensitivity as the minimum picograms of DNA that can be detected (Patiño et al. 2005; Schmidt et al. 2004a), while others refer to it as the minimum percentage of

infected grains in a sample (Schmidt et al. 2004a). To eliminate confusion and uncertainties regarding sensitivity, a single method for sensitivity calculation should be adopted. Sartori et al. (2010) suggested that the number of haploid genomes per gram of sample is the most convenient metric with which to indicate PCR sensitivity.

3.4 Using Molecular Detection of OTA-producing Fungi to Certify Coffee Origin: Is it Possible?

3.4.1 Why is it so Important to Certify Coffee Origin?

Ochratoxin A in coffee beans can come from several sources, from coffee plants to postharvest processing. Since most coffee farms are in tropical and subtropical regions with hot and humid conditions, there are many fungi that thrive in such coffee plantations. In addition, fungi associated with a variety of equipment and processes during coffee manufacturing, storage, and transportation could contribute to OTA in the final coffee products (Bucheli et al. 1998; Durand et al. 2013; Paulino de Moraes & Luchese 2003; Romani et al. 2000; Suárez-Quiroz 2004; Suárez-Quiroz et al. 2004a, b, 2005). In tropical zones, OTA in coffee beans is mainly produced by *Aspergillus* spp.: *A. carbonarius*, *A. niger* (section *Nigri*), *A. westerdijkiae*, and *A. ochraceus* (section *Circumdati*). In temperate zones, *Penicillium verrucosum* and *P. nordicum* are the main species synthesizing OTA in food commodities (O'Callaghan et al. 2003; Pitt et al. 2000).

Ochratoxin A contamination of coffee can occur in the field. Indeed, overripe coffee cherries are often contaminated by filamentous fungi and subsequently by OTA (Duris et al. 2010). Usually, coffees treated with wet processing, especially those from Central and South America, showed a low OTA content. Higher rates were observed for coffee beans treated with dry processing, often from countries in Africa and Asia (Durand et al. 2013).

3.4.2 Interest in Coffee Traceability

Traceability is the information that permits the monitoring (possibly retrospective) of a material or product throughout its production and distribution to the end of life, i.e., “from the farm to the fork” for a food product. This concept became effective in the EU on 1 January 2005, as per Article 18 of EU Regulation 178/2002. Regarding food, the Codex Alimentarius Committee has defined and revised product traceability as follows: “Traceability is the ability to track the movement of food from specific stages of production, processing and distribution” (Codex Alimentarius Commission 2004).

Traceability of coffee has become very important with the application of EC Regulation 1881/2006 (European Commission 2006b) which set the maximum limits of OTA in coffee. Currently, EU regulations require the provision of health certificates which must accompany all food products imported into the EU via Regulation 1664/2006 (European Commission 2006a). This document, which is checked by customs for each shipment, includes four main sections: product identification, product origin, product destination, and health attestation.

Thus, coffee not respecting the standards set regarding OTA would be destroyed at the owner's expense.

3.4.3 Limitations of Current Molecular Techniques

Several molecular biology-based methods, both culture dependent and independent, have been developed to study microbiota dynamics in coffee beans (Masoud & Kalsoft 2006; Masoud et al. 2004; Ramos et al. 2010; Vilela et al. 2010). However, relatively few studies have examined filamentous fungi dynamics. Thus, little is known about the conditions for contamination by OTA-producing fungi and their mycotoxin production as well as their potential interactions with yeasts (Durand et al. 2013).

Many molecular assays have been published for the identification and fast detection of ochratoxigenic species in coffee without the need for isolating pure cultures. These assays include conventional PCR and real-time PCR. Until now, they have been used in research laboratories to detect putative mycotoxin-producing fungi in culture or even in food samples to obtain information on the epidemiology and ecology of ochratoxigenic species or to acquire basic information on gene expression. However, using these molecular assays in routine analyses in the food and feed industries remains a challenge. Specificity, sensitivity, and simplicity of analysis are all areas that must be improved before these assays can become useful for practical applications. Furthermore, OTA biosynthesis is poorly understood relative to the synthesis pathways of other economically important mycotoxins (Sartori et al. 2010).

3.4.4 PCR-DGGE is a Promising Tool to Detect Ochratoxigenic Fungi and Trace Coffee Origin at the Same Time

Several studies have shown that polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) is an efficient, stable, reproducible, rapid, cheap method which can analyze many food samples in one step (El Sheikha & Montet 2012; El Sheikha 2011; El Sheikha et al. 2011a; Ercolini 2004; Le Nguyen et al. 2008; Montet & El Sheikha 2011; Vaz-Moreira et al. 2013). Furthermore, the sensitivity, repeatability, and robustness of PCR-DGGE have already been confirmed by El Sheikha (2010), El Sheikha et al. (2011b), and Durand (2012). At both the qualitative and quantitative levels, PCR-DGGE has been shown to be efficient in monitoring the dynamics of mycotoxigenic fungi in coffee (Durand 2012; Durand et al. 2013; Martins et al. 2003; Nganou et al. 2014).

Figure 3.6 recapitulates how PCR-DGGE works with coffee samples. Briefly, a coffee sample (e.g., fresh cherries, parchment, coffee beans) is subjected to DNA extraction, thus obtaining a mixture containing DNA from the microbial species occurring in the sample. A combined mechanical/enzymatic/chemical extraction method was applied following the protocol of El Sheikha (2010). Then, the DNA mixture is used as a PCR template to amplify and identify fungal species in a unique PCR step. The most commonly employed target for PCR amplification prior to DGGE is ribosomal DNA. This is because ribosomal DNA is considered the most conserved gene in all cells that also includes variable regions (Smit et al. 2007). A fragment of the D1/D2 region of the 28S rDNA gene was amplified using eukaryotic universal primers (U1GC, U2; Sigma, France). The amplification was carried out according to El Sheikha (2010). All the amplicons have the same size (260 bp) but different sequences can be separated by DGGE. The PCR products were analyzed by DGGE by using a Bio-Rad Dcode™ universal mutation detection system (Bio-Rad Laboratories, USA), using the procedure first described by El Sheikha (2010). Thirty microliters of PCR amplicons were loaded into 8% (w/v) polyacrylamide gels (acrylamide/N,N'-methylene bisacrylamide, 37.5/1; Promega, France)

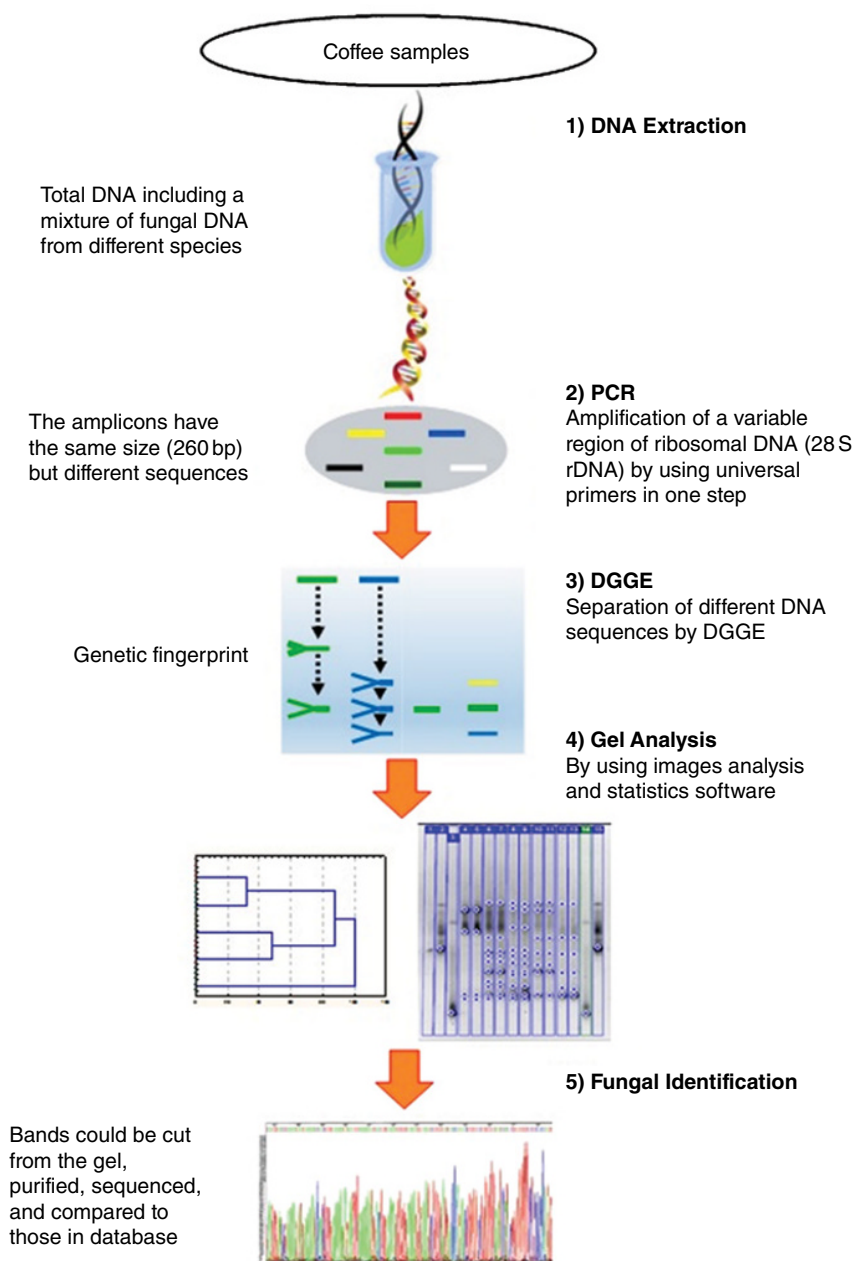


Figure 3.6 How PCR-DGGE works with coffee samples as a traceability and detection tool.
 Source: Adapted from El Sheikha (2015). <http://openventio.org/Special-Edition-1/New-Strategies-for-Tracing-Foodstuffs-Biological-Barcodes-Utilising-PCR-DGGE-AFTNSOJ-SE-1-101.pdf>. Licensed under CC BY 4.0.

in $1 \times$ TAE buffer (40 mM Tris HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na₂-EDTA). Fungi electrophoresis experiments were performed at 60°C using a denaturing gradient range at 40–70% (100% corresponded to 7 M urea and 40% [v/v] formamide, Promega). The gels were electrophoresed at 20 V for 10 min and then at 80 V for 16 h. The final result was a fingerprint specific to the sample analyzed which contained a series of bands relative to the fungal species present in the coffee sample. Identification of the species can be achieved by purifying and sequencing the bands in the DGGE profile (El Sheikh 2010; El Sheikh et al. 2011a, b).

Furthermore, the microbial profiles obtained using PCR-DGGE can be used as a tool to infer coffee origins (Hamdouche et al. 2016; Nganou et al. 2012). Durand et al. (2013) reported that the biodiversity and dynamics of fungal populations linked to OTA production could be studied by PCR-DGGE as the genetic fingerprinting, with the aim of understanding the effects of postharvest processing on the microbiota. PCR-DGGE thus appeared to be a promising tool in order to investigate OTA production in coffee beans. Indeed, relationships could be established between OTA content in green coffee and the fungal DGGE patterns.

As a tool for traceability of coffee origin, Nganou et al. (2012) studied fungal communities using 28S rDNA profiles generated by PCR-DGGE. PCR-DGGE was used to detect variation in fungal populations of coffee from five different locations in the West and the coastal plains in Cameroon. Table 3.3 shows the characteristics of the samples used for this analysis (Nganou et al. 2012). Based on the technique first described by El Sheikh (2010), extraction of fungal DNA was conducted followed by PCR and then DGGE. In Figure 3.7, each vertical line represents a coffee bean sample and each band represents a mold species. The DGGE profile analysis reveals the presence of 5–13 bands. The differences in the band profiles can be attributed to the differences in environment between districts and the type of processing applied could also affect the fungal communities of coffee. In the DGGE gel, some common bands appeared in all the samples independent of location and variety. These bands could be common fungi for both coffee species (Nganou et al. 2012).

A great similarity exists between the fungal profiles of Dschang and Bafoussam, and between those of Santchou and Melong, even though they are separated by approximately 48 km and 33 km from each other respectively (Figure 3.8). The high similarities could be explained either by the proximity of the two sampling sites or by their similar climatic conditions (altitude, rainfall) as Santchou and Melong are located in the plains, while Bafoussam and Dschang are on the plateaus of the West region. This result agrees with that of El Sheikh et al (2011a, b). In those studies, on shea samples and Egyptian physalis, they showed that differences in microbial profiles could be attributed to differences in the environments between regions: geographically close regions have almost the same environmental characteristics, while the differences between regions where the fruits were collected had a major effect on the fungal communities. The types of treatment system applied could also affect the microbial communities on coffee, but no significant difference was observed on their collection sites (Nganou et al. 2012).

As a detection tool, PCR-DGGE can detect the main species of OTA-producing fungi – *A. carbonarius*, *A. ochraceus* and *A. niger* (see Figure 3.7.7) (Nganou et al. 2012). Sequences at the 28S rRNA could be extracted from the gel, sequenced, and compared to those in the GenBank database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi/>) and those of the Ribosomal Database Project (<http://rdp.cme.msu.edu/index.jsp>) using the BLAST program to confirm the results (Altschul et al. 1997). Sequences with a

Table 3.3 Characteristics of the samples used for analysis of fungal communities by PCR-DGGE.

Type	P	G	P	G	G	G	S	G	S	G	C
Variety	A	R	A	R	R	R	R	R	R	R	R
Treatment	WP	DP	WP	DP	DP	DP	DP	DP	DP	DP	DP
	WPD	DPD	WPB	DP1B	DPB	DPS	DP1S	DPA	DP1A	DPM	DP1M
Site	DSH		BFSS			SAN		BAF		MEL	

A, *arabica*; BAF, Bafang; BFSS, Bafoussam; DP, dry process; DSH, Dscang; G, green; MEL, Melong; P, parchment; R, *robusta*; S, shell; SAN, Santchou; WP, wet process.
Source: © *International Journal of Biosciences*. Reproduced with permission of International Network for Natural Sciences, Bangladesh.

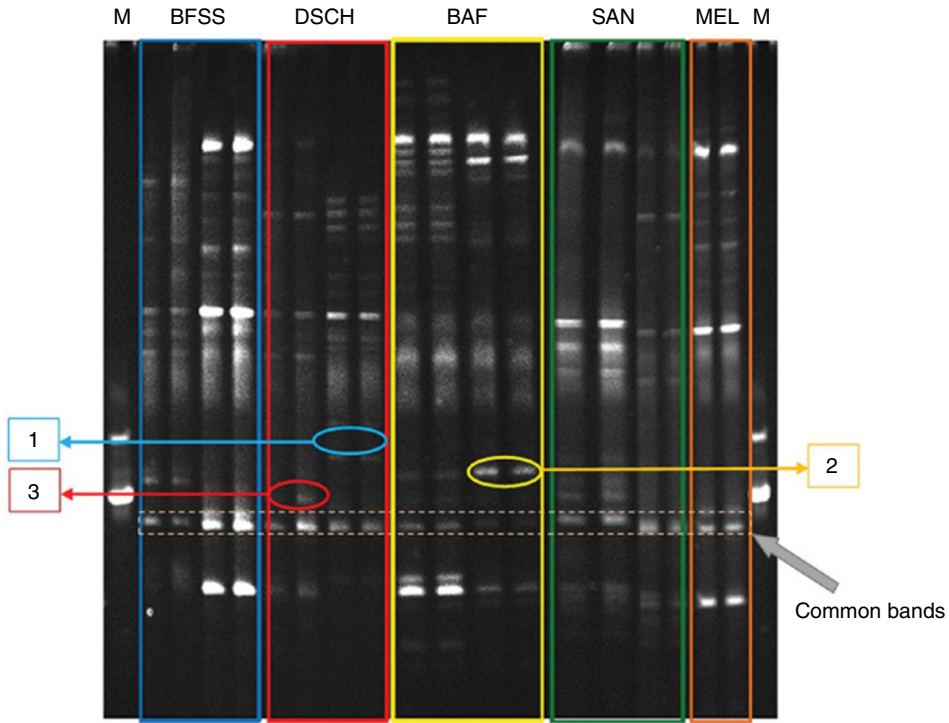


Figure 3.7 PCR-DGGE of 28S rDNA band profiles of both coffee varieties (*arabica*, *robusta*) from five regions of Cameroon. Samples were taken during two treatments (wet and dry). BAF, Bafang; BFSS, Bafoussam; DSCH, Dschang; M, Marker; MEL, Melong; SAN, Santchou. 1: *A. carbonarius*; 2: *A. ochraceus*; 3: *A. niger*. Source: © International Journal of Biosciences. Reproduced with permission of International Network for Natural Sciences, Bangladesh.

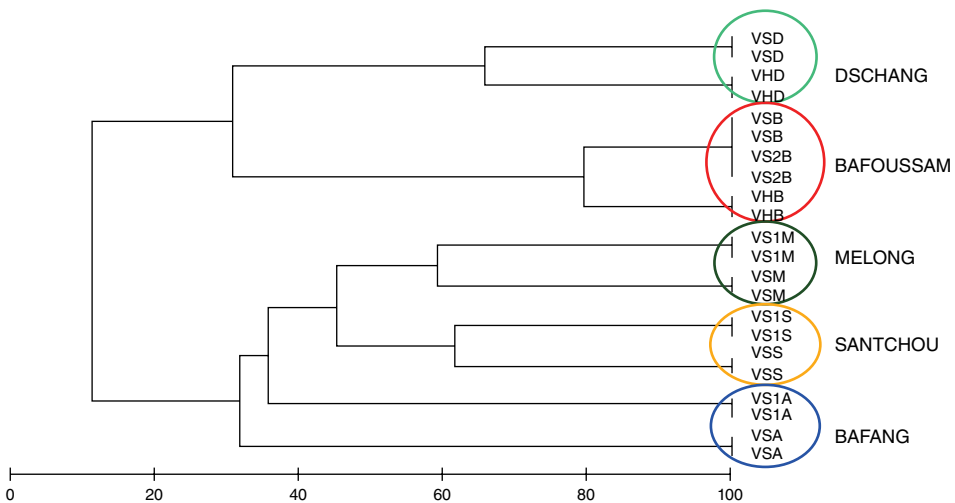


Figure 3.8 Cluster analysis of 28S rDNA band profiles of both coffee varieties (*arabica*, *robusta*) from five regions of Cameroon: Bafoussam, Dschang, Bafang, Santchou, and Melong. Source: © International Journal of Biosciences. Reproduced with permission of International Network for Natural Sciences, Bangladesh.

percentage identity of 97% or more were considered to belong to the same species (Palys et al. 1997; Stackebrandt & Goebel 1994).

On the other hand, PCR-DGGE has several drawbacks, including difficulty in identifying to species level, and the lack of a standardized database from which samples from different runs and labs can be compared. In addition, drawbacks can also occur at different stages of analysis: sampling, preservation of the sample, cell lysis during DNA extraction, amplification of the rDNA by PCR, electrophoretic migration of the DGGE gel and the inability to standardize the gel profiles for comparison in different studies (Renouf 2006). Continuous improvements are needed to make it complementary to the increasingly popular metagenomic approaches analyzing fungal DNA barcodes (Xu 2016).

3.5 Conclusions and Future Perspectives

The serious risk that ochratoxin A (OTA) generates for consumer health has led the sanitary authorities of the European Union to establish maximum allowable limits in several agricultural commodities, particularly coffee. In addition, given the competition, consumer distrust and the globalization of markets, the concept of traceability of various foodstuffs became essential for market access in the European Union and United States. This is consistent with what has been declared by European Regulation 178/2002 which imposed the determination of geographical origin in the process of food traceability in commercial transactions.

Conventional and real-time PCR have been used in research laboratories to detect putative ochratoxin-producing fungi in coffee samples to obtain information on the epidemiology and ecology of ochratoxigenic species or to acquire basic information on gene expression. Specificity, sensitivity, and simplicity of analysis are remaining challenges. Furthermore, OTA biosynthesis is poorly understood relative to the synthesis pathways of other economically important mycotoxins.

The PCR-DGGE technique has been used to determine the presence of viable and non-viable microorganisms. As a detectable and quantitative tool, PCR-DGGE coupled with image analysis software can monitor the dynamics of ochratoxigenic fungi presented in coffee.

The PCR-DGGE technique has also been used for tracing the origin of different samples of coffee. With this tool, it was possible to assign profiles to each site and to show that the common bands at all locations are biological markers that could be used to trace the geographical origin of coffee. These biomarkers are specific for coffee samples from the same locality, which allows regions to be statistically distinguished.

Although PCR-DGGE has several drawbacks, which can be overcome, we believe that it could be a powerful technique to meet the quality and safety needs of coffee producers, consumers, and regulators.

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4

Molecular and “Omics” Techniques for Studying Gut Microbiota Relevant to Food Animal Production

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4.1 Introduction

The mammalian gut microbiota is composed of bacteria, fungi (both unicellular and filamentous), protozoa, viruses, and bacteriophages. Bacteria are the major component of animal gut microbiota with approximately 10^{14} cells/mL of luminal content and 500–1000 species (Lamendella et al. 2011) and there are highly complicated interactions among them and with animal hosts and diets (Xu et al. 2007). The gut microbiota plays critical roles in normal digestive functions (Waititu et al. 2014), maturation of host immunity (Mulder et al. 2011), nutrient utilization (Waititu et al. 2014), growth performance (Stanley et al. 2016), antibiotic resistance (Looft et al. 2012), and defense against pathogens (Stanley et al. 2014). Dysbiotic gut microbiota is associated with several intestinal and extraintestinal diseases and poor animal growth performance, leading to increased risk in food safety and public health as well as low profitability of animal production. Therefore, the gut microbiota and its interactions with animal hosts and diets have long been of research interest.

The composition and diversity of animal gut microbiota can be affected by many different factors, among which feed ingredients and additives have significant impacts (Holman & Chénier 2015). Until recently, the technology was not available to comprehensively monitor the shifts in composition and functionality of microbiota in response to dietary treatments, as many microorganisms cannot be cultured on nutrient media, and polymerase chain reaction (PCR) techniques do not target all microorganisms (Gong & Yang 2012).

Recent advances in sequencing technologies and “omic” tools offer rapid low-cost molecular-based methodologies that can investigate microbial communities as a whole. These advances have enabled more comprehensive studies of the composition and functionality of gut microbiota (referred to as the microbiome) and have significantly enriched our knowledge of its role in animal health and nutrition. Nevertheless, our understanding of the gut microbiome, including the benefits of feed ingredients to animal health and nutrition through the gut microbiota, still remains largely open

to improvement because of the complexity of the gut ecosystem. Integrated use of gnotobiotic and knock-out/transgenic animals, the “omics” tools, and bioinformatics and statistical approaches is useful for advancing our understanding of the gut microbiome of animals. This would particularly enhance our ability to study dietary components and their benefits to the health and nutrition of animals, leading to sustainable food animal production.

There has been a large amount of research in the last decade on the gut microbiota and its functions in food animal production (Burrough et al. 2015; Haenen et al. 2013). The purpose of this chapter is not to summarize the information that has been generated from this field, but rather to critically review the advances in molecular and “omics” techniques recently developed for and applied to microbiota research. The techniques’ potential in improving production efficiency of poultry and swine, the two major species of food animals, is also discussed.

4.2 Methods for Studying Gut Microbiota Composition

The methods for studying gut microbiota composition can be primarily divided into culture-dependent and culture-independent techniques. Combined use of these two approaches may thus provide a better strategy to study the ecology of gut microbiota since both culture-dependent and culture-independent techniques have unique advantages and drawbacks. The advantages of culture-dependent techniques include the ability to:

- study live bacteria
- provide pure cultures of bacteria that allow physiological studies
- detect specific gut bacteria, including pathogens
- genotype cultured isolates (Suchodolski 2011).

However, culture-dependent techniques also have several disadvantages, including the loss of unculturable bacteria (Gong & Yang 2012; Zoetendal et al. 2004), the loss of interactions of bacteria with other microbes and animal cells (Nocker et al. 2007), producing inaccurate ecological results because of the complexity of gut microbiota, and being a time-consuming and laborious task (Zoetendal et al. 2004). With the rapid advances in DNA techniques, more revolutionary technologies characterized by culture-independent, high throughputs with high efficiency and rapid computation have become available for studying gut microbiota. However, there is always a need for pure bacterial cultures for comprehensive studies of the physiology of particular bacteria for better understanding of the gut microbiome.

4.3 Culture-independent Techniques

Culture-independent methods mainly include newly developed DNA techniques including PCR-based DNA profiling, quantitative PCR (qPCR), fluorescent *in situ* hybridization (FISH), DNA sequencing, and DNA microarray (Gong & Yang 2012), which do not require culturing of bacteria, but mainly target molecular markers such as the 16S ribosomal RNA (16S rRNA) gene (Sekirov et al. 2010) and the 60kDa

chaperonin protein subunit gene (*cpn60*) (Hill et al. 2004; Johnson et al. 2015). These techniques can overcome the disadvantages of culture-dependent techniques listed above. With the use of culture-independent techniques, the bacterial diversity and community structure of poultry and swine gut microbiota have been extensively investigated both qualitatively and quantitatively since the initial studies of sequencing 16S rRNA genes (Gong et al. 2002; Park et al. 1995; Zhu et al. 2002). For example, by using DNA profiling and qPCR assays, Gong et al. (2008) found that both postweaning and dietary treatments caused a shift in gut microbiota composition.

16S rRNA-targeted oligonucleotide probes (*in situ* hybridization) were used to investigate the composition of the microbiota of cecal content and mucus from broiler chickens (Zhu & Joerger 2003). Smidt's group studied the effects of resistant starch on gut microbiota composition in pigs using microarray techniques (Haenen et al. 2013). Torok et al. (2011) identified and characterized potential performance-related gut microbiota composition in broilers using high-throughput DNA sequencing.

It should be noted that similar to many other techniques, each culture-independent technique has its own advantages and drawbacks. PCR-based DNA profiling techniques, including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single-strand conformation polymorphism (SSCP), and terminal restriction fragment length polymorphism (T-RFLP) provide a quick but semi-quantitative global assessment of microbiota in multiple samples. In addition, the techniques lack precision for bacterial identification at the lower taxonomical levels, making it difficult to provide robust and accurate quantitative analyses. FISH is commonly used to study the composition of the gut microbiota (Józefiak et al. 2010; Zhu & Joerger 2003), and can provide information on the spatial distribution of target species in the gut (Józefiak et al. 2010). Additionally, FISH can detect uncultured bacteria without enrichment. However, its sensitivity is relatively low. The sequence of target genes must be available in the database and only a few probes can be used simultaneously. DNA microarrays were widely used for high-throughput, quantitative, and systematic studies of gut microbiota (Carey et al. 2007). However, their use has been significantly reduced following recent advances in low-cost DNA sequencing techniques. The use of high-throughput sequencing to study the gut microbiota and its functions has become an obvious trend in the field. Therefore, this chapter mainly discusses the applications of qPCR and DNA sequencing methods for studying animal gut microbiota.

4.3.1 Quantitative Polymerase Chain Reaction (qPCR)

The qPCR is a popular technique for absolute and relative quantification of bacterial populations within animal gut microbiota (Gong et al. 2008; Ott et al. 2004; Snel et al. 2002). The quantification is achieved by detecting the fluorescence emitted by fluorescence-labeled group- or strain-specific probes or with a non-sequence-specific DNA-binding dye (SYBR® green) during the PCR amplification process of a target gene. The fluorescence is monitored each cycle (real-time), as opposed to exclusively at the endpoint as occurs in traditional PCR.

The qPCR can be used for absolute or relative quantification of bacterial populations (Gong et al. 2008; Ott et al. 2004). In absolute quantification, a standard curve (internal) with known amounts of DNA or copy numbers of a target gene can serve for

enumeration of a specific bacterium or bacterial group in a sample (Hill et al. 2005; Ott et al. 2004). In relative quantification, the $2^{-\Delta\Delta C_t}$ method is used to determine the relative abundance (fold changes) of a target bacterial population compared to a baseline (Livak & Schmittgen 2001). The advantages of the qPCR assays include:

- greater sensitivity and accuracy compared to DNA microarray
- automated process
- no need for time-consuming post-PCR procedures, such as gel electrophoresis, staining, and visualization with fluorescence set-up
- reducing contamination in contrast to traditional PCR analyses (Gong & Yang 2012).

However, qPCR assays have several drawbacks including:

- normally limited to the measurement of one or a few target bacterial species per assay
- the target gene sequence needs to be available in advance
- unable to provide a global assessment of bacterial communities (Carey et al. 2007).

Recently, digital PCR (dPCR) was introduced for absolute quantification of bacterial populations within the gut microbiota (Looft et al. 2014a, b). The quantification is achieved by dilution and segregation of the specimen into thousands of parallel PCR mixtures (Baker 2012). Each reaction mixture contains either one or zero copies of the template. With massively parallel PCR, the number of wells with endpoint positivity for an amplification product is a direct estimation of the copy number of templates present in the original samples (Buchan & Ledebor 2014). The advantages of dPCR include:

- no need for calibration or generating a standard curve
- accurate quantification not limited to the log-linear phase of the PCR.

However, the limitations of dPCR are similar to those encountered with traditional qPCR (Baker 2012). Currently, there are several dPCR systems including Quant Studio 3D digital PCR, Bio-Rad Laboratories, and Rain Dance.

4.3.2 DNA Sequencing

DNA sequencing is a commonly used method to identify the composition and diversity of gut microbiota in poultry and swine (Burrough et al. 2015; Gong et al. 2002; Haenen et al. 2013; Hill et al. 2005; Leser et al. 2002). The diversity of the human gut microbiota was thoroughly characterized originally using full-length 16S rRNA gene sequences (Eckburg et al. 2005). However, this approach has largely been limited due to inherent high costs, although it is able to maximize the taxonomic resolution. Due to the quick development of affordable high-throughput sequencing techniques, this method has become popular for studying the gut microbiota, which has greatly enhanced our understanding of the gut microbiome of poultry and swine (Pajarillo et al. 2015; Park et al. 2015). Several high-throughput sequencing techniques have been developed and are widely used, including the Illumina (e.g., HiSeq, MiSeq), Roche 454 GS FLX+, SOLiD 5500 series, and Ion Torrent/Ion Proton platforms (Zhou et al. 2015). Illumina produces short paired-end reads (max. 2×300 bp), whereas 454 produces long read length (600–1000 bp) (Deutsch et al. 2015). As shown in Table 4.1, the high-throughput sequencing techniques produce shorter sequence reads compared to Sanger sequencing and may have higher error rates (~ 0.1 –15%), depending on the technology used

Table 4.1 Comparison of the characteristics of common DNA sequencing technologies.

	Sanger sequencing	454 sequencing	MiSeq (Illumina)	SOLiD	Pacbio RSII
Sequence chemistry	Dideoxy terminator sequencing	Pyrosequencing	Polymerase-based sequence-by-synthesis	Ligation-based sequencing	Single molecular real-time sequencing
Amplification approach	In vivo amplification	Emulsion PCR	Bridge PCR	Emulsion PCR	No amplification
Time per paired end run	2–3 h	10–23 h	4–56 h	4–10 days	4 h
Read length	800 bp	600–1000 bp	2 × 300 bp	50–75 bp	50% of reads > 10 kb
Output per run	0.1 Mb	100–900 Mb	15 Gb	6–15 Gb	5 Gb
Cost per Mb	\$500	\$9–20	\$0.20–0.30	\$0.07–0.13	\$0.4
Accuracy	99.999%	99%	99.9%	99.99%	85%

Adapted from Gong and Yang (2012). Some information was from company sources on the date of online searching (6 June 2016). Reproduced with permission of Elsevier.

(Goodwin et al. 2016; Kircher & Kelso 2010). Sanger sequencing can yield a maximum of approximately 6 Mb of DNA sequences per day at a cost of \$500 per 1 Mb. In contrast, high-throughput sequencing methods can produce a maximum of 750–15 000 Mb per day at a cost of \$0.07–20 per 1 Mb.

The high-throughput sequencing techniques have several advantages, including higher throughput efficiency, increased sensitivity, and simultaneous sequencing of multiple samples, thus providing more comprehensive information (Sekirov et al. 2010). However, extensive bioinformatics analysis is required for processing the enormous amount of sequence data generated by these techniques.

4.4 Tools for Functional Studies of Gut Microbiota

Our knowledge about bacterial diversity in the animal gut has been increased dramatically with the development of different molecular techniques, most notably high-throughput sequencing (Pajarillo et al. 2015; Park et al. 2015). With the use of culture-independent techniques, most studies have shown that the composition of the gut microbiota varies under different physiological conditions and in response to diets. However, these studies have provided minimal insight into the functional characteristics of the microbiota and their influences on the host (Sekirov et al. 2010). In particular, there is a challenge to establish a specific link between particular microorganisms and animal health and nutritional status.

In order to fully understand the contribution of bacterial groups to the host, several molecular techniques have been developed, such as stable isotope probing (SIP) (Dumont & Murrell 2005) and insertion sequencing (INSeq) (Goodman & Gordon 2010). The SIP technique involves the use of labeled substrates (i.e., starch) that are

highly enriched with a stable isotope, such as ^{13}C , and the identification of active microbes by the selective recovery and analysis of isotope-labeled cellular components, such as DNA and RNA (Dumont & Murrell 2005). ^{13}C -labeled DNA or RNA (heavy DNA or RNA) can be separated from unlabeled DNA or RNA (light DNA or RNA) by density-gradient (isopycnic) ultracentrifugation. Retrieved DNA or RNA from the target group of microbes can then be used in further taxonomic and functional gene analyses by gene probing and sequencing (Kovatcheva-Datchary et al. 2009). The INSeq technique uses transposons with an identifiable DNA “barcode” to introduce mutations into tens of thousands of bacteria (Goodman & Gordon 2010; Goodman et al. 2009). After introduction and establishment of the transposon-mutated strains in the guts of animals, e.g., gnotobiotic animals, genomic DNA from the gut microbiota is extracted, digested, and separated by polyacrylamide gel electrophoresis (PAGE). Transposon-sized fragments are then recovered, ligated with sequencing adapters, and subsequently subjected to a limited number of cycles of PCR amplification with transposon-specific and adaptor-specific primers. The PCR amplicons are sequenced with a massively parallel sequencing technique and the relative abundance of each sequence can be used to determine the relative abundance of the corresponding transposon mutant in the microbiota. The genes whose functions are required for fitness *in vivo* can thus be identified by comparison of these relative abundances in input versus output of microbial populations.

Nevertheless, “omics” technologies are the most powerful tool to date, which have been used to study gut microbiome and microbe–host interactions (Simon & Daniel 2011) and demonstrate tremendous potential in revealing microbiota functions. The following discussion will mainly be focused on the applications of “omics” and animal models in functional studies of gut microbiota.

4.5 “Omics”

High-throughput sequencing techniques and other affordable approaches have resulted in the development of “omics” technologies, including metagenomics, metatranscriptomics, metaproteomics, and metabolomics, which allow us to analyze the DNA, mRNA, proteins, and metabolites of the gut microbiota and unravel the complex networks of functions among gut microbes (Deusch et al. 2015).

4.5.1 Metagenomics

Metagenomics has become a commonly used approach to comprehensively study both the structure (composition) and function of gut microbiota by analyzing sequence information from the collective genomes of all members of the microbial community (microbiome) in poultry and swine (Boissy et al. 2014; Singh et al. 2013). With this technique, total DNA is extracted from fecal or gut digesta samples and whole-genome shotgun sequencing is used to characterize the microbiome (Gill et al. 2006; Kurokawa et al. 2007). The predicted metaproteome, based on fragmented sequence data, can be used to identify the functional contributions and biological roles of gut microbiota in animal health and nutrition. The main advantages of metagenomics include its high throughput and ability to identify new functional genes (Hess et al. 2011). However,

major drawbacks of metagenomics include that it is limited to prediction of potential functions, and it is difficult to distinguish the DNA of live versus dead cells (Gong & Yang 2012). These limitations can be overcome by metatranscriptomics and metaproteomics (Wang et al. 2009; Xie et al. 2013).

4.5.2 Metatranscriptomics

Metatranscriptomics relies on the high-throughput sequencing of RNA (RNA-Seq) isolated directly from microbial populations, as opposed to the DNA content which is analyzed in the metagenomics approaches (Sekirov et al. 2010). Metatranscriptomics can be used to obtain functional insights into the gut microbiota as well as information about how changes in the host and diet induce community-wide changes in gene expression. RNA-Seq is a recently developed approach for mapping gene expression using deep-sequencing technologies (Wang et al. 2009). RNA-Seq has been successfully used to profile the gene expression of gut microbiota in swine (Bello-Ortí et al. 2015; Poroyko et al. 2010). The advantages of RNA-Seq include high throughput, high sensitivity, being quantitative and the ability to characterize both known and unknown gene transcripts (Wang et al. 2009). With advances in high-throughput sequencing and the declining sequencing cost, RNA-Seq holds particular promise for studying the function of gut microbiota. However, the drawback of RNA-Seq is the cDNA fragment bias (Roberts et al. 2011), and the analysis of the massive amounts of data generated by large-scale RNA-Seq is still a challenge (Zhao et al. 2016).

4.5.3 Metaproteomics

Metaproteomics is a function-based approach to identify key microbial functions in the whole community (Ram et al. 2005; Wilmes & Bond 2004). This technique needs proteomic measurements with several features including high-throughput processing, sensitive protein/peptide detection, large dynamic range and accurate mass measurements, and ability to structurally characterize peptide sequences (Hettich et al. 2013).

Basically, microbial proteins are extracted from the whole community and then separated by one-dimensional PAGE (SDS-PAGE) or two-dimensional PAGE (2D-PAGE). After separation, the target proteins are recovered from the gels, digested by proteases, and then identified by mass spectrometry (MS) and de novo sequencing. Peptide sequences can then be searched with BLASTP at <http://www.ncbi.nlm.nih.gov/BLAST>. However, a MS-based short gun proteomics approach was proven to be able to detect and identify the diversity and abundance of proteins contained within the gut metaproteome without the need for gel-based separation or de novo sequencing. The whole proteome was digested to peptides by proteases and the peptides were separated by liquid chromatography and infused directly into rapidly scanning tandem mass spectrometers (2D-LC-MS/MS) through electrospray ionization (Hettich et al. 2013).

The advantages of metaproteomics are that microbial protein expression levels can be directly measured, and new functional genes can be identified. Metaproteomics has been successfully used to analyze the complex proteome of the poultry gut microbiota (Tang et al. 2014). However, there are several drawbacks for metaproteomic analyses, including uneven species distribution, purification and detection capabilities for microbial proteins that have a low abundance, and the large genetic heterogeneity of

proteins (Hettich et al. 2013; Tang et al. 2014). Nonetheless, with the development of proteomic techniques, metaproteomics shows a vast potential to link the composition and activities of gut microbiota with the functions of the microbiome.

4.5.4 Metabolomics

Metabolomics is another “omics” technique used to investigate the function of gut microbiota through determination of microbiota and host metabolite profiles with nuclear magnetic resonance (NMR), mass spectroscopy, and other analytical methods (Gong & Yang 2012; Turnbaugh & Gordon 2008). With this technique, multiple small metabolites present in a given sample can be simultaneously analyzed. Metabolomics has been used to analyze the effect of antibiotics or nutrition on the gut microbiota metabolome in feces comprising a variety of different compounds, including short-chain fatty acids (SCFA, e.g., butyrate), amino acids, organic acids (e.g., succinate), trimethylamine, uracil, ethanol, phenolic acids, glycerol, cholate, glucose, and lipid components in the mouse and human (Jacobs et al. 2008; Xie et al. 2013; Yap et al. 2008). The major challenge of metabolomics is the difficulty of analyzing all the metabolites present in a sample due to the high complexity of the gut microbiota.

4.6 Animal Models

Several kinds of animal models, including gnotobiotic and transgenic/knock-out animals, have been developed in recent decades that can be used to investigate the dynamic, ecological diversity and functions of gut microbiota to provide insights into the complexities of the molecular interactions among host, diet, and microbiota (Bry et al. 1996; Heinritz et al. 2013; Mahowald et al. 2009; Samuel & Gordon 2006).

Gnotobiology is the colonization of germ-free (GF) animals with select microbial species/strains or entire bacterial populations. Gnotobiotic animals include GF animals, mono/bi-associated animals, poly-associated animals, and human microbiota-associated animals (Sekirov et al. 2010). The major advantage of GF animal models is the ability to provide a simplified experimental system to study specific members of the gut microbiota. However, the major drawback of the GF animal model is that it might not reflect true changes in the physiology of normal animals since it overlooks the interactions among microbes in the gut microbiota. Hence, care needs to be taken when interpreting results from GF animals. In the mono/bi-associated animal models, only one or two commensal species or probiotics are used to allow the investigation of host–microbe interactions in a simplified ecosystem. In poly-associated animal models, animals are colonized with a standardized defined microbiota referred to as a “cocktail.” Although there are some interactions among the host and a small number of microorganisms in the poly-associated animal model, this model cannot be expected to reflect the complete assortment of host–microbiota interactions. Due to physiological similarity between humans and pigs, human microbiota-associated pig models, colonized with human microbiota, have been used to study how the human gut microbiota interacts with the host as well as how dietary changes can impact the composition and functionality of gut microbiota (Wang & Donovan 2015).

Transgenic/knock-out animal models can be used to discover host genes with the functions of “checking” the gut microbiota, thus leading to elucidation of interactions and mechanisms underlying microbiota functions (Madsen et al. 1999; Malo et al. 2010; Spor et al. 2011; Wang et al. 2016). Several genes have been identified that have significant effects on the gut microbiota (Madsen et al. 1999; Vijay-Kumar et al. 2010). For example, IL-10 knock-out mice decreased the levels of colonic *Lactobacillus* spp. and increased colonic mucosal adherent and translocated bacteria (Madsen et al. 1999). Toll-like receptor 5 (TLR5)-knock-out mice had a decrease in the abundance of certain Bacteroidetes and Lachnospiraceae phylotypes, which led to the development of host metabolic diseases (Vijay-Kumar et al. 2010).

4.7 Bioinformatics

The gastrointestinal (GI) tract is home to a complex and diverse ecosystem of microbes. It is important to note that when we describe the microbiome using “omics” methodologies, where microorganisms are not directly observed/assessed, we often use the term “operational taxonomic units” (OTUs) instead of “species.” In this context, an OTU is defined as a cluster of sequence reads with a given similarity and is assigned to a taxonomical level. For instance, sequences with 97% similarity at the 16S rRNA gene correspond approximately to one species in bacteria.

Different ecological measures, such as richness, abundance, evenness, and diversity, are used to describe and compare microbiota among animals and across treatments (Caporaso et al. 2010). Whereas richness refers to the number of OTUs present in a given community, evenness and diversity also take the abundance of individual OTUs into account. To compare microbiota composition between samples, beta-diversity is usually measured by calculating phylogenetic (e.g., weighted and unweighted UniFrac) or non-phylogenetic-based (e.g., Bray–Curtis) distances matrices.

Bioinformatics in conjunction with advanced multivariate statistical methods have significantly revolutionized the field of microbial ecology. Many bioinformatic tools have been developed recently, such as UniFrac and Python Nearest Alignment Space Termination (PyNAST) (Caporaso et al. 2010; Lozupone & Knight 2005). UniFrac is a phylogenetic method for comparing microbial communities (Lozupone & Knight 2005). PyNAST is a flexible tool for aligning sequences to a template alignment which has become a popular tool for adding new 16S rRNA gene sequences to existing 16S rDNA alignments (Caporaso et al. 2010).

With advances in high-throughput sequencing technologies, thousands of microbial signature sequences have been generated from the gut microbiota and many more are expected to come. The development of advanced computational strategies and databases for phylogenetic comparisons, functional annotations, binning of sequences, phylogenomic profiling, and metabolic reconstructions has become a major focus of bioinformatics research communities (Sun et al. 2010). Several web-based metagenomic annotation platforms have been developed as well, such as the IMG/M server (Markowitz et al. 2008), the metagenomics RAST (mg-RAST) server (Meyer et al. 2008), and the JCVI Metagenomics Reports (METAREP) (Goll et al. 2010). Quantitative insights into microbial ecology (QIIME; <http://qiime.org/>) is an open-source software

recently developed to support a wide range of microbial community analyses, such as choosing OTUs, sequence alignment, inferring phylogenetic trees, and phylogenetic and taxon-based analysis of diversity within and between samples (Caporaso et al. 2010; Munyaka et al. 2016). UPARSE is a very accurate and high-throughput clustering method for generating clusters (OTUs) from reads of marker genes and is available at <http://drive5.com/uparse/> (Edgar 2013; Sun et al. 2015).

Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt; <http://picrust.github.io/picrust/>) is a recently developed computational approach to predict the functional composition of a metagenome using marker gene data and a database of reference genomes (Burrough et al. 2015; Langille et al. 2013). STAMP is a graphical software package that provides statistical hypothesis tests and exploratory plots for analyzing taxonomic and functional profiles and is available at <http://kiwi.cs.dal.ca/Software/STAMP> (Parks et al. 2014). Several protein and nucleotide databases are also available for microbial function analyses, such as the Gene Ontology (GO) database (Ashburner et al. 2000), the Clusters of Orthologous Groups (COG) database (Tatusov et al. 2001), SEED (Overbeek et al. 2014), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2008), NCBI (Sayers et al. 2009), and Pfam (Finn et al. 2010) databases.

Further development of bioinformatics tools and databases to keep pace with the development of high-throughput sequencing techniques will be critical for enhancing our ability in microbiome research and for fully understanding the gut microbiota and its functions.

4.8 Application in Poultry and Swine Research

Bacterial communities colonizing the animal gut are essential for animal physiology and immune development, both of which have profound impacts on animal nutrition, health, and growth. Thus, a full understanding of chicken and pig gut microbiomes and their response to environmental factors, including diets, would promote both basic and applied research to enhance the production efficiency of poultry and swine (Kim & Isaacson 2015; Oakley et al. 2014). The integrated use of “omics” combined with advanced bioinformatics and statistical approaches would enable more comprehensive studies and lead to a better understanding of chicken and pig gut microbiomes. The information could be used for targeted microbiota modulation and restoration of the microbiome.

4.8.1 Understanding of Gut Microbiota and Its Development

The animal gut is generally considered to be relatively sterile prior to birth (or hatch in poultry), but it rapidly becomes colonized with microbes from the environment, diet, and parents (Kim & Isaacson 2015). The colonizing microbes subsequently develop into a highly diverse microbiota and the microbial density and composition can vary among different gut compartments. Our understanding of gut microbiota composition and function has been significantly improved by the application of molecular and “omics” methodologies in combination with bioinformatics and statistical tools. For example, Firmicutes and Bacteroidetes were shown to be the most dominant phyla in pigs

regardless of age, followed by Proteobacteria, Actinobacteria, and Spirochaetes (Kim et al. 2012, 2015; Lu et al. 2014; Slifierz et al. 2015; Zhao et al. 2015). Nevertheless, there were some dynamic shifts in the composition of gut microbiota with age. The phylum Proteobacteria was found to be more abundant in the pig gut prior to weaning (Zhao et al. 2015). In general, the gut microbiota becomes increasingly stable during animal growth and consequently more resistant to dietary perturbations (Kim & Isaacson 2015). This explains why piglets are more susceptible to pathogen infection than adult pigs. It also demonstrates the importance of modulating the gut microbiota of young animals in order to achieve a healthy microbiota for better animal performance.

4.8.2 Modulation of Gut Microbiota by Nutrients

The combined use of DNA sequencing data and bioinformatics tools has possibly allowed us to identify nutrients that can elicit shifts in the gut microbiota, linking their positive effects to animal gut health. Nutrients such as dietary fermentable fiber, resistant starch, wheat and barley, corn, and sorghum have all been studied extensively for their effects on the gut microbiota in poultry and swine (Burrough et al. 2015; Haenen et al. 2013; Lunedo et al. 2014; Sun et al. 2015; Umu et al. 2015; Walugembe et al. 2015; Weiss et al. 2016).

Using 16S rRNA gene sequence data, Burrough et al. (2015) reported that changes in colonic microbiota in pigs fed distillers dried grains with solubles may predispose them to the development of colitis. Using PICRUSt predictions, the authors showed that genes associated with mucin degradation and toxin formation were increased. Metzler-Zebeli et al. (2015) studied the impact of enzymatically modified starch on the microbiota of cecal digesta using MiSeq Illumina sequencing of 16S rRNA genes in combination with qPCR for specific bacterial targets. Their results revealed that enzymatically modified starch reduced cecal short-chain fatty acids through changing the cecal microbiome and was less degradable by cecal bacteria than the control starch indicated by the imputed metabolic functions of the cecal microbiome. Using Illumina sequencing and qPCR, Sun et al. (2015) reported that long-term consumption of resistant starch might result in both positive and negative effects on the gut health of pigs through significantly decreasing the relative abundance of *Clostridium* and increasing proinflammatory cytokine IL-1 beta gene expression, respectively. Using 454 pyrosequencing, Levesque et al. (2014) found that diet complexity had a prolonged impact on the ileal mucosa bacteria profile.

4.8.3 Modulation of Gut Microbiota by Antibiotics

There is an urgent need to develop viable alternatives to in-feed antibiotics due to the threat to public health potentially posed by the use of antibiotics as animal growth promoters (Yang et al. 2015). To identify and implement effective alternatives to in-feed antibiotics, it remains critical to understand the effects of antibiotics on the composition and functions of animal gut microbiota (Allen et al. 2011). An early study with the PCR-DGGE profiling method found that supplementation of lincomycin (110 mg/kg feed) reduced bacterial diversity of ileal microbiota in piglets compared with a basal diet over a 4-week period (Gong et al. 2008). While the DGGE method allowed quick

visualization of large shifts in the microbiota, few subtle changes were identified due to the limitations of the method. This barrier was later overcome by the application of higher resolution and high-throughput sequencing techniques, which has led to a better understanding of gut microbiota changes in response to antibiotic supplementation (Allen et al. 2011; Kim et al. 2012; Looft et al. 2012, 2014a, b). By taking a metagenomic approach, including pyrosequencing of 16S rRNA genes, Allen et al. (2011) revealed that supplementation of ASP250 (chlortetracycline and sulfamethazine, each at 100 mg/kg feed; penicillin at 50 mg/kg feed) changed both fecal bacterial microbiota and fecal bacteriophage community in pigs. The diversity of gut microbiota was also reduced by ASP250, and more phage integrase genes were observed, indicating that antibiotics may have activated prophages (Allen et al. 2011). Moreover, with the same techniques, Looft et al. (2012) reported that in-feed ASP250 increased the diversity of antimicrobial resistance genes, indicating the risk of using in-feed antibiotics in increasing antimicrobial resistance. Holman and Chénier (2015) also reported their trial results obtained using high-throughput sequencing techniques that adult pigs appeared to exhibit fewer changes in their gut microbiota compared with younger piglets in response to in-feed antibiotics, which may explain why antibiotics tend to be most effective in improving feed efficiency and growth rate at early growth stages of animal development.

4.8.4 Modulation of Gut Microbiota by Non-medicated Feed Additives

The use of non-medicated feed additives, such as enzymes, prebiotics, probiotics, organic acids, and sweeteners, has become a topic of interest in animal production because they have the potential to improve gut health, feed utilization efficiency, and growth performance. With advances in DNA techniques, it has been confirmed that beneficial effects of feed additives are at least partially associated with the modulation of gut microbiota (Daly et al. 2016; Huang et al. 2015; Jiao et al. 2015; Park et al. 2016; Ptak et al. 2015; Roto et al. 2015; Ruiz et al. 2015; Thibodeau et al. 2015). For example, by analysis of 16S rRNA gene sequences, Huang et al. (2015) reported that sodium butyrate caused a striking decrease in Lactobacillaceae and a noticeable increase in Clostridiaceae in the ileal and colonic lumen, as well as increases in Ruminococcaceae, Lachnospiraceae, and Bacteroidetes in the colonic lumen, supporting the role of sodium butyrate in improving performance and decreasing diarrhea incidence in weaned piglets. Ptak et al. (2015) used the FISH method to investigate the effect of phytase on the gut microbiota and found that the addition of phytase increased the total numbers of bacteria, as well as *Lactobacillus* spp. and *Enterococcus* spp., which may have been linked with the levels of phosphorus and calcium in the diet. Using MiSeq Illumina sequencing of the V4 region of 16S rRNA genes, the effects of yeast cell wall-based prebiotics on gut microbiota composition in chickens were determined (Park et al. 2016). The results indicated that prebiotics exhibited limited impact on the microbial abundance at the phylum level except for increasing the proportion of Proteobacteria. Non-nutritive artificial sweeteners can improve feed palatability but not nutrition value. More recently, it has been reported that non-nutritive artificial sweeteners can induce the growth of health-promoting gut *Lactobacillus*. The underlying mechanisms are still not clear, but possibly linked with membrane receptors in bacterial and/or gut epithelium cells (Daly et al. 2015, 2016).

The effect of different individual probiotics on animal gut microbiome has been extensively investigated (Molnár et al. 2011; Nakphaichit et al. 2011; Pascual et al. 1999).

For example, it has been shown that several *Lactobacillus* strains decreased the population of *Salmonella*, *Campylobacter*, and some other non-beneficial bacterial groups in the chicken gut (Nakphaichit et al. 2011; Pascual et al. 1999). Similarly, dietary supplementation of *Bacillus subtilis* significantly decreased *E. coli* populations in the ileum of chickens (Molnár et al. 2011). The main advantage of single probiotic supplementation is much easier to apply in animal production both practically and regulatorily, while the disadvantage can be less powerful to restore the microbiota. The effect of defined multispecies probiotics has also been investigated in animals. For example, a multispecies mixture of probiotics containing *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici*, *Lactobacillus salivarius*, and *Lactobacillus reuteri* from chicken gut decreased cecal coliform populations (Mountzouris et al. 2010). Identification of the shifts of gut microbiota composition has become an important approach not only to possibly elucidate the mechanism of beneficial effects of feed additives, but also to guide the selection of proper feed additives for poultry and swine production.

4.9 Integrated Approaches for Studying Gut Microbiome

The GI tract harbors an extremely complex microbial community. Thus, it is necessary to integrate different approaches to study the gut microbiome. Integrated approaches have been used in human gut microbiome research that can serve as the reference for future studies on food animals. Therefore, this section lists several good examples to illustrate how different approaches are integrated to achieve research goals.

The first example is the study by Hsiao et al. (2014) who used 16S rRNA gene sequencing (Copro-Seq) to investigate the fecal microbiota collected during both the acute diarrheal phase caused by *Vibrio cholerae* and the recovery phase afterwards in a cohort of Bangladeshi adults. They found that the recovery was characterized by a pattern similar to the assembly/maturation of the gut microbiota in healthy Bangladeshi children. In order to identify the underlying mechanisms, a gnotobiotic mouse model with an artificial community of 14 sequenced human gut bacterial species was used and one of the species, *Ruminococcus obeum*, was consistently increased in *V. cholerae*-infected mice. With further mono- and co-colonization studies, *R. obeum* was confirmed to have the ability to restrict *V. cholerae* colonization. Using metatranscriptomic analyses of fecal samples, the authors found that *R. obeum* LuxS (autoinducer-2 (AI-2)) synthase and AI-2 production increased significantly following *V. cholerae* invasion and the *R. obeum* AI-2 increase caused quorum sensing-mediated repression of several *V. cholerae* colonization factors through a novel pathway that does not depend on the *V. cholerae* AI-2 sensor, LuxP. Moreover, using ultra-performance liquid chromatography mass spectrometry (UPLC-MS) to characterize bile acid profile in the gnotobiotic mice, they identified that fecal levels of taurocholic acid affected *V. cholerae* gene regulation and that *R. obeum* and/or the other 13 members of the community suppressed the taurocholic acid levels.

The second example is the study by Buffie et al. (2014) on the precision microbiome reconstruction to restore bile acid-mediated resistance to *Clostridium difficile*. *C. difficile* is a major cause of antibiotic-induced diarrhea, which can greatly increase morbidity and mortality in hospitalized patients. However, which gut bacteria could provide resistance to *C. difficile* infection and how the in vivo inhibition works were unclear. Using integrated approaches, including 16S rRNA gene sequencing, bioinformatic analyses, molecular

physiological studies of target bacteria, and a mouse model, the authors revealed that the use of antibiotics did not change the bacterial diversity of gut microbiota, but the microbiota composition. They identified several bacterial species shared between murine and human gut microbiota which could inhibit *C. difficile*. Among these identified bacteria, *C. scindens* has the unique characteristic of producing enzymes crucial for secondary bile acid synthesis. Further studies by inoculating animals with *C. scindens* and the use of different *C. difficile* inhibitors and a bile acid sequestrant confirmed that the *C. scindens*-mediated inhibition of *C. difficile* was associated with secondary bile acid synthesis from primary bile acids. Bile acids are used by *C. difficile* spores as signal molecules in the gut to start their germination.

Recently, Chassaing et al. (2015) reported that emulsifiers (carboxymethylcellulose (CMC) and polysorbate-80 (P80)) did not affect the total levels of fecal bacteria in wild-type, *IL10*^{-/-}, and *TLR*^{-/-} mice. Instead, the emulsifiers led to more than a two-fold increase in bacteria adhering to the colons of the wild-type and *IL10*^{-/-} mice. Moreover, their investigations using 16S rRNA gene sequencing, followed by phylogenetic analysis, and the use of unweighted UniFrac algorithm to compare bacterial community structure revealed that both CMC and P80 dramatically altered microbiota composition in both fecal and mucosa-associated microbiota. These changes in the microbiota were likely responsible for the observed inflammation and metabolic changes. The conclusion was further supported by results from the microbiota transfer experiment where the microbiota from emulsifier-treated mice was transferred to emulsifier-untreated germ-free mice which induced low-grade inflammation, increased adiposity, and dysglycemia in the recipient mice.

Collectively, as shown in Figure 4.1, the integrations of metagenomics, metatranscriptomics, metabolomics, and the use of gnotobiotic animals with bioinformatics and statistical approaches have been critical for unraveling the roles of the gut microbiome in food animal health and production. Such integrated approaches will continue to be essential in addressing the role of gut microbiota in the health and disease of the human/animal.

4.10 Conclusions and Future Directions

Dietary ingredients have profound influences over microbiota diversity and community structure. The functions derived from microbiota shifts, particularly those beneficial to host animals, are yet to be elucidated. Recent advances in methods for studying the gut microbiota have led to considerable progress in understanding the gut microbiome, including its regulation of the host response through microbiota composition changes. These advances (both methods and knowledge) are highly relevant to research on food-producing animals, as they offer a golden opportunity to study the possible impact of dietary ingredients – that are mediated through the gut microbiota – on animal health and productivity. Like other technologies, the molecular and “omics” technologies as well as bioinformatics and statistical analyses, and different animal models described in this chapter have their own advantages and limitations. An integrated use of these methods combined with appropriate experimental designs remains critical in the search to understand the gut microbiome and its response to modifiable factors, such as diets.

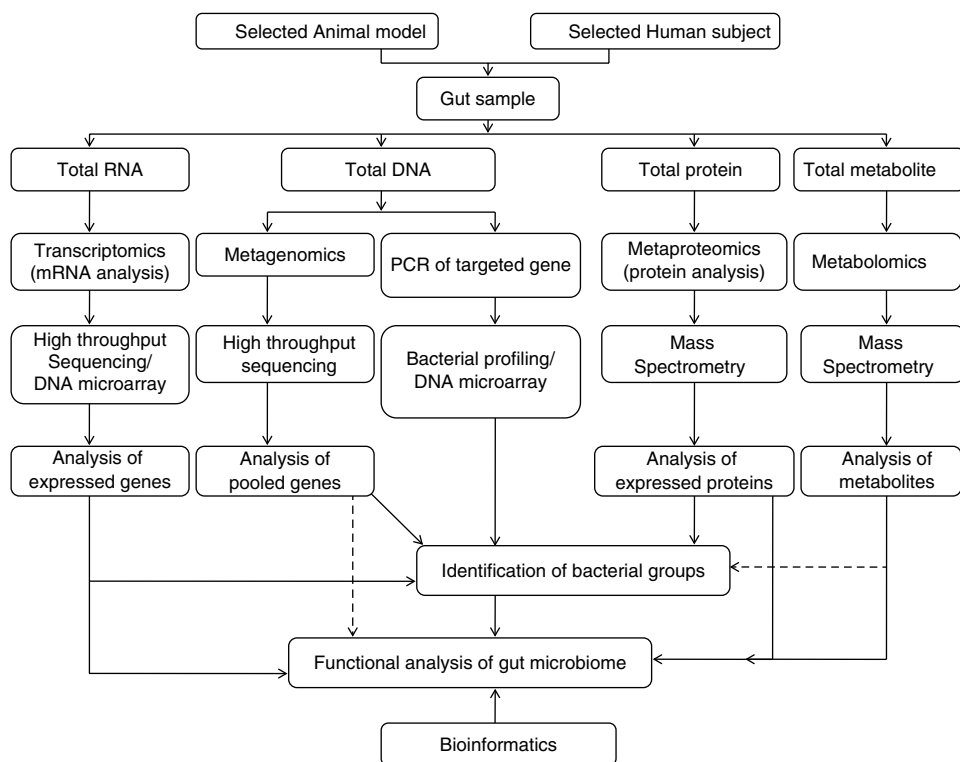


Figure 4.1 Molecular and “omics” approaches for studying the gut microbiome. The culture-independent methods in combination with humanized animal models and bioinformatics provide a more comprehensive view of integrated approaches to study the gut microbiome, including composition, function, and host–microbe interaction. Animal models include gnotobiotic and transgenic/knock-out animals. Human subjects refer to healthy humans or humans associated with acute or chronic diseases. *Source:* Reproduced from Gong and Yang (2012) with permission from Elsevier (License Number: 11606864).

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5

Molecular Techniques for Making Recombinant Enzymes Used in Food Processing

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5.1 Introduction

Enzymes are catalysts produced by living organisms which accelerate numerous natural chemical and biochemical reactions. Compared to normal chemical catalysts that are usually non-specific, each enzyme will only work to facilitate the reactions between very specific substrate molecules. Because enzymes function at lower temperature, they have become an increasingly important tool in modern industry.

The history of enzyme application in food processing can be traced back many centuries. Although not known to people in early times, enzymes were essential for preserving and enhancing the values of foods and beverages, such as cheese and alcoholic drinks. During the 19th and early 20th centuries, several biomolecules that could function as catalysts were mentioned in lab studies. Studies by Swedish scientist Jöns Jacob Berzelius in 1835 and French chemists Anselme Payen and Jean-François Persoz (1833) both mentioned that one type of biomolecule, which they named diastase, could hydrolyze starch into simple sugar (Ullmann 2007). In 1897, Eduard Buchner discovered that yeast extract can convert glucose to ethanol (Bennett & Frieden 1969). However, purification of the first enzyme was not achieved until 1926, by James B. Sumner. A few years later, Dr Sumner crystallized the enzyme urease and won the Nobel Prize (1946). His work formally launched scientific investigation on enzymes, including their commercial applications in modern industry.

At present, many industries, including food manufacturing, textiles, cosmetics, pharmaceuticals, mining, and environmental protection, use enzymes in at least part of their processing. The enzyme market worldwide was estimated to be worth \$8.18 billion in 2015 and is expected to double over the next eight years (Grand View Research 2016). The majority of industrial enzymes are produced by three companies, Novozymes, DuPont, and Roche. At present, about 4000 enzymes are known and characterized. Among these, about 5% (~200) have been commercially applied to different industries. In most of these applications, the original native enzymes were not very efficient in

industrial settings so efforts have been invested to improve the properties of enzymes such as activity, thermal stability, and substrate spectrum.

In this chapter, we summarize the molecular techniques that have been used to produce recombinant enzymes for the food industry. Detailed techniques in the core routes of two strategies will be described first, followed by several common methods used to engineer different enzymes. Considering that the enzymes used in the food industry overlap with those used in other fields, techniques we review here are also applicable to the development of recombinant enzymes used in other industries.

5.2 Molecular Strategies to Produce Recombinant Enzymes Used in the Food Industry

There are two main engineering strategies for enzyme development. One is through directed evolution. In this approach, the genes coding for enzymes of interest are placed in specific environments and allowed to accumulate mutations. The mutants with desirable properties are identified for further analysis and production. The second approach is through targeted/site-directed mutagenesis. For the second approach to work, prior knowledge of the structure and functional properties of the target enzyme as well as appropriate host cells to express the mutated versions of the gene are needed. If our understanding of gene function is limited and recombination techniques are not fully developed, a directed evolution strategy is preferable. The drawback of this strategy is that its success rate is relatively low, with relatively little control by the experimenter. To overcome such problems, multiple cycles of mutation and selection are typically needed in order to achieve the desired goal. In contrast, the rational/semi-rational design strategy provides a clear route to produce enzymes of desired properties. The main processes of these two strategies are presented in Figure 5.1. However, these two strategies are not mutually exclusive and can be applied jointly to develop an efficient enzyme.

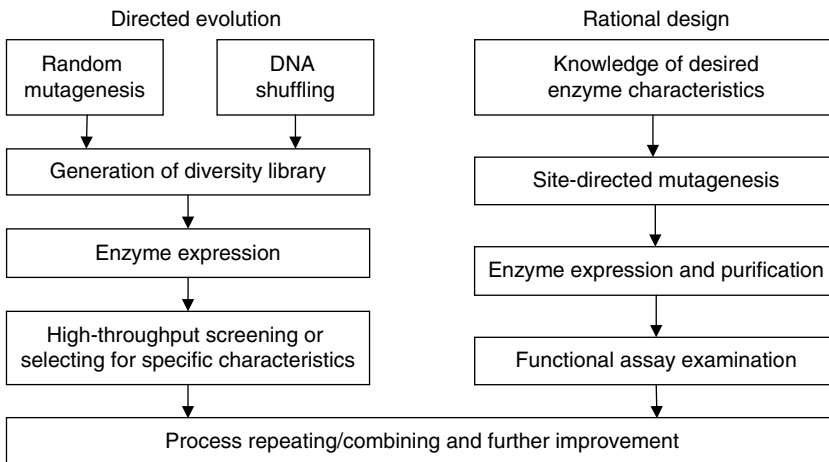


Figure 5.1 Procedural flow sheets of directed evolution and rational design strategies.

5.2.1 Directed Evolution

In the natural environment, spontaneous mutations can happen every time the genome replicates. However, due to the intrinsic proofreading functions of DNA polymerase in the majority of wild-type cells, the rate of these natural mutations is extremely low and not sufficient to generate abundant genetic variations required for selecting enzymes with commercial purposes. Therefore, directed evolution methods are needed, mimicking evolution in nature, and using specific properties as selection pressure to screen and filter the large and diversified gene mutation libraries and to obtain enzymes with the desired characteristics (Packer & Liu 2015). This approach has been used to develop a large number of industrial enzymes. There are two core technical steps in directed evolution: gene diversification and screening/selection.

5.2.1.1 Gene Diversification

Although it is unrealistic to reach all possible mutational types for a typical protein (there are 20^{100} possible sequences for a protein of 100 amino acids long), random mutations of an existing gene can provide a large number of potential variants and some of them may have beneficial properties in industrial applications. This gene diversification process can be approached in two ways: random mutagenesis and DNA shuffling. As the name suggests, the former is a random process and typically involves a mutagen to enhance the genome-wide mutation rate. In contrast, the latter involves randomly mixing and ligating DNA fragments coding for proteins with different *in vitro* properties to produce a large number of recombinant DNA molecules.

Random Mutagenesis

Random mutagenesis is the most straightforward approach to achieve gene sequence diversification with no requirement for structural or mechanistic knowledge of the target enzyme. Traditional random mutagenesis uses chemical agents or physical conditions to damage or increase DNA mutation. These agents include ethyl methane-sulfonate (EMS), nitrous acid, and ultraviolet irradiation. Recently, random mutagenesis through biological means has been developed. For example, the TP-DNA polymerase enzyme from the Φ 29 bacteriophage of the bacterium *Bacillus subtilis* can perform multiple displacement amplification (MDA) *in vivo* and significantly increases the DNA replication error rate to generate diverse gene sequences (Ravikumar et al. 2014).

Compared to the relatively low mutation rate of common *in vivo* mutagenesis, *in vitro* ones typically generate high mutation rates and have the advantages of being easy to implement and control under laboratory and industrial conditions. The most common *in vitro* mutagenesis is the error-prone PCR (ep-PCR). This uses DNA polymerases of low fidelity to amplify target genes with increased Mg^{2+} and additional Mn^{2+} together with unequal dNTPs and dIPT, which usually brings a mutation rate of $10^{-4} \sim 10^{-3}$ per base into encoding sequence (Eckert & Kunkel 1990). In addition, the mutation rate can be controlled by altering reagent concentration, component and processing conditions, such as cycle numbers or initial template concentration. To reach the maximum function of ep-PCR, small improvements and pilot tests are necessary before a formal evolution experiment. However, ep-PCR reactions can lead to biases in library compositions because of the prevalence of GC to AT mutations in such systems. In addition, there may also be a transversion bias of favored base after ep-PCR.

A bacterial lipase from *Pseudomonas aeruginosa* (case 9 in Table 5.2) was successfully modified with this ep-PCR technique and its enantioselectivity (one of the most important enzyme property indexes for industrial application) was increased 25-fold (Liebeton et al. 2000). This technique is also widely used in enzyme development for the baking industry. For example, cyclodextrin glucanotransferases (CGTases) from *Bacillus* spp. (case 11 in Table 5.2), which live optimally at 60°C and pH 6.0, are mainly used to reduce cyclodextrins in starch products. They were mutated by cycles of ep-PCR and gained 15-fold higher hydrolyzing activity than the original enzyme (Shim et al. 2004). Similarly, after modification, an alpha-amylase from *Bacillus licheniformis* (case 3 in Table 5.2) exhibited stronger acid stability than the wild-type enzyme (Liu et al. 2012).

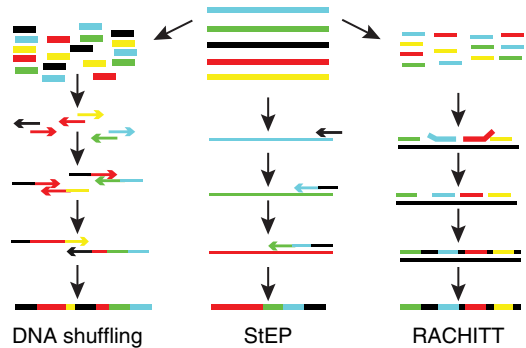
Although random mutagenesis by ep-PCR can generate point mutations at stochastic positions, it is difficult to introduce mutation in a more focused way, such as specific residues involving substrate binding or catalysis. Another mutagenesis approach was developed to deal with this kind of situation: saturation mutagenesis. In this approach, synthetic degenerate oligonucleotides containing one or more mixed populations of nucleotides at a certain position are used to generate all possible mutations at the given site. This process can also incorporate restriction enzymes and gene assemblies to construct a mutagenic library (Gibson et al. 2009; Nour-Eldin et al. 2010). Iterative saturation mutagenesis (ISM) then performs cycles of saturation mutagenesis at target nucleotide sites and can drastically reduce downstream screening. The thermostability of a lipase from *Bacillus subtilis* (case 10 in Table 5.2) was enhanced through ISM and fast screening (Reetz et al. 2006). The downside of this approach is that it can lose the synergistic effects among adjacent sites on enzyme activities (Bougioukou et al. 2009). As a result, targeting iterative mutations involving multiple nucleotide sites may be needed.

DNA Shuffling

DNA shuffling refers to the use of a series of mutant sequences to generate a diversity of recombinant sequences in a gene library. The original parent sequences can be derived from naturally homologous gene families or synthetic ones produced through random mutagenesis methods, like ep-PCR. The shuffling of sequences belonging to homologous gene families is also called family shuffling. DNA shuffling is often applied to accumulate beneficial mutations to one selected sequence. The method was first described by Willem Stemmer when he used DNase I to digest the beta-lactamase gene into smaller fragments and then through a PCR-like process to reassemble them into full length. After selection, a 64-fold increase of beta-lactamase activity was achieved, which was much higher than that obtained through ep-PCR (Stemmer 1994). Later, an enzyme used in the dairy industry (case 13 in Table 5.2), beta-galactosidase, was also successfully modified by this technique to gain higher substrate specificity (Zhang et al. 1997).

Through DNA shuffling, a number of modified or related versions of gene fragments can be developed to meet different purposes. However, family shuffling involving diverged sequences will usually form parental homoduplexes and have a low cross-over rate to generate heteroduplexes and recombinants. A method using single-stranded DNA was reported to overcome this problem and increase the probability of hybrid formation (Kikuchi et al. 2000). Gene family shuffling by random chimeragenesis on transient templates (RACHITT, Figure 5.2) uses a different reassembly strategy instead

Figure 5.2 Comparison of basic schemes of DNA shuffling, StEP, and RACHITT (Neylon 2004). Reproduced with permission of Oxford University Press.



of normal PCR to generate more cross-overs within one shuffling cycle. The key to this method is the formation of transient templates which are generated by hybridizing a temporary scaffold sequence with multiple fragments of another homologous sequences, then filling the gaps and digesting the scaffold (Coco et al. 2001). Nucleotide exchange and excision technology (NExT) utilizes uridine triphosphate (dUTP), an alternative to thymidine, during PCR to help reassemble the fragments in the same way as standard DNA shuffling (Müller et al. 2005). A staggered extension process (StEP, see Figure 5.2) is also applied using a polymerase-catalyzed primer in the PCR annealing step to drive fragments randomly binding to different templates. As a consequence of repeated cycles, full-length genes will contain “pieces” of various parental homologous genes (Zhao et al. 1998). Figure 5.2 illustrates the basic ideas of these technologies.

Other technologies can also be incorporated into DNA shuffling. For example, the RAISE (random insertional-deletional strand exchange mutagenesis) method can help generate random insertion and deletion mutations (Fujii et al. 2006). The method has three steps: digestion by DNase I, random nucleotide introduction at the 3' terminal by deoxynucleotidyl transferase, and PCR extension to full length (Figure 5.3).

5.2.1.2 Screening and Selection Strategies

The screening and selection step is a very important part of directed evolution. This step selects the desirable variant, based on specific phenotypic characteristics, from the variant library generated by the gene diversification methods described above. The phenotypic screening is typically conducted through a phenotype assay for the specific property of interest under a set of conditions, with the aim of identifying the best performer(s). In contrast, selection refers to the use of specific environmental condition(s) to filter out unsuitable individuals. Therefore, the strategies will be separately discussed in two parts: screening and selection.

Screening Strategies

The simplest screening method is to spatially separate the variants. One common approach is to transfer gene libraries into an easily isolated expression host (microorganisms, such as *Escherichia coli*) and dilute them into simple individual colonies or into wells of multiwell plates containing liquid cultures. Such screening can become difficult when dealing with more than 10^4 cultures because of workload. However, it does have advantages in that it is compatible with various assay methods, such as

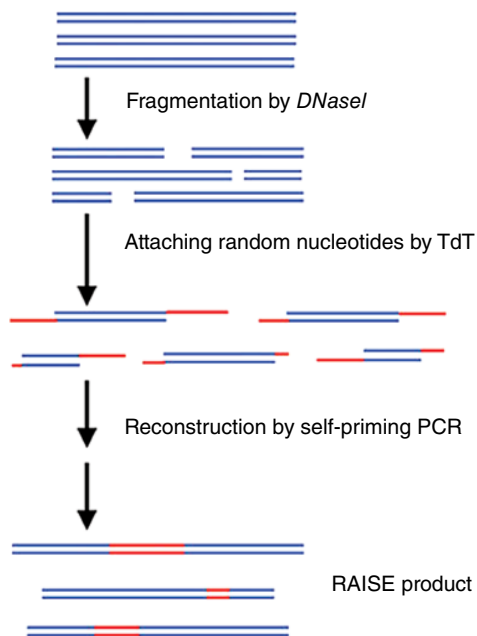


Figure 5.3 Schematic diagram of the RAISE method. Source: Fujii et al. (2006). www.ncbi.nlm.nih.gov/pmc/articles/PMC1380258/. Licensed under Creative Commons CC-BY.

spectroscopy, nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC), gas chromatography (GC), and so on. Among these, optical characteristics are most widely used, like color, fluorescence, and turbidity. These assays can be rapidly conducted, especially for monitoring catalytic activities.

For screening more than 10^4 genetic variants, high-throughput methods are needed and one of those is flow cytometry. The basic technique is called fluorescence-activated cell sorting (FACS), which applies a fluorescent reporter to automatically identify the cells and isolate ones with specific characters. It can sort about 10^8 cells per day (Shapiro 2003). Together with yeast display screening of protein interactions, this technique was recently applied to evaluate enzyme catalytic activities. It enables identification by cell surface epitope that can be specifically bound to a target protein. After cycles of FACS screening, a variant with a 140-fold increase of transpeptidase (protein ligase) activity was obtained (Policarpo et al. 2014; Swee et al. 2015). An advanced version of FACS includes a chip-based microfluidic system, which allows liquid droplets to encapsulate single DNA molecules while the signal of the fluorescent reporter gene still maintains its high quality. This method has proven to be effective for screening cellulase (Ostafe et al. 2014).

If it is not possible to use a cell fluorescent reporter, an alternative plan is screening by an artificial cell-like compartment technique, called in vitro compartmentalization (IVC). IVC was first used by Tawfik and Griffiths (1998) and involved using droplets in water-oil emulsions to encapsulate a single library and a fluorogenic substrate-surrogate reporter that could be picked up by FACS screening.

Selection Strategies

To select the desired protein variant from a library of variants, the essential aspect of selection strategies is to use the different properties of the variant proteins to target immobilization of the desired protein, with the non-desirable ones easily washed away.

This technique requires binding affinity differences among protein variants in each library. To reach this goal, enzymes can be expressed on the surface of the cell or bacteriophage coat and selected by FACS flow cytometry (Boder & Wittrup 1997; Mccafferty et al. 1990). Both methods have an upper capacity of about 10^{10} transformants involved in an intracellular process. Another two extracellular approaches use intermediates to link the expressed proteins and the immobilized molecular together to generate affinity difference among the diversified variants. These are ribosome display and mRNA display. The former utilizes the fact that ribosomes are attached to polypeptides and mRNAs (Hanes & Plückthun 1997) and the latter links expressed proteins with mRNAs by the puromycin analogue (Wilson et al. 2001). Such improved binding affinity methods have been used to screen for improved enzymes, including beta-lactamases (Amstutz et al. 2002).

Another selection strategy is utilizing natural genetic differences in survival under some specific culture conditions, such as resistance to antibiotics. This antibiotic resistance gene can be linked to desirable enzyme activity and works like an expression reporter. Xylose has been used as a selection reporter to distinguish xylose-consuming cells. By the same notion, monosaccharide transporters and xylose isomerase have been used as components in survival selection experiments (Lee et al. 2012; Young et al. 2014).

Limitations of Directed Evolution

As discussed above, directed evolution is a straightforward method that can help obtain significant improvements in enzyme characteristics. The construction of randomly mutated or homologously recombined libraries can be successfully achieved by various methods to be used in future screening for different manufacture conditions (Nannemann et al. 2011). However, there are obvious limitations of directed evolution. For example, high-throughput screening is extremely important to identify the desirable features of an enzyme from a large candidate library, which is also where the main difficulty lies with this approach. Specifically, not all enzyme properties can be effectively tested on a large scale, nor can all the screening and selection methods be implemented in an economic way. On the other hand, when the functional and structural information of a target enzyme is known, it will be more convenient to adopt the rational design strategy (Li et al. 2012).

5.2.2 Rational Design

Fundamentally, the function of an enzyme, such as its substrate specificity, binding affinity, and catalytic efficiency, is determined by its molecular structure. To modify enzymes more effectively than random mutagenesis, we need to take full advantage of the comprehensive knowledge of polypeptide sequences and three-dimensional structures, including identification of the active sites. In return, the improved features need to be assessed by catalysis experiments, which can provide more information about how modified sequence and structure will contribute to specific functional properties.

5.2.2.1 Site-directed Mutagenesis

As mentioned above, the primary requirement of site-directed mutagenesis is a comprehensive understanding of enzyme structure and function, especially how residues contribute to specific catalytic mechanisms. According to the enzyme features, different target sites can be chosen for modification. For example, changes near or in the

active, binding, and catalysis sites can influence substrate specificity, enantioselectivity, and novel activities, while mutations somewhere else are more likely linked to stability and other activity.

Before the start of site-directed mutagenesis, structural information about the original wild-type enzyme is usually required based on high-resolution crystal structure. The general steps used to obtain the crystal structure of an enzyme include:

- the isolation of target enzyme protein with appropriate methods
- purification to remove unwanted metal ions and macromolecules
- the growth of crystals for X-ray diffraction studies
- obtaining and analyzing the crystal structure of the enzyme.

After that, specific plans can be made and implemented to find enzymes with the desired functional properties.

The basic idea behind site-directed mutagenesis approaches is replacement, insertion or deletion of specific amino acid(s) of an enzyme by directly modifying its DNA sequence. For the original ideas and protocols of site-directed mutagenesis, please refer to those summarized by Shortle et al. (1981) at the DNA level and by Mendel et al. (1995) at the amino acid level.

A pioneer method was developed by Kunkel (1985) that could transfer vectors containing site-mutated fragments into double-deficient *E. coli* strains, dUTPase (*dut*) and uracil DNA glycosylase (*ung*), to synthesize heteroduplex DNA between mutated and wild-type strands and allow further replication of the mutated strands. This procedure is widely used in bringing specific mutations to genes by using synthetic oligonucleotides and error-prone polymerase. In the beginning, oligonucleotide-directed mutagenesis was the simplest and most popular technique that utilizes restriction enzyme digestion and M13 vectors to generate point mutations in DNA fragments (Zoller & Smith 1982). Then an improved version emerged which used two primers and a single-stranded DNA template to gain site-specific mutations by polymerase chain reaction (Zoller & Smith 1984), which was later improved by PCR overlap extension (Ho et al. 1989) (Figure 5.4). Cassette mutagenesis is another common method designed for codon-specific mutagenesis. This utilizes restriction sites on plasmid vectors and ligates DNA “cassettes” containing site-specific mutations to the vectors (Wells et al. 1985). Around the early 1990s, amino acid site-directed mutagenesis approaches were also a hot topic. The chemically modified aminoacyl-tRNAs were used to guide the synthesis of desired proteins in vitro (Bain et al. 1991).

Although the experimental techniques of site-directed mutagenesis were developed during the 1980s, to be successful at generating enzymes with desirable properties, valuable information such as active sites and structural specificity of the enzymes is needed to guide enzyme engineering. Thus, method improvements were less reported whereas more structure-based knowledge of enzymes was discussed. Similarly, the enzyme applications of site mutagenesis emphasize key mutation sites rather than the methods. Some industry enzymes modified with this method can be seen in cases # 2, 8, 12, and 15 in Table 5.2.

5.2.2.2 Semi-rational Design

As briefly mentioned before, rational design and directed evolution are not mutually exclusive. The method that combines them both, called semi-rational design, can

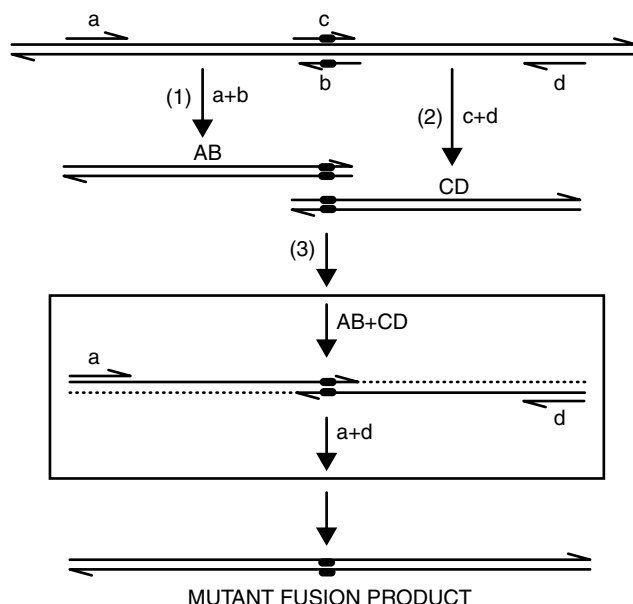


Figure 5.4 Schematic diagram of site-directed mutagenesis by overlap extension. The dsDNA and synthetic oligonucleotides are represented by lines with arrows indicating the 5'-to-3' orientation. The site of mutagenesis is indicated by the small black rectangle. Oligonucleotides are denoted by lower-case letters and PCR products are denoted by pairs of upper-case letters corresponding to the oligo primers used to generate that product. The boxed portion of the figure represents the proposed intermediate steps taking place during the course of reaction (3), where the denatured fragments anneal at the overlap and are extended 3' by DNA polymerase (dotted line) to form the mutant fusion product. By adding additional primers "a" and "d" the mutant fusion product is further amplified by PCR. *Source:* Ho et al. (1989). Reproduced with permission of Elsevier.

enhance the success of yielding desired libraries while optimally decreasing the workload in high-throughput screening. Semi-rational design can be classified into three basic types: sequence based, structure based, and computational model based. Sequence-based semi-rational design utilizes information on amino acid sequence and functional database to find specific nucleotide position(s) to mutate, followed by targeted small-scale random mutagenesis. Structure-based design engineers enzymes with the assistance of a structural topology database, like PDB and homology modeling software. The computational model-based approach mainly depends on algorithms to design proteins and specifically explore the functional improvements of enzyme activities. For example, an algorithm called SCHEMA has been used to estimate disruption by recombination using a pool of parental sequences (see case 6 in Table 5.2). It can generate a library of functional information based on that of parent sequences (Meyer et al. 2003). Another statistical analysis method for protein sequence activity relationships, called ProSARs, was developed to predict functional effect from different mutation sites (Fox et al. 2007).

From the perspective of methodology, the most common semi-rational design is the combinatorial active site saturation testing (CASTing). In this method, screening of libraries generated from a small number of active site residues can be done in an

iterative way. This method is often used to expand the substrate range of enzymes with known structural information and to further probe the precise active sites. The enzyme pyranose 2-oxidase was modified to obtain better substrate selectivity and catalytic activity by saturation mutagenesis of active sites, followed by screening of variant libraries (Spadiut et al. 2009).

5.2.3 Other Techniques

Although the main techniques of the above two strategies are different, there are several shared techniques that are applicable to both. These include screening for enzyme candidate and expression system of target enzymes. Indeed, with the advent of metagenomics, the screening step may directly work with metagenomic libraries built from DNA extracted from extreme environments to discover potentially novel enzymes for industrial purposes. Similarly, efficient protein expression systems can help solve the basic manufacturing problem, which is the production and purification of selected enzymes.

5.2.3.1 Screening of Enzyme Candidates

One common technique uses the combination of computational molecular techniques to explore a wide range of activities for target enzymes, such as at high/low temperature and/or extreme pH environments. To meet such special requirements, metagenomic libraries of DNA samples from such environments are constructed and screened for potential encoding genes with desired functions. The basic steps for metagenomics screening can be summarized as follows: extracting environmental DNA, inserting fragmented DNA into expression vectors, transforming the cloned DNA into host strains, functional screening of the cloned DNA, and DNA sequencing of selected desirable clones (Handelsman 2005).

The most directed screening is based on phenotypical detection. For example, Waschkowitz et al. (2009) constructed small-insert metagenomics libraries from environmental samples and partially screened the libraries for genes encoding proteolytic enzymes. Their screen identified two DNA fragments from genus *Xanthomonas* that possessed the unique modular structure of metalloproteases. Another strategy is using host strains incapable of surviving certain conditions to screen for heterologous sequences that will enable the strains to survive and reproduce in such conditions. This method has been applied to select cold-adapted enzymes in glacial ice environmental samples (Simon et al. 2009). DNA samples from extreme environments such as volcanic vents, deep ocean beds, and arctic tundra likely contain abundant enzymes with novel functions and they should be a very rich source of novel enzymes with potential for wide application. For example, Lee and colleagues (2012) reported that a novel alkaline phospholipase found on the Korean west coast was an intermediate between phospholipase and lipase. Similarly, divergent bifunctional beta-lactamases were first discovered by Allen and colleagues in Alaskan soil (2009).

In general, identifying functional enzymes from metagenomics libraries still suffers from several disadvantages, such as limited background information and functional knowledge of the enzymes, high sensitivity for further mutations and/or rearrangements, and limited information on their hosts and regulations of expression. However, there are concerted efforts under way in developing metagenomics technologies to overcome these problems and stimulate exploration for better biocatalysts.

5.2.3.2 Expression System

The easiest source of enzyme production is extraction from microorganisms. Many microbes can grow on a large scale under relatively manageable conditions. However, some enzymes are not derived from microorganisms - instead they come from macroorganisms, are artificially synthesized, or are a combination of natural and artificial genes. To enable industrial production, it is necessary to have a sustainable production system. At present, a number of species have been used as hosts to express foreign genes from other species. The strains applied in the food industry are mostly microorganisms, including bacteria such as *E. coli* and *Bacillus licheniformis*, and fungi such as *Aspergillus oryzae*, *Aspergillus niger*, *Fusarium venenatum*, *Kluyveromyces marxianus*, *Trichoderma reesei*, etc. Table 5.1 lists the microorganisms used as hosts for recombinant enzymes in the food industry. These host strains have been recognized as non-pathogenic according to FDA regulations (Olempska-Beer et al. 2006).

Aside from having an appropriate host, to enable effective expression, an effective expression vector is also needed. An expression vector is typically a DNA plasmid that carries the target encoding gene sequence into the host strain and expresses the enzyme. The main components of a plasmid include the promoter, insert target sequence, and terminator. For bacteria, the most common plasmid vectors are pUB110, pUC18, and pUC19, which were isolated from *Staphylococcus aureus* and *E. coli* respectively (Keggins et al. 1978; Yanisch-Perron et al. 1985). The pUB110 plasmid has been used for producing enzymes in *B. subtilis*, which contains a kanamycin resistance gene *kan^r* and a phleomycin resistance gene *ph1*, for expression selection. The two pUC plasmids are usually used for expression in *E. coli* with the ampicillin-resistant gene *amp^r* as a selection marker. After ligating the enzyme encoding sequences to the vector, they will be transformed into host strains. Bacterial hosts can be transformed through conjugation, electroporation, or regular transformation, while fungal cells can be transformed by biolistic transformation, electroporation of protoplasts, and T-DNA-mediated gene

Table 5.1 Food-processing enzymes produced from microorganisms.

Host microorganism	Enzyme
<i>Aspergillus niger</i>	Phytase, chymosin, lipase
<i>Aspergillus oryzae</i>	Esterase-lipase, aspartic proteinase, glucose oxidase, laccase, lipase, pectin esterase, phospholipase
<i>Bacillus licheniformis</i>	Amylase, pullulanase
<i>Bacillus subtilis</i>	Acetolactate, decarboxylase, amylase, maltogenic amylase, pullulanase
<i>Escherichia coli</i> K-12	Chymosin
<i>Fusarium venenatum</i>	Xylanase
<i>Kluyveromyces</i>	Chymosin
<i>Pseudomonas fluorescens</i>	Amylase
<i>Trichoderma reesei</i>	Pectin lyase

Source: Adapted from Olempska-Beer et al. (2006). Reproduced with permission of Elsevier.

transfer. Once transformation is completed, the antibiotic resistance markers will be used to select the successfully transformed hosts for further production of the enzymes.

The expression of heterologous proteins will either accumulate within host cells or secrete into extracellular broth. Therefore, the final step is that synthesized enzymes will be collected by lysing cell (for enzymes accumulating intracellularly) and removed from cellular compartments or directly purified from extracellular components (for enzymes that secrete extracellularly). However, not all enzymes can be easily isolated with these methods. Difficulties may exist when breaking cells while keeping the activity of target enzymes. Extracellular enzymes may be prone to degradation. To deal with these problems, several strategies have been developed. For example, as mentioned in section 5.2.1.2, IVC and ribosome display can be applied to cell-free systems, which can minimize the damage and increase the yields of enzymes. Another approach is to modify the host cells to make them easier to break or to reduce their secretion of proteases to minimize degradation of the target extracellular enzymes (Baneyx 1999; Nevalainen et al. 2005).

5.3 Applications and Safety Issues of Enzymes in the Food Industry

Enzymes, although not commonly recognized as such, have played essential roles in the processing of many foods and food products (Figure 5.5). For example, enzymes have been instrumental in making cheese, bread, wine, and beer for thousands of years (Dewdney 1973).

In the modern era, enzymes are widely used in food industries, such as the baking industry, fruit juice and cheese manufacturing, as well as wine making and brewing, to improve flavor, texture, digestibility, and nutritional value (Li et al. 2012) (see also Chapter 6). Representative examples of the enzymes that have been used in several food industry sectors, their roles, and techniques involved are given in Table 5.2.

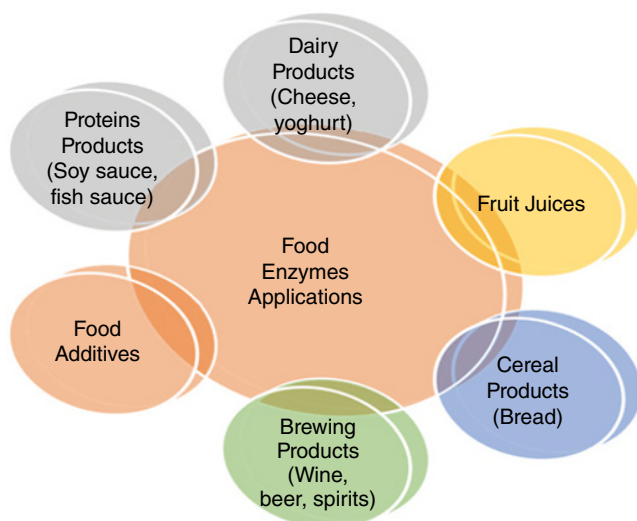


Figure 5.5 An overview of food enzyme applications.

Table 5.2 Food industrial enzymes and technologies involved.

Enzyme	Food application	Functions	No.	Case	Techniques	Improvements	Reference
Amylases	Baking, juice, brewing, starch processing	Degrading starch, controlling the volume and crumb structure of bread, clarifying juice, increasing maltose and glucose in alcohol, starch liquefaction	1	Alpha-amylase expressed in <i>Saccharomyces cerevisiae</i>	High-throughput screening (glucose interference)	Enzyme production increasing with glycerol as carbon source	Wong et al. (2002)
			2	<i>Bacillus subtilis</i> alpha-amylase	Site-directed mutagenesis	Substrate-binding ability retaining	Takase et al. (1992)
			3	<i>Bacillus licheniformis</i> alpha-amylase	Directed evolution: ep-PCR	Resistance to acid environment	Liu et al. (2012)
			4	<i>Bacillus licheniformis</i> alpha-amylase	Error-prone PCR and gene shuffling	pH range extension and enzyme activity promotion (for starch liquefaction)	Shaw et al. (1999)
			5	<i>Thermus</i> sp. maltogenic amylase	DNA shuffling	Improvement of thermostability	Kim et al. (2003)
Cellulases, hemicellulase	Juice	Hydrolyzing soluble pectin and lowering viscosity and maintenance of texture	6	Fungal cellulase	Semi-rational design: SCHEMA recombination	Thermostability improvement	Heinzelman et al. (2009)
Glucoamylase	Starch processing, juice	Saccharification, clarifying juice	7	<i>Aspergillus niger</i> glucoamylase	Directed evolution	Thermostability improvement	Wang et al. (2006)

(Continued)

Table 5.2 (Continued)

Enzyme	Food application	Functions	No.	Case	Techniques	Improvements	Reference
Lipase	Dairy products	Cheese manufacturing	8	<i>Psychrotroph Moraxella</i> lipase	Site-directed mutagenesis	Ability to catalyze lipolysis at low temperature	Feller et al. (1991)
			9	Lipase in <i>Pseudomonas aeruginosa</i>	Directed evolution: ep-PCR	Increasing in enantioselectivity	Liebeton et al. (2000)
			10	Lipase in <i>Bacillus subtilis</i>	Iterative saturation mutagenesis	Thermostability improvement	Reetz et al. (2006)
Cyclodextrin glycosyltransferase	Starch processing	Cyclodextrin production	11	Cyclodextrin glucanotransferases from <i>Bacillus</i> sp.	Directed evolution: ep-PCR	Increasing in enzyme activity	Shim et al. (2004)
			12	Cyclodextrin glycosyltransferase from <i>Bacillus circulans</i>	Rational design	Increase of alpha-cyclodextrin production	Van der Veen et al. (2000)
Galactosidase	Dairy	Glucose hydrolysis in dairy production	13	Beta-fucosidase from <i>Escherichia coli lacZ</i> beta-galactosidase	DNA shuffling	Substrate specificity and enzyme activity improvement	Zhang et al. (1997)
Xylose (glucose) isomerase	Starch processing	Glucose isomerization to fructose	14	<i>Thermotoga neapolitana</i> xylose isomerase (TNXI)	Random mutagenesis	High enzyme activity at low temperature and low pH	Sriprapundh et al. (2003)
			15	Glucose isomerase (GI) from <i>Actinoplanes missouriensis</i>	Site-directed mutagenesis	Stability improvement under industrial conditions	Quax et al. (1991)

Today's consumers demand high levels of quality in their foods in terms of natural flavor and taste, not only in North America and Europe but also in developing countries where consumption is shifting away from staple sources of calories. This trend has triggered the need for the development of enzymatic applications in food processing. The food and beverage enzymes segment reached about \$1.3 billion in 2015 with the highest sales in the milk and dairy market (BCC Research Biotechnology Report 2011). Interestingly, there was a modest decline of enzyme sales in the baking industry in recent years. However, the need for healthy foods has promoted positive growth in the whole food enzyme market.

The key item in evaluating food enzyme safety is the assessment of the host strain involved in enzyme production. Only nine microorganisms are Generally Recognized As Safe (GRAS) based on FDA regulations, primarily *A. oryzae*, *A. niger*, *B. subtilis*, and *B. licheniformis*. In order to increase the enzyme production level, modifications of traits including protease deficient and sporulation deficient were introduced into the wild-type host microorganisms (Koushki et al. 2011). Olempska-Beer et al. (2006) reviewed the microbial strains engineered for food enzyme production from a security point of view.

5.4 Conclusions and Future Perspectives

In this chapter, we described and discussed two main routes of enzyme engineering – directed evolution and rational design. Directed evolution is a well-established approach with a wide range of applications in industrial food enzyme production. We described the typical techniques involved in this approach which include (i) gene diversification methods to generate mutation libraries, such as through error-prone PCR, DNA shuffling, RACHITT, and StEP; and (ii) efficient screening and selecting methods, such as FACS, IVC, ribosome display, and mRNA display. For the rational design approach, both structural and functional knowledge of the target enzyme is needed and the approach includes techniques such as site-directed mutagenesis, restriction site cassette, PCR, and the use of Kunkel's double-deficient strains as hosts. In addition, we discussed the semi-rational design approach that combines techniques in both categories and offers novel strategies for future applications. Aside from identifying the unique characteristics of these approaches, shared features that apply to all approaches were also discussed, including high-level expression systems for target enzymes.

The developments in molecular biology and relevant fields, such as engineering, computational tools, and process optimization, have contributed significantly to the production of industrial enzymes. However, problems and bottlenecks remain, including the general lack of efficient screening of genetic variants derived from random mutagenesis. To increase the efficiency of rational design and make the approach more accessible to broader categories of enzymes, we also need to have an expanded enzyme database with structural and functional information and improved computational algorithms.

Although technological issues are the main topics of this chapter, it is important to note that technologies are useful only when they are directed to help us achieve specific goals. In the field of food production, our purpose is to produce higher quality foods in

greater quantity economically to meet consumer demands. Briefly, the modification goals of industrial enzymes are basically of two types: to make the enzymes more efficient and/or more resistant to environmental stresses. Enzymes that can meet such requirements will always be in demand, to help obtain greater yield, achieve higher catalytic efficiency, and last longer in processing environments. Because industrial conditions are usually less controllable than laboratory ones, enzymes that can maintain better activity in industry-specific environments will be more highly appreciated. Such industry-specific environments may have high/low temperature, suboptimal pH, and excessive/deficient concentrations of substrate or ion supplement. How to engineer enzymes that can meet the varied goals of industrial food production under diverse environments will be a continued challenge for years to come.

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Section II

Fruits and Vegetables

6

Molecular Identification and Distribution of Yeasts in Fruits

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6.1 Introduction

Most of us have heard of yeasts. They are commonly known for being associated with the production of baked goods and alcoholic beverages and for causing infections in humans. However, the yeasts associated with humans and common anthropogenic activities are only a small subset of the yeasts existing across the diverse environments on Earth. Indeed, yeasts are widely distributed not only ecologically and geographically but also functionally, conducting a variety of physiological processes in the biosphere.

Yeasts are unicellular fungi, single-celled microorganisms that are classified together with molds and mushrooms in the kingdom Fungi in the superkingdom Opisthokonta (domain Eukarya) that also includes all the animals. Taxonomically, yeasts are found in two separate phyla – Ascomycota (or the sac fungi) and Basidiomycota that have diverged from each other for about 400 million years. Such ancient divergence between yeasts in these two phyla means that there are significant amounts of genetic variation among many species of yeasts. Ascomycota and Basidiomycota together form the sub-kingdom Dikarya, referring to the life cycle stage where nuclei from two genetically different partners remain separate but co-exist in the same cell. The budding yeast, also commonly referred to as baker's yeast and brewer's yeast, belongs to the phylum Ascomycota and the order Saccharomycetales.

Yeasts are widely dispersed in a variety of habitats. They are commonly found on plant leaves, flowers, and fruits, as well as in soil, air, and aquatic environments. Yeasts are also found on the surface of the skin and in the intestinal tracts of both cold-blooded and warm-blooded animals, where they may live symbiotically or as parasites. For example, specific genotypes of the baker's yeast *Saccharomyces cerevisiae* can cause vaginitis and several species in the genus *Candida* such as *C. albicans*, *C. guilliermondii*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* can cause diaper rash and thrush of the mouth and throat. In immunocompromised patients, these species can cause systemic bloodstream infections and in serious cases, can lead to the deaths of patients.

Due to the significant roles of yeasts in food and in human health, it is important to understand the distribution of yeasts in food. Because fruits are commonly colonized

by microorganisms and are generally considered as healthy food and often eaten raw, our understanding of yeasts in fruits could also have a significant impact on the management of human health. Thus we focus this review of the species and genotype diversities of yeasts in and on fruits.

In the sections below, we first discuss the molecular methods that have been used for distinguishing yeast species and strains. This is then followed by the distribution of yeasts in specific types of fresh fruits, and then processed fruits, including fruit juices. We finish by providing a perspective on potential future research topics.

6.2 Molecular Methods for Distinguishing Yeast Species and Strains

Molecular methods for discriminating species and strains rely on polymorphic molecular markers. A molecular marker refers to any detectable property that identifies a specific region of the genome. Just like phenotypic differences that allow species (for example, between humans and chimpanzees) and individuals (e.g., between two people) to be recognized, to be effective, the molecular marker needs to be polymorphic. That is, different species and individuals need to show differences at the specific genomic region in order for the molecular marker to be informative. Thus, a key step in applying molecular markers for species and individual identification is to find informative markers that can be efficiently assayed and compared at specific taxonomic levels.

The current revolution in genomics and bioinformatics is producing a tremendous amount of DNA sequence information, especially for model organisms such as the baker's yeast where the genomes from multiple strains have been analyzed. The analyses of sequenced genomes have identified large amounts of inter- and intraspecies DNA sequence polymorphisms that could be used for the identification of species and strains. However, the sequenced genomes represent only a small fraction of the known species and strains, even for model organisms. In addition, whole genome sequencing is still costly and not easily accessible by most biologists or food industry workers. Therefore, having one or a few specific molecular markers and a technique that can be easily performed would be very useful. In addition, a large and comprehensive database related to specific molecular markers should be available in order for the obtained data to be compared and to retrieve the required and desired information.

Over the years, many molecular markers and techniques have been developed for species and strain identification. Though methods for identifying chemical signatures based on metabolite profiling have been developed, most methods have focused on polymorphisms in either proteins or DNA sequences. Assays of protein polymorphisms have been primarily conducted through polyacrylamide gel electrophoresis and isozyme staining. For DNA sequence polymorphisms, a large number of techniques have been developed. Some of these techniques, such as electrophoretic karyotyping (also called pulsed field gel electrophoresis), are used to identify large genome structural differences such as chromosome number and size differences. At the other extreme, DNA sequencing allows the identification of base differences at specific nucleotide positions in the genome. Differences in the secondary structure, restriction enzyme digest pattern, and number of repeats in a repetitive sequence region are

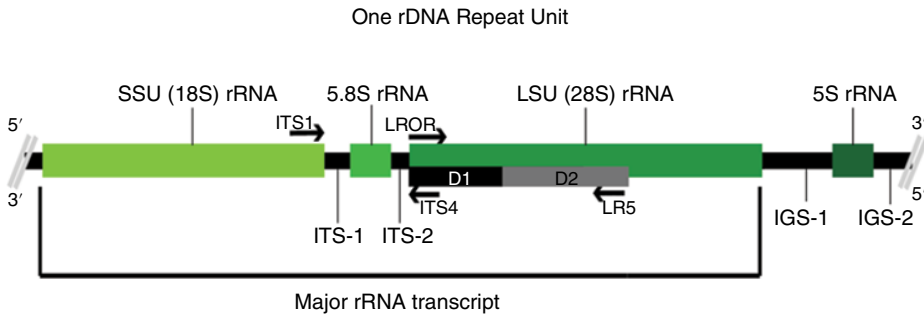


Figure 6.1 Organization of the nuclear ribosomal RNA gene cluster in fungi. Common primers used for sequencing are also shown. IGS, intergenic spacer region; ITS, internal transcribed spacer region; LSU, large subunit; SSU, small subunit.

assayed by other methods such as single-strand conformation polymorphisms, restriction fragment length polymorphisms, polymerase chain reaction (PCR), and high-resolution gel electrophoresis. For details of these and other methods, please refer to the review by Xu (2006).

Many molecular methods mentioned above have been used for yeast species and strain identification. In the last few years, DNA sequencing at two linked fragments of the nuclear ribosomal gene cluster has become routinely used. One fragment is the D1/D2 region of the large subunit ribosomal RNA gene (25–28S rRNA). The second region includes the 5.8S rRNA and its two flanking regions called internal transcribed spacers (ITS), located adjacent to the D1/D2 region and the 3' end of the small subunit ribosomal RNA gene (18S rRNA) (Figure 6.1). Indeed, ITS is the recommended fungal barcode (Schoch et al. 2012; Xu 2016) and sequences at these two fragments are now required information for proposing new taxonomic groups. Due to historical and technical reasons, some of these sequences for certain taxa are not correct in public databases. However, large databases do exist that allow comparisons to be performed for accurate species and strain identification (Xu 2016). In addition, as shown below, many studies have developed more efficient assay methods based on the existing sequence variations at these two DNA regions. For example, restriction enzyme digests of PCR-amplified products of the two gene fragments (especially the ITS) have been commonly used for yeast species identification, with each species having a different digestion profile as shown on agarose gels.

While the D1/D2 regions are typically not variable among strains within a species, within many yeast species, the ITS sequences can vary among strains, allowing the identification of specific ITS genotypes. However, the majority of strains within individual species are distinguished using other more discriminatory methods such as PCR-based fingerprints, restriction fragment length polymorphisms, amplified fragment length polymorphisms, microsatellite markers, and DNA sequencing of single-copy genes. The appropriate strain typing systems differ among species. In addition, the selection of markers also depends on the specific questions that the investigators want to address and how much background information is known about the specific organisms with regard to the different typing methods. As shown below, different markers and methods have been used for different yeast species to analyze intraspecific genetic variations.

6.3 Yeast Diversity in Wild/fresh Fruits

A variety of fruits have been analyzed for the diversity of yeasts associated with them. In the following, we separately describe the yeast diversity associated with each of the fruits.

6.3.1 Grapes

6.3.1.1 Yeast Species Diversity

Several studies have utilized sequence variations at the ITS regions for diversity surveys of yeasts associated with grapes. Results of these studies found that the diversity and frequency of yeast species were influenced by location and climate (Chavan et al. 2009; Li et al. 2010; Raspor et al. 2006). The geographically broadly distributed yeast species include *Hanseniaspora guilliermondii*, *Saccharomyces cerevisiae*, *Issatchenkia orientalis*, *Hanseniaspora uvarum*, *Pichia fermentans*, *Cryptococcus carnescens*, *Cryptococcus flavescens*, *Rhodotorula glutinis*, and *Aureobasidium pullulans*. Interestingly, non-*Saccharomyces* yeasts largely dominated the isolates, particularly *Hanseniaspora* species such as *Hanseniaspora uvarum* (Li et al. 2010; Raspor et al. 2006). Other predominant species were geographically more limited, including *Hanseniaspora guilliermondii* from India (Chavan et al. 2009), *Cryptococcus flavescens*, *Pichia fermentans*, and *Candida zeylanoides* from China (Li et al. 2010), and *Sporobolomyces* and *Cryptococcus* species in Slovenia (Raspor et al. 2006). It has been suggested that using enrichment techniques may allow for greater detection of *Saccharomyces cerevisiae*, which has been isolated at comparatively lower frequencies in grapes than previously thought (Fleet et al. 2002).

In addition to geography and climate, grape variety appears to influence yeast flora on grapes as well (Chavan et al. 2009; Li et al. 2010; Raspor et al. 2006; Sabate et al. 2002). The Merlot variety was reported as possessing a greater yeast diversity compared to Cabernet, Sauvignon, and Chardonnay (Li et al. 2010), and the Bangalore Blue variety as possessing a greater yeast diversity compared to Cabernet, Shiraz, and Zinfandel. In a study by Sabate et al. (2002), *Hanseniaspora uvarum* was predominant in the Carinyena variety, while other, oxidative yeasts, including *Cryptococcus* species, dominated the isolates from the Garnacha variety. However, sampled again a year later, *Aureobasidium pullulans* dominated in the Garnacha variety while *Candida zeylanoides* was most commonly isolated in the Carinyena variety. The Slovenian grape varieties Žametovka, Modra frankinja, and Kraljevina also showed differences in their associated yeast species composition and diversity, though the authors refrained from drawing conclusions at their early stage of research (Raspor et al. 2006).

An additional study by Renouf et al. (2005) found that the level of berry maturation, and not grape variety, was a major factor contributing to yeast species differences on grapes. They found that most yeast species were common between Merlot, Cabernet Sauvignon, and Cabernet Franc grape varieties, but the yeasts were present at different times. At berry set, *Aureobasidium pullulans*, *Rhodospiridium babjevae*, and other *Rhodotorula* yeast species were dominant, but were undetectable at veraison. At maturity, fermentative yeasts like *Saccharomyces cerevisiae*, *Pichia anomala*, and *Candida stellata* were abundant. Basidiomycetous yeasts were more consistently detected

throughout berry developmental stages than ascomycetous yeasts. In general, as the berries matured, the diversity of yeast species and abundance increased (Renouf et al. 2005). One hypothesis for such a correlation was that the softening of the skin associated with maturation allowed for the juice to reach the berry surface and thus provided a competitive advantage for fermentative yeasts such as *Hanseniaspora*, *Candida*, and *Pichia* (Sabate et al. 2002). Consistent with that hypothesis, grapes subjected to damage from *Botrytis* infection possessed a greater diversity and frequency of fermentative or spoilage yeasts (Nisiotou & Nychas 2007).

Table 6.1 summarizes a number of studies investigating yeast species diversity on grapes.

6.3.1.2 Yeast Intraspecific Genetic Diversity

Aa et al. (2006) performed the first multilocus analysis of natural *Saccharomyces cerevisiae* isolates from grapes. The following four loci were sequenced for their isolates: *SSU1*, *FZF1*, *CDC19*, and *PHD1*. Among the 27 isolates analyzed, 15 genotypes were identified. Several isolates from red vineyard grapes, white vineyard grapes, and white table grapes from various locations on the Isle of Elba, Italy, were identical over the four loci studied. Furthermore, one isolate from Tuscany, Italy, possessed the same genotype as another isolate from California, USA (Aa et al. 2006). These results are consistent with long-distance dispersal of *S. cerevisiae* isolates from grapes, likely through anthropogenic routes.

6.3.2 Apples

6.3.2.1 Yeast Species Diversity

Several studies have identified *Meyerozyma guilliermondii* as a dominant yeast species from apples (Mokhtari et al. 2012; Pelliccia et al. 2011; Vadkertiová et al. 2012). Depending on the study, other common yeasts have also been found, including *Rhodotorula glutinis* (Mokhtari et al. 2012; Pelliccia et al. 2011), *Hanseniaspora uvarum* (Pelliccia et al. 2011; Vadkertiová et al. 2012), *Rhodotorula mucilaginosa* (Mokhtari et al. 2012), and *Pichia kluyveri* (Vadkertiová et al. 2012). Other yeasts reported in these studies were *Aureobasidium pullulans* and *Wickerhamomyces anomalus* (Pelliccia et al. 2011; Vadkertiová et al. 2012).

As was found in grapes, the geographic location, apple type, and fruit maturation time influenced yeast species diversity in apples (Mokhtari et al. 2012; Pelliccia et al. 2011; Vadkertiová et al. 2012). Although the distribution of yeast species between the different producers appeared mostly random, the common isolation of *Meyerozyma guilliermondii* and a few other species suggested an association between these yeasts and the surfaces of apple fruit (Pelliccia et al. 2011). Pelliccia et al. (2011) also investigated the influence of industrial cleaning on yeast flora of apples. The isolation of yeast species from cleaned apples, particularly of species not isolated from unwashed fruit, was potentially the result of contamination during the washing process, or the insufficiency of washing treatment in removing yeast from the surface of the fruit.

The importance of molecular methods in accurate species identification was emphasized in a study by Gildemacher et al. (2006), which reidentified a selection of yeast

Table 6.1 Yeast species diversity among isolates obtained from grape berry surfaces.

Method	Yeast species identified	No. or % of isolates	Reference
Morphological identification and BLAST analysis of the ITS regions	<i>Hanseniaspora guilliermondii</i>	14	Chavan et al. 2009
	<i>Saccharomyces cerevisiae</i>	9	
	<i>Issatchenkia orientalis</i>	8	
	<i>Pichia membranifaciens</i>	5	
	<i>Candida azyma</i>	4	
	<i>Issatchenkia terricola</i>	3	
	<i>Hanseniaspora viniae</i>	2	
	<i>Zygoascus steatolyticus</i>	2	
	<i>Candida quercitrusa</i>	1	
	<i>Debaryomyces hansenii</i>	1	
	<i>Hanseniaspora uvarum</i>	1	
PCR-RFLP using <i>Hin6I</i> , <i>BsuRI</i> , and <i>HinfI</i> , followed by sequence analysis of the ITS regions	RFLP analysis:	Not available	Li et al. 2010
	<i>Hanseniaspora uvarum</i> *		
	<i>Pichia fermentans</i> *		
	<i>Candida zemplinina</i> *		
	<i>Pichia guilliermondii</i>		
	<i>Metschnikowia pulcherrima</i>		
	<i>Zygosaccharomyces bailii</i>		
	<i>Candida bombi</i>		
	<i>Issatchenkia terricola</i>		
	<i>Issatchenkia orientalis</i>		
	ITS sequence analysis:		
	<i>Cryptococcus carnescens</i> *		
	<i>Cryptococcus flavescens</i> *		
	<i>Candida inconspicua</i>		
	<i>Candida quercitrusa</i>		
	<i>Cryptococcus magnus</i>		
	<i>Sporidiobolus pararoseus</i>		
	<i>Zygosaccharomyces fermentati</i>		
	<i>Hanseniaspora guilliermondii</i>		
PCR-RFLP using <i>HinfI</i> , <i>HaeIII</i> , <i>HhaI</i> , <i>DraI</i> , and <i>DdeI</i> , and sequence analysis of the ITS regions, as well as D1/D2 domain sequencing	<i>Hanseniaspora uvarum</i> *	Not available	Nisiotou & Nychas 2007
	<i>Hanseniaspora guilliermondii</i>		
	<i>Zygosaccharomyces bailii</i>		
	<i>Issatchenkia terricola</i>		
	<i>Issatchenkia occidentalis</i>		
	<i>Metschnikowia pulcherrima</i>		
	<i>Aureobasidium pullulans</i>		
	<i>Hanseniaspora opuntiae</i>		
	<i>Candida zemplinina</i>		

Table 6.1 (Continued)

Method	Yeast species identified	No. or % of isolates	Reference
PCR-RFLP using <i>CfoI</i> , <i>HaeIII</i> , <i>MspI</i> , and <i>RsaI</i> of the ITS regions; physiological testing performed for strains with identical PCR-RFLP profiles	<i>Rhodotorula glutinis</i>	347	Raspour et al. 2006
	<i>Aureobasidium pullulans</i>	118	
	<i>Cryptococcus laurentii</i>	32	
	<i>Hanseniaspora uvarum</i>	124	
	<i>Sporobolomyces roseus</i>	86	
	<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	8	
	<i>Metschnikowia reukaufii</i>	2	
	<i>Rhodotorula aurantiaca</i>	14	
	<i>Cryptococcus albidus</i> var. <i>albidus</i>	1	
	<i>Dioszegia hungarica</i> (<i>Cry. hungaricus</i>)	1	
	<i>Pichia kluyveri</i>	5	
<i>Pichia membranifaciens</i>	2		
<i>Metschnikowia pulcherrima</i>	12		
RFLP analysis using <i>CfoI</i> , <i>HaeIII</i> , and <i>HinfI</i> , of the ITS regions, and PCR-DGGE analysis of the D1 region	Berry set:		Renouf et al. 2005
	<i>Aureobasidium pullulans</i>	62%	
	<i>Cryptococcus</i> spp.	14%	
	<i>Rhodotorula</i> spp.	12%	
	<i>Rhodospodium</i>	10%	
	<i>Candida</i> spp., <i>Sporobolomyces</i> spp., <i>Hanseniaspora</i> spp., <i>Yarrowia lipolytica</i>	2%	
	Veraison:		
	<i>Candida</i> spp.	24%	
	<i>Sporobolomyces</i> spp.	18%	
	<i>Cryptococcus</i> spp.	16%	
	<i>Rhodotorula</i> spp.	16%	
	<i>Aureobasidium pullulans</i>	14%	
	<i>Rhodospodium</i>	10%	
	<i>Metschnikowia</i> spp.	2%	
	<i>Bulleromyces albus</i>		
<i>Lipomyces spencermartinsaie</i>			
<i>Kluyveromyces lactis</i>			
<i>Pichia</i> spp.			

(Continued)

Table 6.1 (Continued)

Method	Yeast species identified	No. or % of isolates	Reference
	Harvest:		
	<i>Cryptococcus</i> spp.	34%	
	<i>Candida</i> spp.	30%	
	<i>Pichia</i> spp.	16%	
	<i>Rhodotorula</i> spp.	10%	
	<i>Hanseniaspora</i> spp.	8%	
	<i>Debaryomyces hansenii</i>	2%	
	<i>Sporobolomyces</i> spp.		
	<i>Saccharomyces</i> spp.		
	<i>Lipomyces spencermartinsaie</i>		
	<i>Kluyveromyces lactis</i>		
	<i>Issatchenkia</i> spp.		
PCR-RFLP of the ITS regions using <i>Cfo</i> I, <i>Hae</i> III, and <i>Hinf</i> I	1995 vintage		Sabate et al. 2002
	Carinyena variety:		
	<i>Hanseniaspora uvarum</i>	75%	
	<i>Cryptococcus uniguttulatum</i>	10%	
	<i>Candida zeylanoides</i>	10%	
	<i>Aureobasidium pullulans</i>	5%	
	Garnacha variety:		
	<i>Cryptococcus uniguttulatum</i>	65%	
	<i>Cryptococcus ater</i>	15%	
	<i>Cryptococcus laurentii</i>	10%	
	<i>Aureobasidium pullulans</i>	10%	
	1996 vintage		
	Carinyena variety:		
	<i>Candida zeylanoides</i>	90%	
	<i>Aureobasidium pullulans</i>	10%	
	Garnacha variety:		
	<i>Aureobasidium pullulans</i>	100%	

* Most frequently isolated.

BLAST, basic local alignment search tool; DGGE, denaturing gel gradient electrophoresis; ITS, internal transcribed spacer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

isolates from apple fruit surfaces through sequence analysis of the D1/D2 domain and ITS regions. These isolates were previously identified using traditional morphological and physiological methods (Gildemacher et al. 2004). Among the reidentifications were the then recently described *Cryptococcus victoriae*, previously identified as *Cryptococcus laurentii*; *Sporidiobolus pararoseus*, which had been identified as *Sporobolomyces*

roseus; and an undescribed anamorphic stage of a *Tremella* species, previously identified as *Cryptococcus albidus*. Molecular methods of identification also suggested greater species diversity on apple fruits than that based on morphological/physiological methods (Gildemacher et al. 2006). Similar analysis of the D1/D2 domain and ITS regions also allowed for the identification of four strains from apple surfaces as belonging to a novel yeast species, *Cryptococcus spencermartinsiae* sp. nov. (de García et al. 2010).

Table 6.2 summarizes a number of studies investigating yeast species diversity on apple fruits.

6.3.2.2 Yeast Intraspecific Genetic Diversity

Mokhtari et al. (2012) performed ITS sequencing for species identification as yeast strains were largely indistinguishable using only PCR-fragment size polymorphism (FSP). In the case of *Rhodotorula mucilaginosa*, *Rhodotorula glutinis*, and *Pichia guilhermondii*, multiple ITS sequence types were found within each species, suggesting significant intraspecific ITS sequence variation within these species. However, no or very limited variation was found among strains within the apple-associated yeasts for the D1/D2 domain (Pelliccia et al. 2011).

6.3.3 Citrus Fruits

Several studies have analyzed yeasts from citrus fruits. Heras-Vazquez et al. (2003) found that *Saccharomyces cerevisiae* was the most commonly isolated species from orange fruit surfaces and juice, followed by *Candida tropicalis* and *Hanseniaspora uvarum*. *Clavispora lusitaniae* and *Trichosporon asahii* were isolated at lower frequencies and *Saccharomyces unisporus*, *Pichia anomala*, *Rhodotorula mucilaginosa*, and *Pichia fermentans* were also isolated but very infrequently. Restuccia et al. (2006) also performed RFLP analysis of the ITS regions and similarly identified *Saccharomyces cerevisiae* as the most commonly isolated species on minimally processed orange slices. However, in contrast with results in the previous study, *Pichia anomala* and *Rhodotorula mucilaginosa* were the next most commonly isolated species and *Hanseniaspora uvarum* was isolated at a lower frequency. Other species not found in the study by Heras-Vazquez et al. (2003) included *Cryptococcus macerans*, *Rhodotorula acuta*, *Candida ergastensis*, and *Cryptococcus laurentii*. Restuccia et al. (2006) also found that packaging permeability and atmosphere influenced yeast species diversity on spontaneously fermenting orange fruits and juice. Arias et al. (2002) obtained yeasts from freshly squeezed orange juice and, consistent with the study by Heras-Vazquez et al. (2003), identified *Hanseniaspora uvarum* as a dominant species; however, other common species identified on citrus fruit surfaces and juice were not detected. Instead, another *Hanseniaspora* species (*Hanseniaspora occidentalis*) was also frequently isolated, though it was not found in other studies that used similar identification methods (Heras-Vazquez et al. 2003; Restuccia et al. 2006). The differences in yeast species between these studies were likely the result of differences in harvesting times, geographical location and climate, fruit processing environment, and/or other factors discussed in previous sections.

Several common yeasts have been isolated from other types of citrus fruits, including sweet lemon, sour lemon, and tangerine. These yeasts included *Rhodotorula glutinis*, *Hanseniaspora opuntiae*, *Hanseniaspora occidentalis*, *Cryptococcus flavescens*, *Cryptococcus magnus*, and *Sporidiobolus pararoseus* (Mokhtari et al. 2012). Interestingly,

Table 6.2 Yeast species diversity in isolates obtained from apple fruit surfaces.

Method	Yeast species identified	No. of isolates				Reference
Analysis of the D1/D2 domain and ITS regions	<i>Cryptococcus spencermartinsiae</i> sp. nov.	Not available				de García et al. 2010
Sequence analysis of the D1/D2 domain and ITS regions	<i>Metschnikowia pulcherrima</i> <i>Candida sake</i> <i>Rhodosporeidium babjevae</i> <i>Erythrobasidium hasegawianum</i> <i>Rhodotorula glutinis</i> <i>Rhodotorula</i> aff. <i>pinicola</i> <i>Rhodotorula fragaria</i> <i>Sporidiobolus pararoseus</i> <i>Cryptococcus victoriae</i> Undescribed anamorphic stage of a <i>Tremella</i> species	Not available				Gildemacher et al. 2006
Fragment size polymorphism followed by sequencing and BLAST analysis of ITS regions of representative isolates	<i>Metschnikowia pulcherrima</i> <i>Cryptococcus adeliensis</i> <i>Rhodotorula mucilaginosa</i> <i>Rhodotorula glutinis</i> <i>Pichia guilliermondii</i> <i>Candida membranifaciens</i>	No. of isolates sequenced				Mokhtari et al. 2011
Sequencing of the D1/D2 domain		Fuji	Fuji (clean)	Golden	Golden (clean)	Pelliccia et al. 2011
	<i>Aureobasidium pullulans</i>		1	1	1	
	<i>Candida oleophila</i>				2	
	<i>Debaryomyces hansenii</i>	2	1		1	
	<i>Hanseniaspora uvarum</i>		1	3	1	
	<i>Metschnikowia pulcherrima</i>				3	
	<i>Wickerhamomyces anomalus</i>			1		
	<i>Meyerozyma guilliermondii</i>	3	2	3	4	
	<i>Rhodotorula glutinis</i>	2	1	1	1	
	Total strains isolated	7	5	9	12	
Total species isolated	3	5	5	7		

Table 6.2 (Continued)

Method	Yeast species identified	No. of isolates			Reference
		Sampling time 1	Sampling time 2	Total	
RFLP-PCR of the ITS regions using <i>Hae</i> III, <i>Taq</i> I, <i>Alu</i> I, and <i>Hin</i> fI	<i>Aureobasidium pullulans</i>	7		7	Vadkertiová et al. 2012
	<i>Cryptococcus</i> sp.	7		7	
	<i>Galactomyces candidus</i>		10	11	
	<i>Hanseniaspora guilliermondii</i>		23	23	
	<i>Hanseniaspora uvarum</i>	40		40	
	<i>Metschnikowia pulcherrima</i>	13	7	20	
	<i>Pichia kluyveri</i>	7	43	50	
	<i>Pichia kudriavzevii</i>	17		17	
	<i>Pichia membranifaciens</i>	3	7	10	
	<i>Saccharomyces cerevisiae</i>		10	10	
	<i>Saccharomycopsis crataegensis</i>	3	7	10	
	<i>Wickerhamomyces anomalus</i>		3	3	

BLAST, basic local alignment search tool; ITS, internal transcribed spacer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

no *Metschnikowia* or *Candida* species were isolated from citrus fruits, though these species were commonly isolated from apple fruits in the same study.

Table 6.3 summarizes a number of studies investigating yeast species diversity on citrus fruits.

6.3.4 Yeast Species Diversity in Other Fruits

6.3.4.1 Pears

Pelliccia et al. (2011) investigated the yeast species composition found on pears and the impact of industrial washing on yeasts on pears. *Meyerozyma guilliermondii* was the most commonly isolated species, followed by *Aureobasidium pullulans*, both of which were isolated from both unwashed and washed samples (Pelliccia et al. 2011). While *Candida oleophila* and *Pichia kluyveri* were found exclusively on unwashed samples, *Metschnikowia pulcherrima* and *Debaryomyces hansenii* were only isolated from washed fruits. *Hanseniaspora uvarum* and *Metschnikowia fructicola* were found on two varieties of pears (Abate Fetel and Kaiser) and their presence did not appear to be influenced by industrial washing (Pelliccia et al. 2011). Vadkertiová et al. (2012) also investigated the yeast organisms associated with mature pear fruits and found a similar group of yeast species using RFLP-PCR of the ITS regions. They also found that

Table 6.3 Yeast species diversity in isolates obtained from various citrus fruits.

Method	Yeast species identified	No. of isolates	Reference
Fresh-squeezed juice 5.8S-ITS restriction profiles (obtained using <i>Cfo</i> I, <i>Hae</i> III, and <i>Hinf</i> I) and partial sequencing of the 26S rRNA gene	<i>Candida stellata</i>	1	Arias et al. 2002
	<i>Hanseniaspora occidentalis</i>	14	
	<i>Hanseniaspora uvarum</i>	24	
	<i>Pichia fermentans</i>	2	
	<i>Pichia kluyveri</i>	9	
Spontaneously fermenting oranges and fresh juice Restriction pattern analysis of the ITS regions using <i>Cfo</i> I, <i>Hae</i> III, and <i>Hinf</i> I, and sequence analysis of the ITS regions	<i>Saccharomyces cerevisiae</i>	35	Heras-Vazquez et al. 2003
	<i>Candida tropicalis</i>	17	
	<i>Clavispora lusitaniae</i>	12	
	<i>Trichosporon asahii</i>	10	
	<i>Saccharomyces unisporus</i>	2	
	<i>Pichia anomala</i>	1	
	<i>Rhodotorula mucilaginosa</i>	1	
	<i>Hanseniaspora uvarum</i>	17	
Various citrus fruits Fragment size polymorphism followed by sequencing of the ITS regions for final identification of representative isolates		No. of isolates sequenced	Mokhtari et al. 2012
	<i>Rhodotorula glutinis</i>	1	
	<i>Hanseniospora opuntiae</i>	1	
	<i>Hanseniospora occidentalis</i>	1	
	<i>Cryptococcus flavescens</i>	1	
	<i>Cryptococcus magnus</i>	1	
	<i>Sporidiobolus pararoseus</i>	1	
Minimally processed orange slices Amplification of the ITS regions and PCR/RFLP analysis using <i>Hha</i> I, <i>Hae</i> III, and <i>Hinf</i> I	<i>Saccharomyces cerevisiae</i>	118	Restuccia et al. 2006
	<i>Pichia anomala</i>	82	
	<i>Rhodotorula mucilaginosa</i>	69	
	<i>Cryptococcus macerans</i>	30	
	<i>Rhodotorula acuta</i>	25	
	<i>Candida ergastensis</i>	7	
	<i>Cryptococcus laurentii</i>	7	
	<i>Hanseniaspora uvarum</i>	7	
<i>Pichia scolytii</i>	5		
Clementine and orange isolates antagonistic to <i>P. digitatum</i> Sequencing of the D1/D2 domain	<i>Hanseniaspora guilliermondii</i>	7	Taqaort et al. 2008
	<i>Hanseniaspora guilliermondii</i> isolate G1	4	
	<i>Debaryomyces hansenii</i> strain CECT 1066	1	
	<i>Hanseniaspora</i> sp. YS DN19	1	
	<i>Hanseniaspora uvarum</i> isolate G8p1	1	
	<i>Pichia anomala</i> strain VTT C-04565	1	

ITS, internal transcribed spacer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

sampling time seemed to influence species diversity. For example, *Pichia kudriavzevii* was a common species in the first set of samples, but was not detected a year later. In contrast, *Galactomyces candidus* and *Saccharomyces* species were isolated exclusively in the second year (Vadkertiová et al. 2012).

6.3.4.2 Nectarines

Janisiewicz et al. (2010) investigated yeast species diversity on nectarine fruits. Their analyses identified 17 yeast genera and 23 species. In addition, they found several new species and an influence of fruit maturity on yeast species composition. The dominant yeasts were *Aureobasidium pullulans*, *Cryptococcus* spp., *Sporodiobolus* spp., and *Rhodotorula* spp., which were present from early to late stages of fruit development. *Erythrobasidium*, *Pseudeurotium*, and *Tremella* species were mainly isolated during early stages of fruit development. Species in the genera *Hanseniaspora*, *Candida*, *Acremonium*, *Metschnikowia*, *Pichia*, *Sporobolomyces* and *Zygosaccharomyces* were more commonly detected during later stages of development (Janisiewicz et al. 2010).

6.3.4.3 Pineapples

Hanseniaspora uvarum and *Pichia guilliermondii* were the dominant yeast species isolated from pineapple skins, freshly crushed pineapple juice, and their fermentative products (Chanprasartsuk et al. 2010). Of the two yeast species, *Pichia guilliermondii* was the dominant species during the early stages of the fermentation, whereas *Hanseniaspora uvarum* was more prevalent later in the 6-day fermentation period. Other species included *Aureobasidium pullulans*, *Issatchenkia orientalis*, *Candida sorboxylosa*, *Issatchenkia occidentalis*, *Candida apicola*, and other *Candida* spp. *Saccharomyces* and other wine yeasts were notably absent from the ferments of pineapple juices (Chanprasartsuk et al. 2010). *P. guilliermondii* is an opportunistic human pathogen. The result suggests that immunocompromised patients should refrain from consuming fermented pineapple juice.

6.3.4.4 Plums

Vadkertiová et al. (2012) found that *Hanseniaspora guilliermondii* was the yeast most commonly isolated from plum fruits. Yeasts of the species *Hanseniaspora uvarum*, *Pichia kluyveri*, and *Pichia kudriavzevii* were also commonly isolated. Similar to temporal studies of yeast diversity, sampling time influenced yeast species composition. For example, *Hanseniaspora guilliermondii* dominated the isolates from the first year and *Pichia kluyveri* dominated those in the second year (Vadkertiová et al. 2012).

6.3.4.5 Jujube Fruits

Xue et al. (2006) analyzed yeasts from jujube fruits. Known yeast species were characterized using non-molecular methods based on morphological and physiological features. Eight yeasts with novel morphological and physiological features were further analyzed through sequencing the D1/D2 domain and the ITS regions. Their analyses revealed three novel *Metschnikowia* species (Xue et al. 2006).

Table 6.4 summarizes a number of studies investigating yeast species diversity in isolates obtained from a variety of other fruit, some of them not described in previous sections.

Table 6.4 Yeast species diversity in isolates obtained from a variety of other fruit.

Method	Yeast species identified	No. of isolates	Reference
Pineapple Sequence analysis of the ITS regions and D1/D2 domain primarily, as well as RFLP analysis of ITS region amplicons using <i>CfoI</i> , <i>HaeIII</i> , and <i>HinfI</i>	Main yeast species:	Not Available	Chanprasartsuk et al. 2010
	<i>Hanseniaspora uvarum</i>		
	<i>Pichia guilliermondii</i>		
	Early fermentation species:		
	<i>Pichia guilliermondii</i>		
	Late fermentation species:		
	<i>Hanseniaspora uvarum</i>		
	Other species:		
	<i>Aureobasidium pullulans</i>		
	<i>Issatchenkia orientalis</i>		
	<i>Candida sorboxylosa</i>		
	<i>Issatchenkia occidentalis</i>		
	<i>Candida apicola</i>		
<i>Candida</i> spp.			
Nectarine BLAST search of GenBank using D1/D2 sequences and, where available, confirmed with ITS sequences	<i>Aureobasidium pullulans</i>	43	Janisiewicz, Kurtzman, & Buyer 2010
	<i>Cryptococcus weiringae</i>	23	
	<i>Cryptococcus</i> sp. nov.	16	
	<i>Sporidiobolus pararoseus</i>	15	
	<i>Sporidiobolus pararoseus</i> – probable undescribed sister sp.	9	
	<i>Rhodotorula himmulea</i>	15	
	<i>Rhodotorula himmulea</i> or undescribed sister sp.	4	
	<i>Rhodotorula</i> sp. near <i>Rhodotorula larynges</i>	10	
	<i>Rhodotorula pinicola</i>	5	
	<i>Erythrobasidium hasegawianum</i>	5	
	<i>Crypt</i> VKMY2958	4	

<i>Rhodotorula glutinis</i>	4
<i>Auriculibuller fuscus</i>	3
<i>Cryptococcus magnus</i>	3
<i>Cryptococcus victoriae</i>	2
Near <i>Rhodotorula philya</i>	2
<i>Taphrina carnea</i>	2
<i>Taphrina deformans</i>	2
<i>Acremonium strictum</i> or related sp.	1
<i>Candida</i> sp. nov. 1	1
<i>Candida</i> sp. nov. 2	1
<i>Dothichiza pithyophila</i>	1
<i>Hanseniaspora uvarum</i>	1
<i>Metschnikowia fructicola/pulcherrima</i>	1
Near <i>Metschnikowia kunwiensis</i>	1
Near <i>Pseudewrotium zonatum</i>	1
<i>Pichia kluyveri</i>	1
<i>Rhodotorula philya</i>	1
<i>Sporobolomyces phaffi</i>	1
<i>Sporobolomyces roseus</i>	1
<i>Tremella globispora</i> or a sister species	1
<i>Zygosaccharomyces bailii</i>	1

(Continued)

Table 6.4 (Continued)

Method	Yeast species identified	No. of isolates				Reference
		AF	AF (clean)	K	K (clean)	
Pear						Pelliccia et al. 2011
Abate Fetel (AF) and Kaiser (K) varieties of pear were analyzed for surface yeasts before and after cleaning treatment. The D1/D2 domain was amplified and sequenced	<i>Aureobasidium pullulans</i>	1	1	1	3	
	<i>Candida oleophila</i>	2		2		
	<i>Debaryomyces hansenii</i>				1	
	<i>Hanseniopsis uvarum</i>		1	4		
	<i>Metschnikowia fructicola</i>	1			4	
	<i>Metschnikowia pulcherrima</i>		1		2	
	<i>Wickerhamomyces anomalus</i>					
	<i>Meyerozyma guilliermondii</i>	1	4	5	2	
	<i>Pichia kluyveri</i>	1		1		
	<i>Rhodotorula glutinis</i>	1				
	Number of strains	7	7	13	12	
	Number of yeast species	6	4	5	5	
Pear, Plum						Vadkertiová et al. 2012
RFLP-PCR of the ITS regions using restriction endonucleases <i>Hae</i> III, <i>Taq</i> I, <i>Alu</i> I, and <i>Hinf</i> I	Pear	7			7	
	<i>Aureobasidium pullulans</i>					
	<i>Cryptococcus</i> spp.	7			7	
	<i>Galactomyces candidus</i>		13		13	
	<i>Hanseniopsis guilliermondii</i>	57	30		87	
	<i>Hanseniopsis uvarum</i>	3			3	
	<i>Lindnera sargentensis</i>		3		3	
	<i>Metschnikowia pulcherrima</i>	27	47		74	
	<i>Pichia kluyveri</i>	3	7		10	
	<i>Pichia kudriavzevii</i>	23			23	

<i>Pichia membranifaciens</i>	7	13	20
<i>Saccharomyces cerevisiae</i>		3	3
<i>Saccharomycopsis crataegensis</i>		7	7
<i>Wickerhamomyces anomalus</i>		3	3
<i>Zygosaccharomyces bailii</i>		3	3
Plum			
<i>Aureobasidium pullulans</i>	3		3
<i>Candida tropicalis</i>	3		3
<i>Cryptococcus</i> spp.	3		3
<i>Galactomyces candidus</i>		20	20
<i>Hanseniaspora guilliermondii</i>	37	30	67
<i>Hanseniaspora uvarum</i>	37		37
<i>Metschnikowia pulcherrima</i>	3		3
<i>Pichia fermentans</i>		3	3
<i>Pichia kluyveri</i>	17	47	64
<i>Pichia kudriavzevii</i>	27		27
<i>Rhodotorula mucilaginosa</i>	3		3
<i>Saccharomyces cerevisiae</i>	3	43	46
<i>Saccharomycopsis crataegensis</i>	3	3	6
<i>Wickerhamomyces anomalus</i>		7	7

Jujube

Sequence comparisons of the D1/D2 domain and ITS regions

Study focused on three novel species of <i>Metschnikowia</i> :			
<i>Metschnikowia sinensis</i> sp. nov.	2		
<i>Metschnikowia zizyphicola</i> sp. nov.	5		
<i>Metschnikowia shanxiensis</i> sp. nov.	1		

Xue et al. 2006

6.4 Yeast Diversity in Processed Fruits

6.4.1 Processed Grapes (Wines and Musts)

Restriction fragment length polymorphism analysis of the mitochondrial DNA (mtDNA) and the nuclear ITS regions showed a dominance of winery *Saccharomyces cerevisiae* strains immediately following inoculation of musts in a new winery (Constanti et al. 1997). This suggested that early conditions of fermentations allowed starter *S. cerevisiae* strains to inhibit growth of indigenous *Saccharomyces* strains. mtDNA restriction digest patterns of isolates from musts ferment spontaneously without artificial starters and from musts inoculated with a commercial starter showed a total of 18 profiles and a high degree of diversity for indigenous *S. cerevisiae* strains. These profiles also supported a degree of population structure, as strains from the same region appeared more closely related (Constanti et al. 1997). Non-*Saccharomyces* species were characterized by RFLP analysis of 5.8S rDNA, which showed a strong presence of *Candida stellata* (50%) and *Hanseniaspora uvarum* (45%) in the beginning of spontaneous fermentation (Beltran et al. 2002; Constanti et al. 1997). The source of the latter species was likely the berry surface (Constanti et al. 1997), though grape variety did not appear to greatly influence yeast flora on the whole (Beltran et al. 2002). Wild yeasts were completely replaced by winery *S. cerevisiae* strains in middle and later fermentation stages (Constanti et al. 1997), and continuous inoculation of commercial strains gradually reduced the diversity and frequency of indigenous strains (Beltran et al. 2002).

In a similar study, Sabate et al. (2002) also identified *Hanseniaspora uvarum* and *Candida stellata*, with *Aureobasidium pullulans* and *Pichia kluyveri* at greater frequencies than *Candida stellata*. *Rhodotorula mucilaginosa* and *Metschnikowia pulcherrima* were also isolated.

Hierro et al. (2004) used a different set of methods to identify indigenous yeast strains isolated from wine fermentations. The three PCR techniques used primers that targeted intron splice sites, repetitive extragenic palindromic (REP) elements, and enterobacterial repetitive intergenic consensus (ERIC) elements to rapidly and reliably create species-specific, though not strain-specific, fingerprints. These methods identified *Candida stellata* and *Hanseniaspora uvarum* as the most frequent yeasts, followed by *Issatchenkia terricola* and *S. cerevisiae*. Through random application of polymorphic DNA (RAPD) assay, Baleiras Couto et al. (1995) identified the common spoilage yeasts *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *S. cerevisiae*, *Candida valida* and *C. lipolytica* from wine. However, Hierro et al. (2004) identified only three of the five in their study (*Candida valida*, *S. cerevisiae*, and *Zygosaccharomyces bailii*) as spoilage yeasts.

Table 6.5 summarizes a number of studies investigating yeast species diversity on wine fermentations and musts.

6.4.2 Processed Apples (Apple Ciders and Cider Musts)

Similar to that in processed grapes, non-*Saccharomyces* yeasts generally dominated the early stages of apple processing and were then gradually succeeded by strongly fermentative *Saccharomyces* yeasts (Valles et al. 2007). Non-*Saccharomyces* species included apiculate yeasts (*Kloeckera apiculata* and *Hanseniaspora uvarum*), weakly fermentative yeasts (*Hanseniaspora valbyensis*, *H. uvarum*, and *H. osmophila*), and oxidative yeasts

Table 6.5 Yeast species diversity in isolates obtained from wine fermentations and musts.

Method	Yeast species identified	No. or % of isolates	Reference			
Restriction analysis of ITS regions using <i>CfoI</i> , <i>HaeIII</i> , and <i>HinfI</i> , and partial sequencing of the 26S rRNA gene	<i>Hanseniaspora vineae</i>	1	Arias et al. 2002			
	<i>Rhodotorula minuta</i>	1				
RAPD assay and restriction enzyme analysis of PCR amplified rDNA, targeting the common spoilage yeasts <i>Zygosaccharomyces bailii</i> , <i>Z. rouxii</i> , <i>Saccharomyces cerevisiae</i> , <i>Candida valida</i> and <i>C. lipolytica</i>	<i>Candida valida</i>	2	Baleiras Couto et al. 1995			
	<i>Saccharomyces cerevisiae</i>	5				
	<i>Zygosaccharomyces bailii</i>	5				
RFLP analysis of the ITS regions using <i>HinfI</i> , <i>CfoI</i> , and <i>HaeIII</i> , and RFLP analysis of mitochondrial DNA using <i>HinfI</i>	Garnacha must	Day of fermentation		Beltran et al. 2002 (included is only a subset of data)		
	1995	0	2/3		5	End
	<i>Saccharomyces cerevisiae</i>		5%		90%	100%
	<i>Hanseniaspora uvarum</i>	40%	45%		10%	
	<i>Candida stellata</i>	60%	50%			
	2000					
	<i>Saccharomyces cerevisiae</i>		76%		100%	100%
	<i>Hanseniaspora uvarum</i>	10%	12%			
<i>Candida stellata</i>		75%				
Other		15%	12%			
mtDNA restriction analysis using the restriction endonucleases <i>HinfI</i> and <i>AluI</i> , and RFLP analysis of the ITS regions using the restriction endonucleases <i>HinfI</i> and <i>CfoI</i>	Winery <i>Saccharomyces cerevisiae</i>	Not available		Constanti et al. 1997		
	Wild <i>Saccharomyces</i> sp.					
	<i>Hanseniaspora uvarum</i>					
	<i>Candida stellata</i>					
Analysis of PCR fingerprints obtained from primers complementary to intron splice sites, REP, and ERIC elements. RFLP analysis of the ITS regions using <i>CfoI</i> , <i>HaeIII</i> , and <i>HinfI</i> was applied for ambiguous fingerprints	<i>Candida stellata</i>	21	Hierro et al. 2004			
	<i>Hanseniaspora uvarum</i>	8				
	<i>Issatchenkia terricola</i>	4				
	<i>Saccharomyces cerevisiae</i>	3				
	<i>Dekkera bruxellensis</i>	4				
Restriction analysis of the ITS regions using <i>CfoI</i> , <i>HaeIII</i> , and <i>HinfI</i> . mtDNA restriction, using <i>HinfI</i> , was applied for <i>Saccharomyces</i> strain characterization		20 colonies total		Sabate et al. 2002		
	<i>Hanseniaspora uvarum</i>	60%				
	<i>Aureobasidium pullulans</i>	15%				
	<i>Pichia kluyveri</i>	10%				
	<i>Candida stellata</i>	5%				
	<i>Metschnikowia pulcherrima</i>	5%				
<i>Rhodotorula mucilaginosa</i>	5%					

ERIC, enterobacterial repetitive intergenic consensus; ITS, internal transcribed spacer; PCR, polymerase chain reaction; RAPD, random application of polymorphic DNA; REP, repetitive extragenic palindromic; RFLP, restriction fragment length polymorphism.

(*Metschnikowia pulcherrima* and *Pichia guilliermondii*). In contrast, *Saccharomyces bayanus* and *S. cerevisiae* are strongly fermentative (Valles et al. 2007).

The study by Coton et al. (2006) also indicated *Saccharomyces bayanus* and *S. cerevisiae* as predominant species in apple cider. Other frequently isolated species were *Lachancea cidri* and *Dekkera anomala*. The yeasts *Candida sake*, *C. stellata*, *C. tropicalis*, and *Kluyveromyces marxianus* were also identified, though they had never been isolated from apple musts previously (Coton et al. 2006). Three new *Candida* species were discovered from apple cider isolates through sequencing of the D1/D2 domain (Kurtzman et al. 2001).

Table 6.6 summarizes a number of studies investigating yeast species diversity on apple ciders and cider musts.

6.4.3 Processed Olives

Using RFLP analysis of the ITS regions as well as sequence analysis of the D1/D2 domain, Arroyo-López et al. (2006) identified *Saccharomyces cerevisiae* and *Candida boidinii* as the dominant species isolated from green seasoned olives and processed black olives, respectively. These molecular methods also allowed for the identification of *Issatchenkia occidentalis*, *Geotrichum candidum*, and *Hanseniaspora guilliermondii*, none of which had been identified previously in table olives. Physiological and morphological tests were also performed but were insufficient methods of identification on their own; however, those tests consistently confirmed the results of molecular identification (Arroyo-López et al. 2006).

A similar study by Coton et al. (2006) also established *Candida boidinii* as a dominant species in black olive fermentation; however, *Pichia anomala* and *Debaryomyces etchellsii* were also dominant, while *Saccharomyces cerevisiae* was not isolated. Other yeasts included *Candida atlantica*, *C. pararugosa*, *C. diospyri*, *C. ishiwadae*, *Zygoascus hellenicus*, *Citeromyces matritensis*, and *Saccharomycopsis malanga*. The RFLP method also allowed for the identification of six species that had not been previously identified in black olive fermentations: *Candida atlantica*, *C. pararugosa*, and four unknown species (Coton et al. 2006).

Table 6.7 summarizes a number of studies investigating yeast species diversity on processed olives.

6.4.4 Processed Fruit Juices

Recontaminated pasteurized single-strength orange juice possessed a greater diversity of yeasts than recontaminated and pasteurized single-strength grapefruit juice or apple juice (Arias et al. 2002). Among orange juice isolates, *Candida intermedia* and *C. parapsilosis* were the predominant species. *Clavispora lusitaniae* and *Saccharomyces cerevisiae* were also isolated. Distribution of species differed between the types of juice. *Saccharomyces uvarum* was isolated from grapefruit and apple juice, but not orange juice. This study, which used a range of methods for identification, found that partial sequencing of the 26S rRNA gene produced the most accurate results, followed by classic techniques and ITS region analysis. Arias et al. (2002) concluded that analysis of the ITS regions against the extensive ITS database, to which their research contributed six new profiles, was the optimal method for identifying orange juice yeast isolates.

Table 6.6 Yeast species diversity in isolates obtained from apple ciders and cider musts.

Method	Yeast species identified	% of isolates	Reference			
ITS region restriction analysis using <i>CfoI</i> , <i>HinfI</i> , and <i>HaeIII</i> . Representative PCR-RFLP profiles were identified by sequencing of the D1/D2 domain	<i>Candida oleophila</i>	1.0	Coton et al. 2006			
	<i>Candida sake</i>	0.5				
	<i>Candida stellata</i>	1.5				
	<i>Candida tropicalis</i>	1.0				
	<i>Dekkera anomala</i>	10.5				
	<i>Hanseniaspora uvarum</i>	3.5				
	<i>Hanseniaspora valbyensis</i>	6.5				
	<i>Kluyveromyces marxianus</i>	1.0				
	<i>Lachancea cidri</i>	15.0				
	<i>Metschnikowia pulcherrima</i>	2.5				
	<i>Pichia delftensis</i>	3.5				
	<i>Pichia misumaiensis</i>	1.5				
	<i>Pichia nakasei</i>	1.5				
Sequencing of the D1/D2 domain	The study examined only the three previously unidentified isolates:	Not available	Kurtzman et al. 2001			
	<i>Candida anglica</i>					
	<i>Candida cidri</i>					
	<i>Candida pomicola</i>					
RFLP analysis of the ITS regions, using <i>CfoI</i> , <i>HaeIII</i> , and <i>HinfI</i> , as well as <i>DdeI</i> and <i>HpaII</i>	800 isolates total, combining data for two sampling times (2001 and 2002) and pneumatic and traditional pressing techniques	% of yeast species during spontaneous fermentations at four sampling stages:	Valles et al. 2007			
		Day 1		Day 4	Day 7/16	Day 20/28
	<i>Hanseniaspora talbyensis</i>	21		12	20.5	32.5
	<i>Hanseniaspora uvarum</i>	27.5		2	3	
	<i>Hanseniaspora osmophila</i>	15.5		2.5	3	0.5
	<i>Metschnikowia pulcherrima</i>	18		4.5	1	
	<i>Pichia guillermondii</i>	0.5				
	<i>Saccharomyces bayanus</i>	16		50	53.5	17
	<i>Saccharomyces cerevisiae</i>	1.5		29	19	50

ITS, internal transcribed spacer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Table 6.7 Yeast species diversity in isolates obtained from processed olives.

Method	Yeast species identified	% of isolates					Reference			
		Seasoned green	Aerobic processed black	Anaerobic processed black	black	black				
RFLP analysis of the ITS regions, using <i>CfoI</i> , <i>HaeIII</i> , <i>HinfI</i> , and <i>ScrFI</i> . For some species, sequence analysis of D1/D2 domain was necessary	<i>Saccharomyces cerevisiae</i>	58				28	Arroyo-López et al. 2006			
	<i>Issatchenkia occidentalis</i>	20								
	<i>Geotrichum candidum</i>	10	7							
	<i>Zygosaccharomyces bailii</i>	5								
	<i>Candida diddensiae</i>	5								
	<i>Candida holmii</i>	2								
	<i>Candida boidinii</i>		70		27					
	<i>Hanseniaspora guilliermondii</i>		15		9					
	<i>Rhodotorula glutinis</i>		8		9					
	<i>Dekkera bruxellensis</i>				18					
	<i>Rhodotorula graminis</i>			9						
RFLP analysis of the ITS regions using <i>CfoI</i> , <i>HinfI</i> , and <i>HaeIII</i> . Representative strains of each PCR-RFLP profile were identified at the species level by sequencing of the D1/D2 domain		Day					Coton et al. 2006			
	Isolates from black olives	1	8	15	29	68		124	173	276
	<i>Pichia anomala</i>		50	100	63.9	58.3		24.6	0.8	
	<i>Candida boidinii</i>				25.6	41.6		40.5	1.6	
	<i>Debaryomyces etchellsii</i>							15.9	52.9	50.0
	<i>Zygoascus hellenicus</i>								27.6	25.0
	<i>Candida pararugosa</i>	100								
	Unknown (closest relative <i>Candida diospyri</i>)		50							
	Unknown (closest relative <i>Citeromyces matritensis</i>)				10.5					
	<i>Candida atlantica</i>							19.0		
	Unknown (closest relative <i>Candida ishiwadae</i>)								5.3	
	Unknown (closest relative <i>Saccharomycopsis malanga</i>)								11.8	
	<i>Pichia membranifaciens</i>									25.0

ITS, internal transcribed spacer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Sancho et al. (2000) used species-specific PCR primers to target the ITS region of *Zygosaccharomyces bailii*, *Z. bisporus*, *Z. rouxii*, and *Torulasporea delbrueckii*. Among these dangerous contaminants, *Z. rouxii* and *Torulasporea delbrueckii* were detected in orange and apple concentrates, as well as in pear pulp. However, the authors used only species-specific primers and thus only known species could be identified.

Based on previous findings, Casey and Dobson (2004) investigated the potential of a real-time PCR system to identify common spoilage yeasts in fruit juice. The species targeted for study were *Zygosaccharomyces bailii*, *Z. rouxii*, *Candida krusei*, *Rhodotorula glutinis*, and *Saccharomyces cerevisiae*. Melting peak analyses of the 5.8S rDNA subunit and the ITS2 regions were found to be rapid and highly discriminatory. Using this real-time PCR method targeting the citrate synthase gene, identification and quantification of *Candida krusei* growth in artificially contaminated apple juice were possible. In a similar study, Renard et al. (2008) investigated the potential of quantitative PCR to identify and quantify common spoilage yeasts in orange juice using melting point analysis of the ITS region. This method was able to reliably distinguish pure cultures of two major spoilage yeasts, *Saccharomyces cerevisiae* and *Hanseniaspora uvarum*. However, a potential amplification bias favored the appearance of the peak associated with *Hanseniaspora uvarum* in samples contaminated by both species. It was concluded that melting peak analysis with universal primers was not the best method for direct identification or quantification (Renard et al. 2008).

Table 6.8 summarizes a number of studies investigating yeast species diversity in processed fruit juices.

6.4.5 Other Processed Fruits

Restriction analysis of the ITS-18S rRNA gene identified *Metschnikowia pulcherrima* as the dominant species isolated from homogenized blackcurrant samples (Senses-Ergul et al. 2006). RAPD analysis was performed within this species to differentiate strains. Two of the five *Metschnikowia pulcherrima* strains possessed different profiles. Other yeast species isolated in common with studies of other processed fruits included *Pichia anomala* and *Rhodotorula mucilaginosa* (Senses-Ergul et al. 2006).

Other studies targeted common spoilage yeasts in processed fruits to investigate the potential of molecular methods in tracking contamination and spoilage yeasts. Martorell et al. (2005) found that *Zygosaccharomyces bailii* and *Z. rouxii* were dominant in samples taken from syrup and nougats of candied pumpkin, cherry, and orange, throughout the manufacturing process. Results indicated that *Z. bailii* was responsible for the fermentation of the spoiled nougats (Martorell et al. 2005). Baleiras Couto et al. (1995) used RAPD assays and restriction enzyme analysis to identify and differentiate the common spoilage yeasts *Zygosaccharomyces bailii*, *Z. bailii*, *Saccharomyces cerevisiae*, *Candida valida*, and *C. lipolytica* from citrus syrup and tomato sauce. Similar to the previous study, *Z. bailii* and *Z. rouxii* were identified. Both molecular methods were able to identify these yeasts (Baleiras Couto et al. 1995).

Table 6.9 summarizes a number of studies investigating yeast species diversity in isolates obtained from other processed fruits that were not described in previous sections.

Table 6.8 Yeast species diversity in isolates obtained from fruit juice processing.

Method	Yeast species identified	No. of isolates	Reference	
RFLP analysis of ITS regions using <i>CfoI</i> , <i>HaeIII</i> , and <i>HinfI</i> , and partial sequencing of the 26S rRNA gene		<ul style="list-style-type: none"> ● PSOJ: pasteurized and recontaminated single-strength orange juice ● SSGJ: pasteurized and recontaminated single-strength grapefruit juice ● SSAJ: pasteurized and recontaminated single-strength apple juice 	Arias et al. 2002	
		PSOJ SSGJ SSAJ		
		<i>Candida intermedia</i>	7	
		<i>Candida parapsilosis</i>	6	
		<i>Candida tropicalis</i>	2	
		<i>Candida zeylanoides</i>	2	
		<i>Clavispora lusitaniae</i>	3	
		<i>Geotrichum citri-aurantii</i>	1	
		<i>Issatchenkia orientalis</i>	1	
		<i>Hanseniaspora uvarum</i>	1	
		<i>Metschnikowia pulcherrima</i>		2
		<i>Pichia anomala</i>	1	
		<i>Pichia jadinii</i>	1	
		<i>Pichia stipitis</i>	1	
		<i>Rhodotorula mucilaginosa</i>	1	1
	<i>Saccharomyces cerevisiae</i>	3		
	<i>Saccharomyces uvarum</i>		1 2	
	<i>Torulaspota delbrueckii</i>	2		
A real-time PCR system, utilizing melting peak analysis of the 5.8S rDNA subunit and the ITS2 region of certain spoilage yeasts	Study targeted only the spoilage yeasts <i>Zygosaccharomyces bailii</i> , <i>Zygosaccharomyces rouxii</i> , <i>Candida krusei</i> , <i>Rhodotorula glutinis</i> , and <i>Saccharomyces cerevisiae</i>	Not available	Casey & Dobson 2004	
Melting point analysis of PCR-amplified ITS region	Study targeted only the spoilage yeasts <i>Saccharomyces cerevisiae</i> and <i>Hanseniaspora uvarum</i>	Not available	Renard et al. 2008	
Species-specific PCR primers for amplification of the ITS region to detect certain dangerous species: <i>Zygosaccharomyces bailii</i> , <i>Z. bisporus</i> , <i>Z. rouxii</i> , and <i>Torulaspota delbrueckii</i>	<i>Saccharomyces</i> sp.	5	Sancho et al. 2000	
	<i>Torulaspota delbrueckii</i>	1		
	<i>Zygosaccharomyces rouxii</i>	9		

ITS, internal transcribed spacer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

Table 6.9 Yeast species diversity in isolates obtained from other processed fruits.

Method	Yeast species identified	No. of isolates	Reference
Citrus syrup and tomato sauce RAPD assay and RFLP analysis of PCR-amplified rDNA targeting the common spoilage yeasts	Citrus syrup		Baleiras Couto et al. 1995
	Tomato sauce		
<i>Zygosaccharomyces bailii</i> , <i>Z. rouxii</i> , <i>Saccharomyces cerevisiae</i> , <i>Candida valida</i> , and <i>C. lipolytica</i>	<i>Zygosaccharomyces rouxii</i>	1	
	<i>Zygosaccharomyces bailii</i>	1	
Spoiled nougat of candied pumpkin, cherry, orange Restriction analysis of ITS regions using <i>CfoI</i> , <i>HaeIII</i> , and <i>HinfI</i> , as well as sequencing the D1/D2 domain.	<i>Zygosaccharomyces rouxii</i>	4	Martorell et al. 2005
	<i>Zygosaccharomyces bailii</i>	7	
	<i>Sporobolomyces roseus</i>	2	
Homogenized blackcurrant RFLP analysis of ITS regions using <i>MspI</i> and <i>HaeIII</i> . RAPD analysis allowed for discrimination of <i>Metschnikowia pulcherrima</i> strains	<i>Debaryomyces hansenii</i>	2	Senses-Ergul et al. 2006
	<i>Cryptococcus albidus</i>	1	
	<i>Aureobasidium</i> sp.	1	
	<i>Hanseniaspora valbyensis</i>	1	
	<i>Metschnikowia pulcherrima</i>	5	
	<i>Lachancea thermotolerans</i>	1	
<i>Pichia anomala</i>	1		
<i>Rhodotorula mucilaginosa</i>	1		

ITS, internal transcribed spacer; PCR, polymerase chain reaction; RAPD, random application of polymorphic DNA; RFLP, restriction fragment length polymorphism.

6.5 Conclusions and Future Perspectives

This chapter reviewed the application of DNA-based molecular methods in strain and species identification of yeasts from fruits. Both fresh and processed fruits from a variety of plants have been investigated. A large diversity of yeast species has been reported, including many novel species. Some of these yeasts, such as *Hanseniaspora guilliermondii*, are commonly found in fresh fruits while others such as *Saccharomyces cerevisiae* are dominant in fermenting juices. Spoilage yeasts are also frequently found in processed juices. Some of these yeasts (e.g., *Pichia guilliermondii* and *Candida krusei*) are opportunistic pathogens and care should be taken before they are given to immunocompromised hosts.

As shown above, much remains to be investigated about yeasts from many common fruits such as mango, strawberries, and blackberries. Due to their structural features, complex fruits such as strawberries and blackberries may contain significant microbes (including yeasts). Indeed, bacterial pathogen contamination has been frequently reported in strawberries.

At present, most molecular surveys have used PCR-RFLP of the ITS regions for their yeast species identification. While useful for a specific known group of yeasts,

this method cannot detect many species, including new species. With the rapid accumulation of ITS sequence data in public databases and the broad acceptance of ITS as a fungal barcode, ITS sequencing is becoming the standard method for species recognition. Similarly, sequencing highly polymorphic markers should be widely used for differentiating strains within individual yeast species.

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7

Current and New Insights on Molecular Methods to Identify Microbial Growth in Fruit Juices

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7.1 Introduction

Fruit juices are acidic products often containing fermentable sugars, making them exposed to spoilage not only by yeasts and molds but also by acid-tolerant bacteria (Stratford et al. 2000). Due to the low pH and water activity as well as the presence of weak-acid preservatives, the number of microorganisms able to grow in fruit juices is restricted (Roberts et al. 2005). Despite these inhibiting conditions, numerous fungi are able to grow in fruit juices, whereas only lactic and acetic acid bacteria have this feature. Among yeasts, *Zygosaccharomyces bailii* is known for its resistance to high acidic conditions, preservatives, and salts (Thomas & Davenport 1985). Some thermoacidophilic spore-forming microorganisms (e.g., *Alicyclobacillus acidoterrestris*) are also able to grow in fruit juices (Walls & Chuyate 2000). Traditional detection methods are mainly based on morphological and phenotypic behaviors of microorganisms growing in fruit juices by plating them in specific media. This conventional methodology usually requires the implementation of up to 90 tests, which results in complexity and long processing times (Barnett et al. 2000). Rapid kit identification techniques (e.g., API 20C AUX system) have been developed to overcome the limitations of using such traditional methods (Arias et al. 2002). Although offering a faster handling time compared to conventional methods, the application of these identification kits remains limited in the food industry. Faster methods based on molecular identification techniques have been developed which also result in higher accuracy and sensitivity. Although these newer molecular techniques present few applications for fruit juice contamination analyses, they represent the potential to replace the use of traditional methods in the food industry.

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7.2 Microorganisms in Fruit Juices

7.2.1 Resistant Yeasts in Fruit Juices

Due to their ability to grow anaerobically as well as their tolerance of high acidic conditions (low pH, presence of acidic preservatives), yeasts are the most important microorganisms spoiling fruit juices (Arias et al. 2002). Moreover, they possess limited biochemical pathways compared to filamentous fungi and their requirements for sugar and nitrogen sources are usually present in fruit juice products (Roberts et al. 2005). It was previously reported that strains of *Saccharomyces cerevisiae* have the highest heat resistance in fruit juices and they can survive 10 minutes at 65°C (Put et al. 1976). Compared to vegetative cells, ascospores were found to be more heat resistant (25–350 times) and the highest $D_{60^{\circ}\text{C}}$ -value for ascospores was 19.2 minutes (among 21 strains tested).

As yeasts have the ability to grow anaerobically, their action on the utilization of carbohydrates leads to the formation of CO_2 and alcohol. Their presence in fruit juices may also enhance turbidity, as well as flocculation, pellicles, and clumping. Another important parameter influencing yeast growth consists of degrading the natural pectin when pectinesterases are produced, leading to enhancing the spoilage process. Fermented flavors can also be tasted when organic acids and acetaldehyde are formed. Table 7.1 shows the most important identified yeasts frequently growing in fruit juices.

Table 7.1 Most frequently identified yeasts in fruit juices.

Yeast species	References
<i>Candida intermedia</i>	(Arias et al. 2002)
<i>Candida parapsilosis</i>	(Arias et al. 2002; Deák & Beuchat, 1993a,b)
<i>Candida tropicalis</i>	(Arias et al. 2002; Deák & Beuchat 1993a; Put et al. 1976; Sen et al. 2013; Suresh et al 1982)
<i>Clavispora lusitaniae</i>	(Arias et al. 2002; Deák & Beuchat 1993a)
<i>Debaryomyces hansenii</i>	(Dragoni & Comi 1985; Put et al. 1976; Suresh et al. 1982)
<i>Hanseniaspora uvarum</i>	(Arias et al. 2002; Dragoni & Comi 1985; Suresh et al. 1982; Walker & Ayres 1970)
<i>Issatchenkia orientalis</i>	(Deák & Beuchat 1993a,c; Suresh et al. 1982)
<i>Lachancea thermotolerans</i>	(Deák & Beuchat 1993a,c; Put et al. 1976)
<i>Pichia fermentans</i>	(Put et al. 1976)
<i>Pichia guilliermondii</i>	(Dragoni & Comi 1985)
<i>Pichia kluyveri</i>	(Arias et al. 2002)
<i>Pichia manshurica</i>	(Pina et al. 2005)
<i>Rhodotorula glutinis</i>	(Casey & Dobson 2004)
<i>Saccharomyces cerevisiae</i>	(Deák & Beuchat 1993a,c; Dragoni & Comi 1985)
<i>Torulaspora microellipsoides</i>	(Casey & Dobson 2004; Deák & Beuchat 1993a; Put et al. 1976; Put & Jong 1982)

7.2.2 Filamentous Fungi (Molds)

Due to the low oxygen tension and/or low redox potential, most molds are not able to grow in fruit juices, as they are strict aerobes, unlike yeasts, with a few exceptions. Spoilage caused by molds occurs mainly on the surface of juices in contact with air by forming colonies and also by flocculation, floating, and clarification by breaking down the pectin. Molds are extremophilic organisms growing and reproducing in conditions with a low availability of water, known as low water activity products. Spoilage of jams and some preserves is usually caused by *Eurotium* species, especially *Penicillium corylophilum* (Pitt & Hocking 2009). According to these authors, the most prominent heat-resistant fungal species growing on fruit-based products are *Byssoschlamys fulva*, *Byssoschlamys nivea*, *Neosartorya fischeri*, and *Talaromyces*. Soil represents the primary source of heat-resistant fungal ascospores, as they usually do not develop in food-processing plants. Screening therefore concerns mainly juice produced from fruits having direct or indirect contact with soil (e.g., grapes, passion fruits, pineapples, mangoes).

7.2.3 Bacterial Growth in Fruit Juices

As reported above, acid-tolerant bacteria can develop in fruit juices (Stratford et al. 2000). Common spoilage bacterial species growing in fruit juices include *Acetobacter*, *Alicyclobacillus*, *Bacillus*, *Clostridium*, *Gluconobacter*, *Lactobacillus*, *Leuconostoc*, *Saccharobacter*, *Zymomonas*, and *Zymobacter* (Eiroa et al. 1999; Foster & Vasavada 2003; Jay 2000; Morton 1998; Pettipher et al. 1997; Splittstoesser et al. 1994; Stratford et al. 2000; Walls & Chuyate 1998).

The main aerobic spoilage species, due to their ability to grow at low pH (3–3.5) with low nutrient amounts, are acetic acid bacteria (e.g., *Gluconobacter (Acetomonas)* spp. and *Acetobacter* spp.). *Lactobacillus* and *Leuconostoc* spp. have also been identified in spoiled fruit juices (Rushing et al. 1956). They are associated with several organoleptic changes resulting from extensive fermentation and CO₂ production (heterofermentative lactobacilli), slime production, buttermilk-like off-flavor (due to the formation of diacetyl), as well as generation of cloudiness and turbidity (Jay 2000). Acetic acid and gluconic acid produced during the development of *Gluconobacter* and *Acetobacter* are the cause of changes of product flavors. Increasing the amount of oxygen, when packaging fruit juices, constitutes a primary source of enhancing this bacterial spoilage, as these microorganisms are strict aerobes.

Four strains of *Lactobacillus plantarum* have been isolated from fresh orange juice (Parish & Higgins 1988). One study demonstrated that in a reconstituted orange juice of pH 3.9, the population of *L. plantarum* did not decrease after 14 days at 4°C (Parish et al. 1990). It has also been demonstrated that *L. plantarum* is able to grow in an orange juice skimmed milk beverage, causing its spoilage (Sampedro et al. 2006).

Alicyclobacillus acidoterrestris is a thermoacidophilic spore-forming bacterial species able to grow on fruit juices and lemonades, causing their spoilage. Products contaminated with *A. acidoterrestris* show visible growth as well as the production of off-flavors, thus changing the organoleptic properties (Baumgart et al. 1997; Stratford et al. 2000). This bacterium was first isolated from spoiled orange juice and classified as

Bacillus acidocaldarius (Cerny et al. 1984), then as *Bacillus acidoterrestris* (Deinhard et al. 1987), before being assigned to the genus *Alicyclobacillus* (Wisotzkey et al. 1992). Numerous studies reported the identification of this microorganism in a wide variety of commercial fruit juices (apple, orange, mango, and pineapple) (Baumgart et al. 1997; Chang & Kang 2004; Danyluk et al. 2011; Orr et al. 2000; Parish 1997; Splittstoesser et al. 1994; Yamazaki et al. 1996).

Application of conventional techniques (i.e., selective media, phenotype) or implementing more rapid and sensitive methods such as molecular techniques for facilitating the identification of microorganisms causing fruit juice spoilage have gained more attention. Due to the importance of yeast contamination and the occurrence of *Alicyclobacillus* spp. in fruit juices, both conventional and molecular methods are discussed below.

7.3 Conventional Identification Techniques

7.3.1 Description

Counting techniques are the most commonly used methods for routine microbiological analysis of fruit juices. Enumeration of aerobic viable microorganisms in fruit juices is usually performed using plate count agar (PCA) medium in order to evaluate global contamination. Traditional yeast identification and characterization are based on standard procedures detailed in the literature (Lodder 1970; van der Walt & Yarrow 1984), and in practical manuals (Barnett et al. 1990; Kreger-van Rij 1987; Kurtzman & Robnett 2003). Lactic acid bacteria are frequently enumerated using the Man–Rogosa–Sharpe medium whereas total fungal contamination is usually estimated using acidified potato dextrose agar, Sabouraud agar media or dichloran-glycerol agar (for xerotolerant molds) (Falguera & Ibarz 2014). Pathogenic bacteria analysis, on the other hand, requires more specific media and more sample pre-enrichment steps.

Generally, media used for the isolation and enumeration of foodborne molds are similar to those applied for foodborne spoilage yeasts (Loureiro & Querol 1999). These complex and nutritionally enriched media are usually supplemented with antibiotics against bacteria as well as with mold growth inhibitors (e.g. rose bengal or dichloran), thus giving a global estimation of total yeast contamination. In addition to the media reported above, other specific media have been used for selective yeast identification. These media include assessment of resistance to ethanol, preservatives, and reduced water activity, as well as the use of differential media to identify yeasts degrading some macromolecules (e.g., polysaccharides, proteins, pectins, lipids) (Loureiro & Querol 1999).

Molds (including heat-resistant species) have been identified during concentrated apple juice processing (de Cássia Martins Salomão et al. 2014), using identification media. Strains were first isolated on PDA media then transferred to malt extract agar (MEA), Czapek yeast extract agar (CYA), and 25% glycerol nitrate agar (G25N) (for xerophilic strain identification) media. After 7 days incubation at 25°C (except CYA incubated for 7 days more at 5°C and 37°C), the authors reported the identification of 13 mold strains, 12 belonging to the genus *Penicillium*. Acidothermophilic

spore-forming bacteria, on the other hand, were isolated using BAT broth, revealing the presence of *Alicyclobacillus acidoterrestris* in the samples. A similar study for the identification of acidothermophilic bacteria in orange juice entering processing facilities was performed (Parish & Goodrich 2005). The authors compared results from three common isolation agars (acidified potato dextrose agar, Ali agar, and K agar), and concluded that more than one-third of all sampled fruits were contaminated with presumptive *Alicyclobacillus* strains.

Many other studies have been described in the literature for conventional identification of yeasts cultured from fruit juices (Chang et al. 2013; Henczka et al. 2013; McNamara et al. 2011; Spinelli et al. 2009; Steyn et al. 2011; Tianli et al. 2014). Conventional detection methods are thus based on morphological behaviors and physiological traits. Although conventional techniques offer a high level of discrimination of yeasts and enumeration and detection of low contamination levels, these techniques require long incubation times (usually 3–7 days), and still present limitations for the relevant identification of contamination strains (Falguera & Ibarz 2014).

Fast rapid kit identification methods have been commercialized for the identification of microorganisms. These kits include mainly those commercialized by Biomerieux (France) (e.g., API 20 C AUX, and API rapid ID32 C) and by Remel (USA) (e.g., RapID Yeast Plus System). The RapID Yeast Plus System kit, although based on physiological properties, does not require yeast growth for biochemical test evaluation, and the identification time is reduced to only 4 hours. These kits have been applied to identify yeast contamination in different citrus juices (Arias et al. 2002). The authors compared five different identification methods (26S rRNA gene, internal transcribed spacer region restriction pattern, classic methodology, RapID Yeast Plus system, and API 20C AUX), and reported a total of 99 strains identified. However, only 35% and 13% of the isolates were correctly identified using the RapID Yeast Plus and API 20C AUX systems, respectively.

Candida species have been identified in freshly squeezed fruit juices (Uhtil et al. 2009). The authors determined the degree of yeast contamination in 84 juice samples (orange, lemon, grapefruit, and apples) by isolation on Sabouraud 4% glucose agar plates, followed by identification using the API 20C AUX yeast kit. The common identified strains were *Candida guilliermondii*, *C. krusei*, *C. famata*, *C. spherica*, *C. colliculosa*, *C. albicans*, *Trichosporon mucoides*, *Kloeckera* spp., and yeast-like fungus *Cryptococcus neoformans*. Although application of the RapID Yeast Plus system and API 20C AUX kits for fast yeast identification in fruit juices is described in the literature, these methods were originally designed and commercialized for clinical diagnosis purposes, and their application is generally restricted to a few yeast species (Arias et al. 2002).

7.3.2 Benefits and Limitations

Traditional methods based on morphological and phenotypic identification are cost-effective techniques that are easy to perform for industrial applications for microbiota identification in fruit juices. However, they are time consuming and difficult to handle if a quick result is needed. To overcome these problems, alternative methods consisting mainly of molecular techniques have been developed.

7.4 Non-conventional Identification Techniques

To overcome the limitations occurring when using traditional methods, many techniques for microbial identification have been developed in recent years. These include identification according to the fatty acid composition of the cell membrane, Fourier transform infrared spectroscopy spectra, polymerase chain reaction fingerprinting, real-time polymerase chain reaction, restriction fragment length polymorphism, and sequencing of ribosomal DNA.

7.4.1 Analytical Biochemistry Methods

7.4.1.1 Cell Membrane Fatty Acid Analysis

Fatty acid analysis has been used as a non-conventional method for the identification of growing microorganisms in fruit juices. After a strain's isolation, fatty acids constituting the cell membrane are extracted and analyzed using gas chromatography-mass spectrometry, which generally characterizes unique species. In this line, omega-alcyclic fatty acids (e.g., omega-cyclohexane and omega-cycloheptane) have been associated with the presence of *Alicyclobacillus* species, as they constitute the major components of the cellular membranes (Duvenage 2006). Omega-cyclohexyl fatty acid, obtained by cell membrane fatty acid analysis, has been reported as the main fatty acid of *Alicyclobacillus acidoterrestris* cell membranes, growing in fruit juices (Jensen & Whitfield 2003).

Cell membrane fatty acid analysis has been used to characterize yeasts (Augustyn & Kock 1989; Oosthuizen et al. 1987; Razes et al. 1992; Tredoux et al. 1987). The technique has shown potential to differentiate fermenting yeasts from spoilage yeasts (Razes et al. 1992) which represents a great advance for facilitating identification. However, it has been shown that this method was not able to differentiate between the three major fermentative *Saccharomyces* species: *S. cerevisiae*, *S. bayanus*, and *S. pastorianus* (Augustyn & Kock 1989).

7.4.1.2 Fourier Transform Infrared Spectroscopy Identification

Changes in the dipole moment due to vibrations constitute the Fourier transform infrared (FT-IR) spectrum, which is characteristic for any chemical molecule. Each biological material may present a complex and specific FT-IR spectrum representing a characteristic fingerprint (Kümmerle et al. 1998). Based on this technique, the identification of several microorganisms has been described in the literature (Naumann 1985; Naumann et al. 1988). The principle of the method consists of comparison between the FT-IR spectrum of an unknown isolate and the FT-IR spectra database of known isolates. When the unknown spectrum is very similar to that of a known strain in the database, identification is possible. The application of the FT-IR method therefore depends primarily on the reference library database, and has been successfully applied to identify some species belonging to the genera *Lactobacillus* (Curk et al. 1994), *Actinomyces* (Haag et al. 1996), *Listeria* (Holt et al. 1995), and many other species.

The FT-IR method for discrimination between *Bacillus* and *Alicyclobacillus* isolates has been successfully applied to apple juice (Al-Holy et al. 2015). The authors described the identification and discrimination between four *Alicyclobacillus* strains and four *Bacillus* isolates inoculated individually into the analyzed samples. They reported that

FT-IR spectroscopy, in combination with multivariate analysis, constitutes a rapid and powerful tool for differentiation between *Bacillus* and *Alicyclobacillus* in fruit juice, for industrial applications.

7.5 Molecular Techniques

Molecular techniques have emerged as the most important investigation tool for microbial identification. These techniques revolutionized all fields of microbiology. In this chapter, due to the vast literature on the progress made in these techniques, only selected examples of each described technique will be cited, giving an overview of molecular techniques used for the detection and identification of microorganisms in fruit juices.

7.5.1 Polymerase Chain Reaction Fingerprinting

The polymerase chain reaction (PCR) is a powerful technique that has provided new opportunities to develop faster and more convenient methods for microbial detection and identification. Among the different applications of PCR, random amplified polymorphic DNA (RAPD) analysis and amplified fragment length polymorphism (AFLP) analysis were the first developed (Baleiras Couto et al. 1994, 1995, 1996; van der Vossen et al. 2003; Vos et al. 1995). These methods are able to analyze a large section of the genome and reveal polymorphisms differentiating the species and strains present in a sample. When performing RAPD analysis, DNA is first amplified by PCR using single and short primers (10–15 bp) hybridizing a set of arbitrary loci in the genome.

PCR-fingerprinting and RAPD approaches have been applied for tracing yeast contamination in a carbonated orange juice production chain (Pina et al. 2005). The authors analyzed the presence of 58 yeast isolates in carbonated orange juice samples. They used RAPD with the primer P24 and the PCR-fingerprinting technique with the microsatellite primers (GTG)₅ and (GAC)₅. The most frequent isolates were identified at the end stage of orange juice preparation as well as before and after pasteurization of the juice. The contaminant strains were identified as *Pichia galeiformis* by D1/D2 26S rRNA gene sequence analysis. *Alicyclobacillus acidoterrestris*, an acidophilic thermophilic spore-forming microorganism contaminating fruit juices, has been identified using RAPD in pasteurized exotic Brazilian fruit (passion fruit and pineapple) juices (McKnight et al. 2010). *Alicyclobacillus* spp. were detected in passion fruit juice but not found in any pineapple juice samples. The strains were identified using RAPD-PCR, revealing high genetic similarity between *Alicyclobacillus acidoterrestris* DSM 2498 and the detected strains in passion fruit juice.

7.5.2 Real-time Polymerase Chain Reaction

Real-time polymerase chain reaction (real-time PCR) and quantitative polymerase chain reaction (qPCR) methods have been successfully applied to detect the presence of spoilage yeasts in fruit juices (Casey & Dobson 2004). This method is based on the selection of a targeted yeast population in the analyzed sample, thus reducing the time consumed by traditional detection and identification methods. Similarly, a real-time PCR system

has been used for differentiation between *Saccharomyces cerevisiae* and some frequent spoilage yeasts (*Zygosaccharomyces bailii*, *Z. rouxii*, *Candida krusei*, *Rhodotorula glutinis*) in artificially contaminated apple juice samples (Casey & Dobson 2004). The real-time PCR method applied to these yeasts was based on analysis of the 5.8S rDNA subunit and the adjacent ITS2 region. The authors targeted the *C. krusei* citrate synthase gene, using real-time PCR, and reported the sensitivity of the method for both identification and quantification of this yeast in the analyzed samples.

Zygosaccharomyces bailii, a major spoilage organism in food and beverages, has also been identified by applying real-time PCR to fruit juices (Rawsthorne & Phister 2006). The authors targeted the gene coding for the D1/D2 loop of the 26S ribosomal RNA subunit to identify this microorganism. Using this method, contaminations as low as two cells per mL were detected in cranberry, raspberry, and apple juices, whereas 22 cells per mL were quantified in grape juice. The correlation between real-time PCR and total *Z. bailii* cell count was high as determined by fluorescent microscopy. Application of this assay, using double-stranded DNA binding dye SYBR Green, resulted in detection of *Z. bailii* in contaminated samples, thus providing a rapid and accurate method to determine both viable and non-viable cells.

By the application of whole genome amplification and real-time PCR for spoilage yeast detection and identification in orange juice, the detection level of *Saccharomyces cerevisiae* was decreased from 10^6 to 10^2 CFU/mL (Renard et al. 2008). The authors reported the distinction between the PCR products of the 5.8S internal transcribed spacer (ITS) region of two major spoilage yeasts in orange juice: *S. cerevisiae* and *Hanseniaspora uvarum*. The method, although sensitive, showed limitations in mixed-contaminated samples. In fact, preferential appearance of the melting peak coincidence with *H. uvarum* has been observed, except when high levels of *S. cerevisiae* are present.

7.5.3 Restriction Fragment Length Polymorphism

Restriction fragment length polymorphism (RFLP) represents a detected difference in homologous DNA sequences after digestion with specific restriction endonucleases generating different length fragments. The RFLP technique is considered a molecular marker which specifically addresses a single clone/restriction enzyme combination. The recognition is based on the hybridization of a labeled DNA sequence (RFLP probe) with fragment(s) of digested DNA, and gel electrophoresis separation.

Given the reliability of this technique, it has been applied to identify microorganisms in fruit juices. Genetic diversity of yeasts from fermented orange juice was successfully determined using PCR-RFLP and ITS sequence regions analysis (Soka & Susanto 2010). The authors conducted the analysis using RFLP on the ITS region (including ITS1, 5.8S rRNA gene, and ITS2), amplified with PCR. Three fermented orange juices (Indonesian Medan orange, Sunkist orange, and Indonesian Pontianak orange) were analyzed for total yeast contamination. A total of 24 yeast isolates were analyzed using RFLP and PCR amplification, giving a restriction profile for each type of orange juice. Although all yeasts isolates from the same type of orange juice showed identical restriction patterns, ITS region sequences showed the presence of three species in each type of juice (*Pichia veronae*, *Cryptococcus albidosimilis*, and *Issatchenkia orientalis*).

Similarly, yeast species have been identified from orange fruit and juice using two molecular techniques: RFLP analysis of PCR-amplified 5.8S rRNA gene and the two ITS

regions, and the analysis of the ITS region sequences (Las Heras-Vazquez et al. 2003). The authors reported the identification of nine different restriction profiles corresponding to *Candida tropicalis*, *Clavispora lusitaniae*, *Hanseniaspora uvarum*, *Pichia anomala*, *Pichia fermentans*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, *Saccharomyces unisporus*, and *Trichosporon asahii*. The results were checked and compared to the morphological properties of each strain, giving good correlation.

7.5.4 Sequencing of Ribosomal DNA

Although ribosomal RNA (rRNA) is highly conserved throughout nature, some segments are species variable. This property led to the development of rDNA sequencing for the identification of microorganisms. Consequently, rDNA sequences are well known for a wide range of microorganisms, including yeasts, and constitute a routine application for diagnosis and identification purposes (Valente et al. 1999). Tandem and repeat units in rDNA consist of conserved and variable regions of several hundred copies per genome. These regions encode small 18S, 5.8S, 5S, and large 25–28S subunits. ITS are variable regions between the different subunits, containing information to differentiate genera and species (Kurtzman 2003; Valente et al. 1999).

Based on 16S rDNA sequencing, *Alicyclobacillus acidocaldarius* has been isolated and identified from mango juice and concentrate (Gouws et al. 2005). The authors tested the presence of *Alicyclobacillus* in mango concentrate, as well as other manufacturing ingredients, using PCR and sequencing analyses. After incubation at 55°C on YSG agar medium, spore-forming, acid-dependent, and thermotolerant bacteria were detected. *A. acidocaldarius* identification was performed by amplifying the 16S rDNA variable region and its sequencing. The authors concluded that with these newer molecular techniques, *A. acidocaldarius* was able to survive the acidic conditions and pasteurization, and grow in mango juice after aseptic packaging.

Using the same methodology, spoilage yeasts were identified during the production of candied fruit nougats (Martorell et al. 2005). The authors analyzed the presence of four yeast species (*Zygosaccharomyces bailii*, *Z. rouxii*, *Sporobolomyces roseus*, and *Debaryomyces hansenii*) as well as a filamentous fungus (*Nectria mauriiticola*). The strains were identified according to the restriction analysis of 5.8S-ITS rDNA. *Z. rouxii* and *Z. bailii* were isolated and identified as the main spoilage strains of candied fruits. These molecular methods have therefore been found suitable for differentiating *Zygosaccharomyces* species isolates.

7.5.5 Benefits and Limitations of Molecular Methods

Molecular diagnostic methods represent advantageous alternatives to traditional immunoassays and culture techniques and provide faster and more sensitive identification results. These molecular techniques, replacing the traditional methods, are currently limited to a few targeted genera and species in the food industry. Several factors have hindered the widespread application of molecular diagnostic assays in replacing traditional phenotypic and morphological characterization.

Besides the high cost of molecular diagnosis methods, as well as the highly-qualified personnel required to monitor such experiments, the abundance of false-positive and false-negative results constitutes one of the major issues. False-positive detections are

mainly caused by the presence of exogenous DNA (from the instruments used for the preparations, the laboratory equipment, and the environment). This DNA is different from the living cell contaminations, as it is difficult to remove by wiping surfaces and lab equipment. The presence of inhibitors causes false-negative results. When the analyzed sample contains chemical compounds, they may interfere with the enzyme activity. To overcome this problem, some matrices need to be treated to remove inhibitors before nucleic acid processing.

7.6 Conclusions and Future Perspectives

Different methods have been suggested for the identification of microorganisms in fruit juices. Traditional techniques, based on morphological and phenotypic identification, are cost-effective and easy to perform for industrial applications for microbiota identification. However, these techniques require long incubation times, are difficult to handle if a quick result is needed, and have limitations for the relevant identification of contamination strains. To overcome these limitations, other techniques such as non-conventional identification techniques and molecular techniques for microbial identification have been suggested. Compared to traditional immunoassays and culture techniques, these methods have some advantages such as providing faster and more sensitive identification results. However, they also have some drawbacks such as high costs, requirement for highly-qualified personnel to monitor the experiments and the abundance of false-positive and false-negative results. Thus, more research is needed to prepare new high-accuracy, quick, and cost-effective methods for identification of microorganisms in the food and beverage industries.

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Section III

Fish and Meat Products (Non-Fermented)

8

Molecular Techniques Related to the Identification of the Bacterial Flora of Seafood

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8.1 Introduction

The bacterial flora of fish and shellfish can be divided into two major groups, those microorganisms involved in refrigerated spoilage and those involved in human infections. Among the psychrotrophic seafood spoilage bacteria, relatively few genera and species are considered intense spoilage organisms.

An early study was undertaken by Castell and Anderson (1948) to identify the major intense fish spoilage bacterial genera and species. Three categories of pure cultures were described. The first group of organisms represented by enteric bacteria, bacilli, and micrococci yielded no off-odors at 3°C because they were unable to grow at this low temperature. The second group produced musty, sour or sweetish odors at 3°C and consisted of flavobacteria, *Achromobacter*, and micrococci. The third group consisted of organisms that produced offensive odors rapidly at 3°C: *Pseudomonas* spp., *Achromobacter* spp., *Serratia marcescens*, and *Proteus vulgaris*. *Proteus vulgaris* is never found on seafood and the isolate used was from the American Type Culture Collection (ATCC) (C. Castell, personal communication). This early study reduced the intense spoilage organisms primarily to members of the genera *Pseudomonas* and *Achromobacter*. A later study, to identify the major psychrotrophic bacterial genera on freshly caught cod, was by Georgala (1958). Among a total of 727 isolates, the following were identified: 51.5% *Pseudomonas*, 41.8% *Achromobacter*, 3.3% *Vibrio*, 1.5% *Flavobacterium*, 0.7% *Micrococcus*, and 0.7% miscellaneous.

More recent molecular studies have confirmed these earlier taxonomic conclusions regarding the major psychrotrophic spoilage groups. Since these early studies, various attempts have been made to further elucidate the major intense fish spoilage bacterial genera and species. This has resulted in expansion and taxonomic alterations of the two originally recognized intense spoilage genera, *Pseudomonas* and *Achromobacter*, which this chapter elucidates.

Human infectious and toxigenic bacteria associated with seafood include *Vibrio cholerae*, *V. parahaemolyticus*, *Vibrio vulnificus*, *Aeromonas hydrophila*, *Plesiomonas*

shigelloides, *Listeria monocytogenes*, and *Clostridium botulinum* type E which can now be rapidly detected using one or more molecular techniques which are described in this chapter.

8.2 Major Seafood Spoilage Bacteria

8.2.1 The Genus *Pseudomonas*

The genus *Pseudomonas* consists of obligately aerobic Gram-negative rods with polar flagella. The molar G + C content for members of this genus is recognized as being from 58% to 70%. Any organism outside this range is considered to not be a member of the genus *Pseudomonas*. The intense fish spoilage psychrotrophic species of the genus *Pseudomonas* can be divided into two convenient major groups consisting of fluorescent and non-fluorescent isolates. Among the fluorescent pseudomonads, we find that isolates of *P. fluorescens* are protease positive while isolates of *P. putida* are protease negative which constitutes the major distinction between these two intense fish spoilage fluorescent species. Stanier et al. (1966) established seven biotypes for isolates of *P. fluorescens* (A–G) and two biotypes (A and B) for isolates of *P. putida* based on metabolic characteristics which Gennari and Fragotto (1992) employed for distinguishing fluorescent isolates from seafood and other food products. However, when it comes to the non-fluorescent fish spoilage pseudomonads, little is known regarding their species designations.

Shewan et al. (1960a) established a broad grouping of Gram-negative organisms found on fish and in other habitats which was based on 10 phenotypic characteristics. They then applied the Hugh–Leifson test to distinguish the various Gram-negative organisms prevailing on fish which yielded four distinguishable metabolic groups of *Pseudomonas* (Figure 8.1). This grouping of *Pseudomonas* isolates from seafood is still used since many such isolates do not adhere to recognized species of *Pseudomonas*.

Shewan et al. (1960b) presented a dichotomous key for the screening of cultures from seafood involving all the major genera. This diagrammatic outline made use of the Gram stain, pigmentation, flagellation, the cytochrome oxidase test (Kovács 1956), and the medium of Hugh and Leifson (1953) for the determination of oxidative versus fermentative metabolism and is presented in Figure 8.2.

8.2.1.1 *Pseudomonas fragi*

Among the off-odors that frequently develop during the early stages in the spoilage of refrigerated fillets are those that have been described as “sweet” and “fruity.” The responsible organism was identified as *Pseudomonas fragi*, which has been characterized as producing a “sweet, ester-like odor resembling that of the flower of the May apple” (Castell et al. 1959). Isolates of *P. fragi* from seafood are characterized as being non-fluorescent, non-proteolytic, do not produce trimethylamine or H₂S, but are capable of producing ammonia from amino acids, are lipolytic and are isolated from both fresh and spoiled fillets (Castell et al. 1959).

8.2.1.2 *Pseudomonas perolens*

During the early stages of fish spoilage, a “musty” odor is sometimes noted. When the implicated organisms are in pure culture they give rise to a “stored potato” odor.

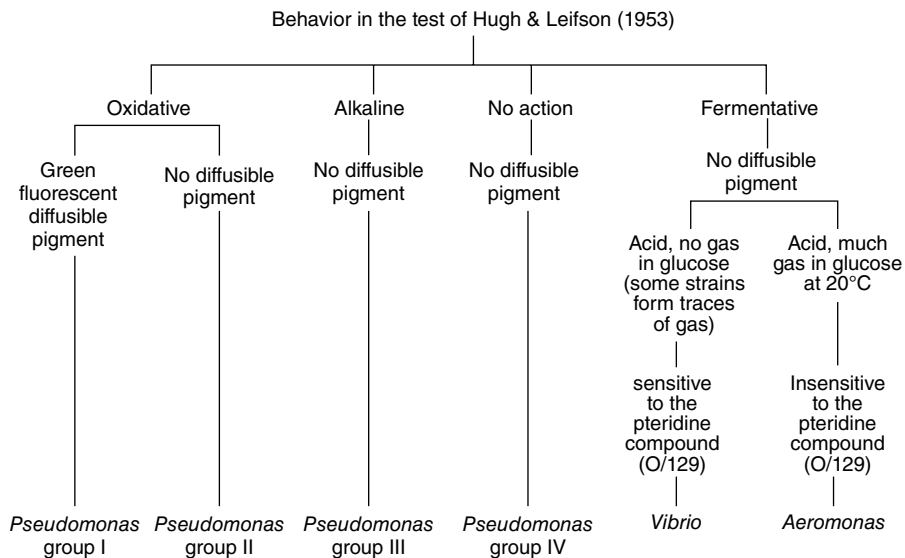


Figure 8.1 A grouping of the Gram-negative asporogenous rods, polar-flagellate, oxidase positive and not sensitive to 2.5 i.u. of penicillin, on the results of four other tests. Redrawn from Shewan et al. (1960a) with permission. Reproduced with permission of John Wiley and Sons.

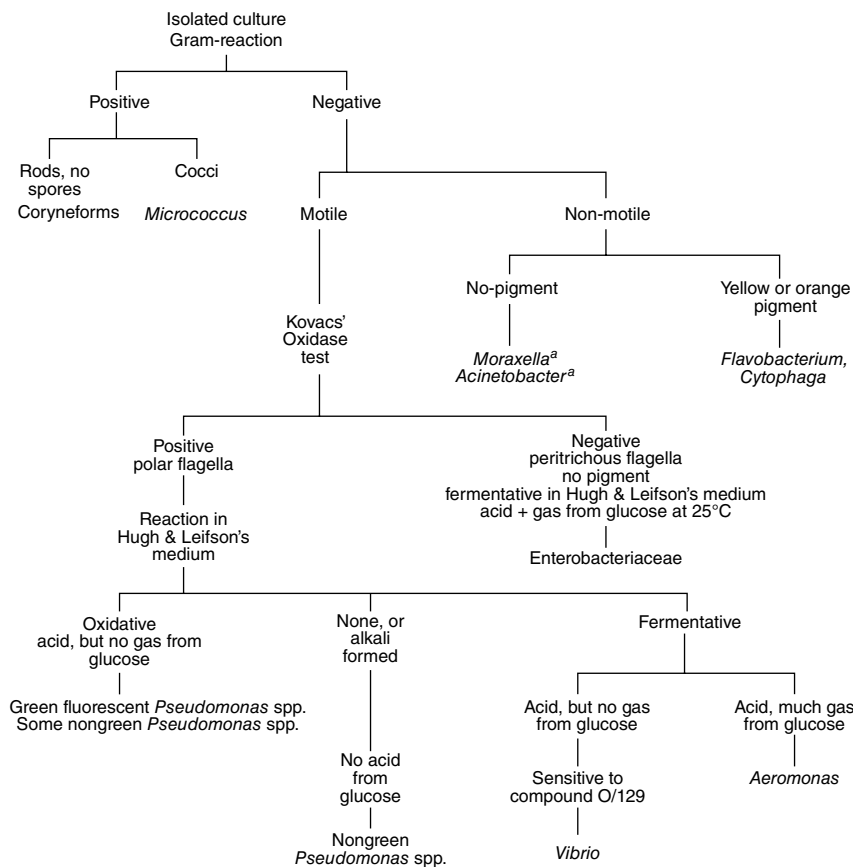


Figure 8.2 Outline of the sequence of tests used in the screening of bacterial cultures from seafood. Redrawn from Shewan et al. (1960b) with permission. ^aAllocated to the genus *Achromobacter* in original figure. Reproduced with permission of John Wiley and Sons.

The responsible organism has been found to be *P. perolens*. Isolates of this organism species are neither proteolytic nor lipolytic, do not produce trimethylamine, and produce little or no change in milk, but do produce ammonia from amino acids, and produce H₂S (Castell et al. 1957).

8.2.2 The Genus *Alteromonas*

The genus *Alteromonas* was originally created to accommodate organisms having typical phenotypic characteristics of the genus *Pseudomonas* but which have a molar G + C content of less than 58%, thereby excluding them from the genus *Pseudomonas*.

8.2.2.1 *Alteromonas nigrifaciens*

Alteromonas nigrifaciens was originally known as *Pseudomonas nigrifaciens* in the older literature. This is an extremely intense fish spoilage organism characterized by producing an intense black melanin type of pigment. The organism is often overlooked in that maximum pigment production occurs only with 1.5–2.5% NaCl added to the culture medium with incubation from 4°C to 15°C. The presence of tyrosine (0.1%) has been found to be essential for pigment production (Ivanova et al. 1996). In the absence of pigment production, this organism appears as a typical pseudomonad. Cells are motile by means of a single polar flagellum. Cultures are obligately aerobic, cytochrome oxidase positive, gelatinase positive, lipase positive, amylase positive, and produce putrescine, cadaverine, and spermidine. Sodium ions are required for growth. The molar G + C content is 39–41%.

8.2.3 The Genus *Shewanella*

Members of the genus *Shewanella* were formerly considered pseudomonads. Venkateswaran et al. (1999) reviewed the taxonomy of this genus in detail. All isolates are Gram-negative, non-spore-forming rods, motile by means of a single polar flagellum, and are 2–3 μm in length. There are presently 12 recognized species in this genus, some of which produce salmon or pink-colored colonies. All species are cytochrome oxidase and catalase positive and negative for the production of amylase. Most species are gelatinase positive and lipase is produced by several species. All species reduce trimethylene N-oxide (TMAO) to trimethylamine (TMA) and reduce nitrate to nitrite and the majority produce H₂S from thiosulfate. Several species reduce elemental sulfur.

8.2.3.1 *Shewanella putrefaciens*

The organism currently known as *Shewanella putrefaciens* was first isolated from tainted butter and classified as a member of the genus *Achromobacter* by Derby and Hammer (1931). It was transferred to the genus *Pseudomonas* in 1941 by Long and Hammer (1941). In 1972, it was allocated to the genus *Alteromonas* by Lee et al. (1977) on the basis of its much lower mols % G + C DNA content than the accepted range of 58–70 mols % G + C for members of the genus *Pseudomonas* (Baumann et al. 1972). In 1985, it was transferred to the newly established genus *Shewanella* under the family Vibrionaceae due to its perceived closer relationship with the genus *Vibrio* (MacDonell & Colwell 1985). Strains in this species vary from 43% to 48% G + C (Levin 1972; Nozue et al. 1992; Vogel et al. 1997).

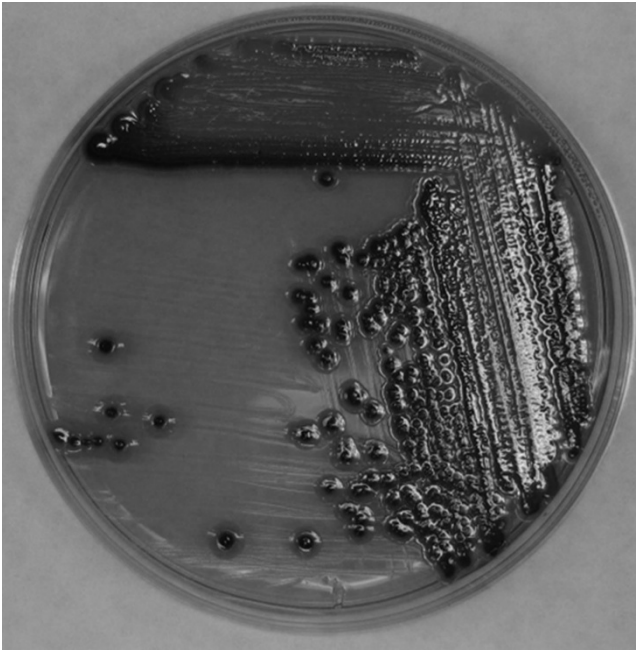


Figure 8.3 Typical colonies with black centers of *Shewanella putrefaciens* on peptone iron agar.

Isolates of *S. putrefaciens* are intense psychrotrophic fish spoilers. On peptone iron agar (IA) well isolated colonies usually produce salmon pigmented colonies with intense black centers while crowded surface colonies produce uniformly black colonies (Figure 8.3). With pour plates of PIA, intensely black pinpoint subsurface colonies develop. All such isolates produce an extracellular DNase (Sadovski & Levin 1969) in addition to an extracellular protease and lipase. Isolates of *S. putrefaciens* have been found on occasion to dominate at the time of intense fish spoilage (Chai et al. 1968). Mg ions are a critical requirement for maintaining the integrity of the cell membrane (van Sickle & Levin 1978). If one prepares decimal dilutions of fish tissue in saline for plate counts, the organism will rupture unless at least 0.001 M Mg^{++} ions are added to the saline by way of $MgCl_2$. This requirement is not widely recognized. Phosphate buffer enhances the lytic phenomenon by presumably pulling Mg^{++} ions out of the membrane or sacculus.

8.2.4 The Genera *Moraxella* and *Acinetobacter*

The genera *Moraxella* and *Acinetobacter* are Gram-negative, non-pigmented, non-flagellated, obligately aerobic coccobacilli that were originally allocated to the genus *Achromobacter* in the older literature. The genus *Achromobacter* was eventually eliminated so that now all such Gram-negative coccobacilli from seafood are placed into either *Moraxella* or *Acinetobacter*. Without species differentiation, such bacterial isolates have often been placed into the “*Moraxella-Acinetobacter*” group. The molar G + C value for isolates of *Moraxella* varies from 40% to 46% and for *Acinetobacter* varies from 40% to 47%. These two genera are distinguished primarily on the basis that the *Moraxella*

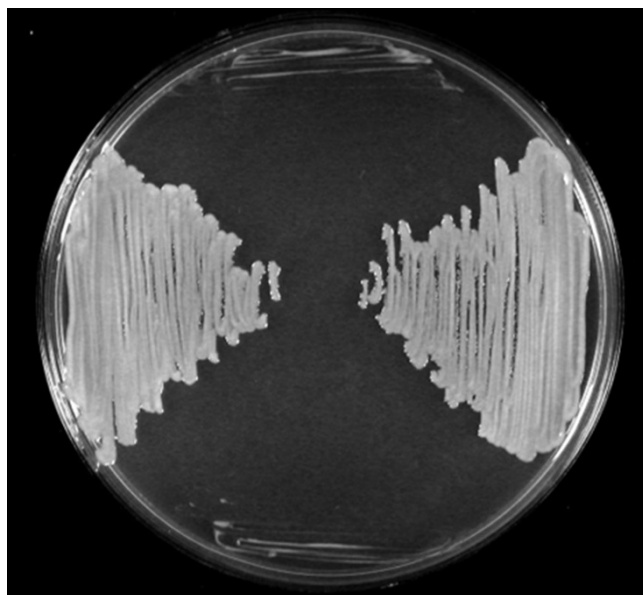


Figure 8.4 Genetic transformation of *Psychrobacter immobilis* whereby growth of recipient A351-Hyx-7 is genetically transformed by DNA from a fishery isolate of *P. immobilis*, allowing growth on an M9A plate lacking hypoxanthine.

are sensitive to penicillin (1 i.u. disk) and are cytochrome oxidase positive while members of the genus *Acinetobacter* are resistant to penicillin and are cytochrome oxidase negative. However, Juni and Hyme (1980) found that fishery isolates of both *Acinetobacter* and *Moraxella* were cytochrome oxidase positive.

A major metabolic distinction between these two genera results from the ability of *Moraxella* isolates to produce significant amounts of phenylethanol from the amino acid phenylalanine (Chen & Levin 1974) while *Acinetobacter* isolates produce little or no phenylethanol. Juni and Hyme (1980) developed a genetic transformation assay whereby the DNA from members of both of these psychrotrophic genera isolated from fish and meat products is able to transform a recipient unable to synthesize hypoxanthine to hypoxanthine synthesis (Figure 8.4). In a subsequent report by Juni and Hyme (1986), the designation *Psychrobacter immobilis* was proposed for all psychrotrophic Gram-negative, aerobic, cytochrome oxidase-positive coccobacilli on the basis of genetic compatibility (genetic transformation).

Quadrants without growth have been streaked with the recipient A351-Hyx culture that requires hypoxanthine without prior contact with exogenous DNA and serve as controls. The procedure as described by Juni and Hyme (1980) is as follows. Several large loops of cell growth from an unknown slant are transferred to a vial of sterile lysing solution (0.05% sodium dodecyl sulfate in 0.15 M NaCl and 0.015 M trisodium citrate) and the cells are dispersed by vigorous agitation. The vials are then held at 65°C for 1 hour to lyse the cells and achieve sterility. A brain heart infusion agar (BHI-A) plate is divided into four sections and a full loop of DNA from each sample is applied as a 1 inch diameter circle to a separate quadrant. A duplicate control plate is prepared for

determining the sterility of the DNA samples. To one set of BHI-A plates, apply a loop of the recipient 135-Hyx-7 culture to each area smeared with DNA and the other plate is left as a DNA sterility control. In addition, one quadrant of the BHI-A plate is inoculated with just the recipient to detect spontaneous revertants. The plate is incubated overnight at 20°C. A loop of cell growth from each area of the BHI-A plate that has grown up is streaked to a quadrant of an M9A agar plate (lacking hypoxanthine) followed by incubation at 20°C for 3 days and observed for the development of extensive growth (genetic transformants) at the end of the streaks (see Figure 8.4). Solid areas of growth at the initial areas of the streaks are discounted due to hypoxanthine carry-over from the BHI-A plate.

8.2.5 The Genera *Flavobacterium* and *Cytophaga*

Members of both these genera are characterized as producing yellow, orange, or red carotenoid pigments. The flavobacteria may be motile by peritrichous flagella or non-motile. The *Cytophaga*, if motile, exhibit gliding motility and lack flagella. Both genera are characterized as being obligately aerobic and weakly active on carbohydrates. Not all isolates of both genera are capable of utilizing glucose. Castell and Maplebeck (1952) examined 245 isolates of *Flavobacterium* (132 yellow and 113 orange) from fish for the ability to exhibit fish spoilage activities. Seventy-eight percent of the yellow isolates and 92% of the orange isolates grew at 2–3°C; 36% of the yellow isolates and only 4% of the orange isolates produced trimethylamine. Forty percent of the yellow isolates and 84% of the orange isolates were proteolytic. When isolates were inoculated onto sterile fish tissue incubated at 3°C, the orange cultures of *Flavobacterium* began to develop disagreeable odors after 5–8 days and many became quite putrid by the 10th or 11th day. Sterile fish tissue inoculated with the yellow cultures yielded no perceptible spoilage odors, even after 15 days, but did discolor the fish tissue yellow, indicative of growth. In contrast, fish tissue inoculated with *Pseudomonas* isolates became offensive after 48 and 72 hours. It is a widely recognized observation that members of the brightly pigmented genera *Flavobacterium* and *Cytophaga* are frequently encountered on fresh fish where they may constitute 10–30% of the initial flora and are rarely among the dominant flora of stale fish.

Although isolates of these pigmented organisms have been found under non-competitive conditions (heavy pure culture inoculation of fish tissue) to eventually spoil fish tissue, on a practical basis, under commercial conditions they are generally outgrown by the more intense spoilage pseudomonads that grow more rapidly under refrigerated conditions than members of these two pigmented genera. As a result, many workers group such isolates into the “*Flavobacterium-Cytophaga*” group rather than attempting to clearly and arduously determine which pigmented genus they belong to.

8.2.6 The Genus *Brochothrix*

These are Gram-positive non-spore-forming rods closely related to the genus *Lactobacillus* and are considered hetero-fermentative with regard to lactic acid production. Log-phase cells are typically rods, while older cells are coccoids, a feature common to coryneforms. Only two species are recognized in this genus: *B. thermosphacta* and

B. campestris. These organisms are important in the spoilage of modified atmosphere (MA) stored seafood. In contrast to *B. thermosphacta*, *B. campestris* is rhamnose and hippurate positive. Both species have a molar G + C content of 36%.

8.2.7 The Genus *Photobacterium*

These are Gram-positive non-spore-forming, peritrichously flagellated rods possessing fermentative metabolism with sugars as carbon and energy sources and are therefore facultative anaerobes. Isolates are luminescent (glow in the dark). The molar G + C for the genus is 39–42%. Because of their facultatively anaerobic metabolism, they have been frequently found to be among the major spoilage organisms in MA storage when oxygen is excluded. The marine species associated with spoiled seafood is *P. phosphoreum*. Sivertsvik et al. (2002) reviewed the relationship between this organism and MA storage.

8.2.8 The Genus *Lactobacillus*

These organisms are Gram-positive, non-spore-forming rods 2–9 μ long. All species of lactobacilli produce at least 1.0% lactic acid from 3–5% glucose, are nutritionally fastidious and catalase negative. Members of the genus *Lactobacillus* do not predominate when seafood is stored under normal iced or refrigerated conditions. However, under conditions of MA storage, lactobacilli can dominate at the termination of storage. They are most readily enumerated from seafood products as dominant members of the prevailing flora with the use of lactobacilli MRS agar which is designed to favor luxuriant growth of all lactobacilli but is not a selective medium. Identification is therefore based on the phenotypic properties of isolated colonies.

8.2.9 The Genus *Aeromonas*

Members of the genus *Aeromonas* are straight rod-shaped Gram-negative, polarly flagellated cells. They are facultative anaerobes exhibiting fermentative metabolism in the absence of oxygen with the production of acid and gas ($H_2 + CO_2$). Isolates are proteolytic, produce extracellular DNase, are cytochrome oxidase positive, and insensitive to the vibriostatic agent 2,4-diamino-6,7-diisopropyl pteridine (O/129). The molar G + C content ranges from 57% to 63%. The genus contains several species pathogenic to fish such as *A. hydrophila* and *A. salmonicida*. Isolates of *Aeromonas* have on occasion been implicated in gastroenteritis (Kirov 1997).

8.2.10 Molecular Techniques for Detection and Enumeration of Seafood Spoilage Bacteria

The lowest number of bacterial colony-forming units (CFU) per gram of fresh raw seafood tissue is about 1×10^4 . On a more practical basis, commercially processed fresh fish fillets will usually have an initial CFU count of about 1×10^5 CFU/gram of tissue. Counts in the range of 10^7 to 10^8 per gram are usually associated with some degree of spoilage and poor quality.

Universal primers have been successfully applied to the quantification of the total bacterial population on fish tissue (Lee & Levin 2006a,b, 2007). The universal forward

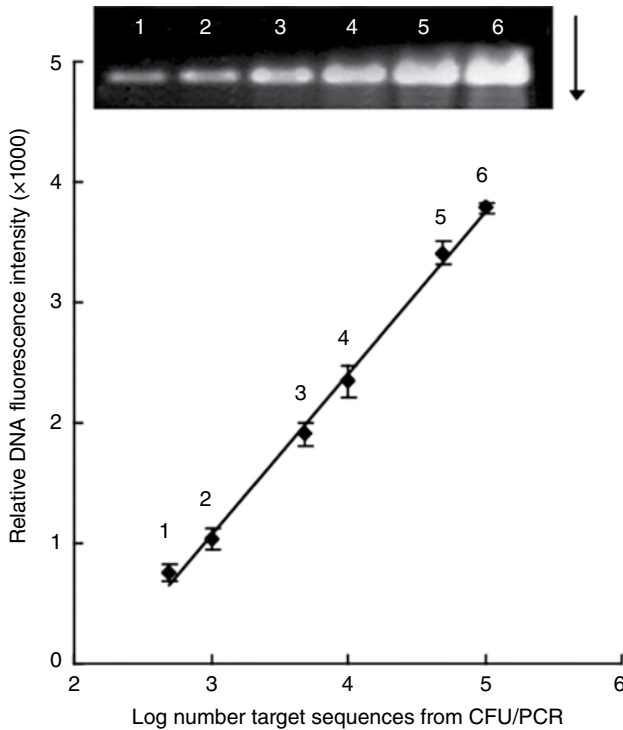


Figure 8.5 Relationship between the relative fluorescent intensity of DNA bands derived from PCR amplification and the number of DNA target sequences derived from CFU. Plotted values are the means and standard deviations derived from three independent assays. *Inset*: Image of PCR-amplified product of mixed culture of bacterial fish flora with varying CFU/PCR: lanes 1–6, 5×10^2 , 1×10^3 , 5×10^3 , 1×10^4 , 5×10^4 , and 1×10^5 CFU respectively). Numbers above plotted points correspond to insert lanes. From Lee and Levin (2006b). Reproduced with permission of Taylor & Francis.

primer DG74 5'-AGG-AGG-TGA-TCC-AAC-CGA-A-3' and the universal reverse primer RW01: 5'-ACC-TGG-AGG-AAG-AAG-GTG-GGG-AT-3' (Greisen et al. 1994) amplify a 370 bp sequence of the 16S rRNA gene derived from all bacteria. With the use of this pair of universal primers and conventional PCR (Lee & Levin 2006b), a notably close linear relationship was found between the log of CFU of mixed fish flora per PCR determined from plate counts and the relative fluorescent intensity of resulting DNA bands (Figure 8.5). When these universal primers were then applied to haddock and cod fillets stored at 4°C for 14 days, an extremely close linear relationship was obtained between the log of total CFU per gram of fish tissue and relative fluorescence of the resulting DNA bands (Figure 8.6).

In addition, this methodology has been extended to the use of PCR for distinguishing the total number of dead and viable bacteria on fish tissue with the use of the selectively permeable DNA binding dye ethidium bromide monoazide (Lee & Levin 2006c). Seafood isolates of *S. putrefaciens* can be confirmed as such by PCR with the use of the primers SP-1: 5'-TTC-GTC-GAT-TAT-TTG-AAC-AGT and SP-2r: 5'-TTC-TCC-AGC-AGA-TAA-TCG-TTC which amplify a 422 bp section of the Gyr B sequence (Venkateswaran et al. 1999).

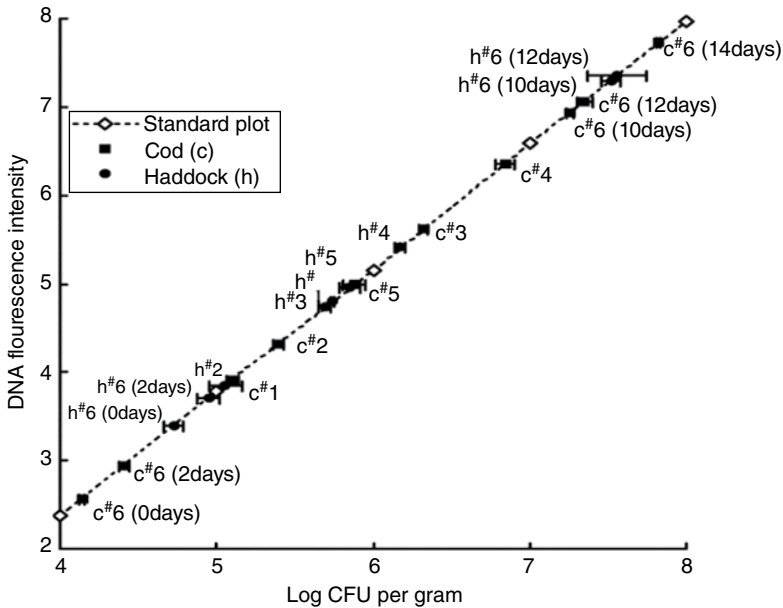


Figure 8.6 Relationship between the relative fluorescent intensity of DNA bands and corresponding CFU derived from plate counts per gram of fish tissue. The dotted line corresponds to the extrapolated standard curve from Figure 8.5. The mean values from three independent assays for each fillet were plotted. From Lee and Levin (2006b). Reproduced with permission of Taylor & Francis.

The development of a primer pair specific for members of the genus *Pseudomonas* (Locatelli et al. 2002) has allowed the use of PCR for the identification of *Pseudomonas* isolates. This PCR assay is ideally suited for the confirmation of presumptive isolates of *Pseudomonas* from seafood and has the potential to be used to numerically quantify the total number of *Pseudomonads* per gram of seafood tissue. The assay is based on the presence of two *Pseudomonas*-specific and conserved sequences, one at the middle of the 16S rDNA sequence and the other at the beginning of the 23S rDNA sequence. As a result, the amplified region includes the 3'-half of the 16S rDNA with the whole 16S-23S rDNA internal transcribed spacer (ITS1) sequence plus the first 25 nucleotides of the 23S rDNA sequence from the 5'-end. The *Pseudomonas*-specific primers generated amplicons of 1300bp. The sequence of the forward primer fPs16S is 5'-ACT-GAC-ACT-GAG-GTG-CGA-AAG-CG and that of the reverse primer rPs23S is 5'-ACC-GTA-TGC-GCT-TCT-TCA-CTT-GAC-C. All 33 *Pseudomonas* strains representing 14 species yielded amplicons while none of the 13 Gram-negative, non-*Pseudomonas* species did, with the exception of *Azotobacter chroococcum*. In addition, several of the *Pseudomonas* yielded two or three bands varying from 1100 to 1300 bp. The multiple bands are thought to reflect the number and variation in the length of ITS1 sequences among strains within a given species.

Spilker et al. (2004) used 16S rDNA sequence data to design PCR assays for identification of members of *Pseudomonas* and for identification of *P. aeruginosa* isolates. The primers PA-GS-F/PA-GS-R (Table 8.1) amplified a 618bp sequence from 28 different species of *Pseudomonas*. Furthermore, a different pair of primers, PA-SS-F/PA-SS-R, amplified a 956bp sequence of all 14 isolates of *P. aeruginosa* tested.

Table 8.1 PCR primers and DNA probes.*

Species or genus	Primer or probe	Sequence (5' → 3')	Size of amplified sequence (bp's)	Gene or DNA target sequence	References
<i>Pseudomonas</i>					
<i>Pseudomonas</i> spp.	P16sf	TGA-AGT-CGT-AAC-AAG-GTA-GC	650	16S-23S rDNA	Jaturapahu et al. (2005) from Sawada et al. (1997)
	P23sr	ATC-GCC-TCT-GAC-TGC-CAA-GG			
<i>P. aeruginosa</i>	pAeru1	CGG-CGA-ATG-TCG-TCT-TCA-CAG		" "	" " " "
<i>P. putida</i>	pPuti 1	GCG-GTA-GAT-GTT-GCT-GC		" "	" " " "
<i>P. fluorescens</i>	pFluo1	GCA-TTC-CAT-TGT-GAT-GAT-GGT-G		" "	" " " "
<i>P. diminuta</i>	pDim2	GAT-ACA-AGT-ATA-CGA-ATA-GAG-CC		" "	" " " "
<i>Pseudomonas</i> spp.	PA-GS-F	GAC-GGG-TGA-GTA-ATG-CC-T	618	16S rDNA	Spilker et al. (2004)
	PA-GS-R	CAC-TGG-TGT-TCC-TTC-CTA-TA			
<i>P. aeruginosa</i>	PA-SS-F	GGG-GGA-TCT-TCG-GAC-TCA	956	" "	" " " "
	PA-SS-R	TCC-TTA-GAG-TGC-CCA-CCG-G			
<i>V. cholerae</i>					
	CTX2	CGG-GCA-GAT-TCT-AGA-CCT-CCT-G	564	<i>ctxA</i>	Fields et al. (1992)
	CTX3	CGA-TGA-TCT-TGG-AGC-ATT-CCC-AC		"	" " " "
	P1	TGA-AAT-AAA-GCA-GTC-AGG-TG	778	<i>ctxB</i>	Koch et al. (1993)
	P3	GGT-ATT-CTG-CAC-ACA-AAT-CAG		"	" " " "
	rtxA-F	CTG-AAT-ATG-AGT-GGG-TGA-CTT-ACG	417	<i>rtxA</i>	Chow et al. (2001)
	rtxA-R	GTG-TAT-TGT-(C/T)CG-ATA-TCC-GCT-ACG			
	rtxC-F	CGA-CGA-AGA-TCA-TTG-ACG-AC	263	<i>rtxC</i>	" " " "
	rtxC-R	CAT-CGT-CGT-TAT-GTG-GTT-GC			

(Continued)

Table 8.1 (Continued)

Species or genus	Primer or probe	Sequence (5' → 3')	Size of amplified sequence (bp's)	Gene or DNA target sequence	References
ctxB ₂		GAT-ACA-CAT-AAT-AGA-ATT-AAG-GAT	460	<i>ctxB</i>	" " " "
ctxB ₃		GGT-TGC-TTC-TCA-TCA-AAC-CAC		"	" " " "
VHMF		TGG-GAG-CAG-CGT-CCA-TTG-TG	519	<i>lolB</i>	Lalitha et al. (2008)
VHA-AS5		CAA-TCA-CAC-CAA-GTC-ACT-C		"	" " " "
Outer ctxA-F		TCT-ATC-TCT-GTA-GCC-CCT-ATT-ACG	–	<i>ctxA</i>	Mendes et al. (2008)
Outer ctxA-R		ATA-CCA-TCC-ATA-TAT-TTG-GGA-G		"	" " " " from Li et al. (2002)
Inner ctxA-F		CTC-AGA-CGG-GAT-TTG-TTA-GGC-ACG	302	<i>ctxA</i>	" " " " from Keasler & Hall (1993)
Inner ctxA-R		TCT-ATC-TCT-GTA-GCC-CCT-ATT-ACG		"	" " " " " " " " " " "
rfbN-F		GTT-TCA-CTG-AAC-AGA-TGG-G	198	<i>rfbN</i>	" " " " from Islam et al. (2004)
rfbN-R		GGT-CAT-CTG-TAA-GTA-CAA-C		"	" " " " " " " " " "
CT-F		ACA-GAG-TGA-GTA-CTT-TGA-CC	308	<i>ctx</i>	" " " " from Nair et al. (1994)
CT-R		ATA-CCA-TCC-ATA-TAT-TTG-GGA-G		"	" " " " " " " " " "
RAPD 1		GGT-GCG-GGA-A	–	–	Coelho et al. (1995)
RAPD 2		AAC-GGT-GAC-C	–	–	" " " "
RAPD 3		CCA-GAT-GCA-C	–	–	" " " "
RAPD 4		AAG-ACC-CCT-C	–	–	" " " "
RAPD 5		CTT-CAG-AGT-AGA-ACG-CAA-TG	–	–	" " " "
RAPD 6		GAC-ATA-AGA-ACA-AGT-TAC-AG	–	–	" " " "
RAPD 7		CGC-TAG-CAA-TTA-ATG-TGC-ATC	–	–	" " " "
RAPD 8		GCT-CTA-GAT-AAG-AAC-AAG-TTA-CAG-ACG	–	–	" " " "
RAPD 9		CGC-GGA-TCC-ATA-AGA-ACA-AGT-TAC-AGA-CG	–	–	" " " "
RAPD 10		AGA-GGG-CAC-A	–	–	" " " "

NA1	GGA-TCA-GAA-TGC-CAC-GGT-G	-	16S/23S rRNA	" " "
NB2	TCG-CTC-GCC-GCT-ACT-GG	"	-	" " " "
O139-F2	AGC-CTC-TTT-ATT-ACG-GGT-GG	449	<i>rfb</i> O139	Hoshino et al. (1998)
O139-R2	GTC-AAA-CCC-GAT-CGT-AAA-CG		"	" " " "
O1F2-1	GTT-TCA-CTG-AAC-AGA-TGG-G	192	<i>rfb</i> O1	" " " "
O1F2-2	GGT-CAT-CTG-TAA-GTA-CAA-C		"	" " " "
VCT1	ACA-GAG-TGA-GTA-CTT-TGA-CC	308	<i>ctxA</i>	" " " "
VCT2	ATA-CCA-TCC-ATA-TAT-TTG-GGA-G			" " " "
RAPD ERIC1	ATG-TAA-GCT-CCT-GGG-GAT-TCA-C	-	-	Pazzani et al. (2006)
RAPD ERIC2	AAG-TAA-GTG-ACT-GGG-GTG-AGC-G	-	-	" " " "
RAPD VCR1	CAG-CTC-CTT-AGG-CGG-GCG-TTA-G	-	-	" " " "
RAPD VCR2	ACA-GTC-CCT-CTT-GAG-GCG-TTT-G	-	-	" " " "
RAPD ATX1	AAG-CGA-TTG-AAA-GGA-TGA	-	-	" " " "
RAPD ATX2	CCG-CGA-GTG-CTT-GGT-TAG	-	-	" " " "
tcpA-R (El Tor)	AAG-AG-TTT-GTA-AAA-GAA-GAA-CAC	472	<i>tcpA</i>	Khuntia et al. (2008) from Keasler & Hall (1993)
tcpA-R (El Tor)	GAA-AGG-ACC-TTC-TTT-CAC-GTT-G		"	" " " " " " " " " "
<i>V. vulnificus</i>				
VVp1	CCG-GCG-GTA-CAG-GTT-GGC-C	519	<i>cth</i>	Hill et al. (1991)
VVp2	CGC-CAC-CCA-CTT-TCG-GGC-C		"	" " " "
RAPD - Gen 1-50-3	AGG-AYA-CGT-G	-	-	Radu et al. (1998)
RAPD Gen 1-50-09	AGA-AGC-GAT-G	-	-	" " " "

(Continued)

Table 8.1 (Continued)

Species or genus	Primer or probe	Sequence (5' → 3')	Size of amplified sequence (bp's)	Gene or DNA target sequence	References
	RAPD primer	GGA-TCT-GAA-C	–	–	Hoi et al. (1997)
	Choi-1	GAC-TAT-CGC-ATC-AAC-AAC-CG	704	<i>vvhA</i>	Lee et al. 1997
	Choi-2	AGG-TAG-CGA-GTA-TTA-CTG-CC		"	" " " "
	P1	GAC-TAT-CGC-ATC-AAC-AAC-CG	704	<i>vvh</i>	Lee et al. (1998)
	P2	AGG-TAG-CGA-GTA-TTA-CTG-CC		"	" " " "
	P3	GCT-ATT-TCA-CCG-CCG-CTC-AC	222	<i>vvh</i>	" " " "
	P4	CCG-CAG-AGC-CGT-AAA-CCG-AA		"	" " " "
	Vv oligo 1	CGC-CGC-TCA-CTG-GGG-CAG-TGG-CTG	386	<i>cth</i>	Brauns et al. (1991)
	Vv oligo 3	CCA-GCC-GTT-AAC-CGA-ACC-ACC-CGC		"	" " " "
	VV1	GAC-TAT-CGC-ATC-AAC-AAC-CG	704	<i>vvh</i>	Fischer-Le Saux et al. 2002, from Lee et al. 1997
	VV2R	AGG-TAG-CGA-GTA-TTA-CTG-CC		"	" " " " " " " " " " " "
	VV3	GCT-ATT-TCA-CCG-CCG	604	"	" " " " " " " " " " " Lee et al. 1998
	L-CTH	TTC-CAA-CTT-CAA-ACC-GAA-CTA-TGA-C	205	<i>vvh</i>	Brasher et al. (1998)
	R-CTH	GCT-ACT-TTC-TAG-CAT-TTT-CTC-GC		"	" " " "
	P-CTH probe	GAA-GCG-CCC-GTG-TCT-GAA-ACT-GGC-GTA-ACG		"	" " " "
	L-vvh	TTC-CAA-CTT-CAA-ACC-GAA-CTA-TGA-C	205	<i>vvh</i>	Lee et al. (2003)
	R-vvh	GCT-ACT-TTC-TAG-CAT-TTT-CTC-GC		"	" " " "
	PP-vvh probe	GAA-GCG-CCC-GTG-TCT-GAA-ACT-GGC-GTA-ACG-GAT-TT		"	" " " "
	BP-vvh probe	GTT-CTT-CCT-TCA-GCG-CTG-TTT-TCG-GTT-TAC		"	" " " "
	RAPD primer	TAT-CAG-GCT-GAA-AAT-CTT	–	–	Vickery et al. (1998)
	R-PSE420				

Probe 610	A(K)A-(R)TT-GGC-GCC-GAC-GA	-	16S rDNA	Aznar et al. (1993)
Probe 1038	GCT-GTT-CCT-TTA-AGC-GAT-G	-	23S rDNA	" " " "
M13	GAA-ACA-GCT-ATG-ACC-ATG	-	-	" " " "
T3	ATT-AAC-CCT-CAC-TAA-AGG	-	-	" " " "
T7	AAT-ACG-ACT-CAC-TAT-AGG	-	-	" " " "
M13	GAA-ACA-GCT-ATG-ACC-ATG	-	-	Arias et al. (1995)
T3	ATT-AAC-CCT-CAC-TAA-AGG	-	-	" " " "
1038 probe	UAG-CGA-AAU-UCC-UUG-UCG	-	-	" " " "
Vvu1 probe	CAT-AGA-ACA-TTG-CCG-CAG	-	-	Aznar et al. (1994)
Vvu2 probe	ACT-CAA-TGA-TAC-TGG-CTT-A	-	-	" " " "
Vvu3 probe	ACC-GTT-CGT-CTA-ACA-CAT	-	-	" " " "
Vvu4 probe	TCA-AAG-AAC-ATT-GCC-GCA	-	-	" " " "
L-vvh	TTC-CAA-CTT-CAA-ACC-GAA-CTA-TGA	205	<i>vvh</i>	Panicker et al. (2004)
R-vvh	ATT-CCA-GTC-GAT-GCG-AAT-ACG-TTG	-	"	" " " "
VVAP probe	GAG-CTG-TCA-CGG-CAG-TTG-GAA-CCA	-	<i>vvha</i>	Wright et al. (1993)
<i>recA-1</i>	GAC-GAG-AAT-AAA-CAG-AAG-GC	543	<i>recA</i>	Gutacker et al. (2003)
<i>recA-2</i>	TCG-CCG-TTA-TAG-CTG-TAC-C	-	"	" " " "
<i>glnA-1</i>	TGA-CCC-ACG-CTC-TAT-CGC	402	<i>glnA</i>	" " " "
<i>glnA-2</i>	GCG-TGT-GCA-ACG-TTG-TG	-	"	" " " "
<i>V. parahaemolyticus</i>				
D1	CCA-TCT-GTC-CCT-TTT-CCT-GC	373	<i>tdh</i>	Tada et al., (1992)
D2	CCA-AAT-ACA-TTT-TAC-TTG-G	-	"	" " " "
D5	GGT-ACT-AAA-TGG-CTG-ACA-TC	199	<i>tdh</i>	" " " "
D2	CCA-AAT-ACA-TTT-TAC-TTG-G	-	"	" " " "

(Continued)

Table 8.1 (Continued)

Species or genus	Primer or probe	Sequence (5' → 3')	Size of amplified sequence (bp's)	Gene or DNA target sequence	References
D5		GGT-ACT-AAA-TGG-CTG-ACA-TC	251	<i>tdh</i>	" " " "
D3		CCA-CTA-CCA-CTC-TCA-TAT-GC		"	" " " "
R3		GCC-TCA-AAA-TGG-TTA-AGC-GC	210	<i>trh1</i>	" " " "
R5		TGG-CGT-TTC-ATC-CAA-ATA-CG		"	" " " "
R2		GGC-TCA-AAA-TGG-TTA-AGC-G	250	<i>trh1, trh2</i>	" " " "
R6		CAT-TTC-CGC-TCT-CAT-ATG-C		"	" " " "
VP-1		CGG-CGT-GGG-TGT-TTC-GGT-AGT	285	<i>gyrB</i>	Venkateswaran et al. (1998)
VP-2r		TCC-GCT-TCG-CGC-TCA-TCA-ATA		"	" " " "
toxR-F1		GTC-TTC-TGA-CGC-AAT-CGT-TG	350	<i>toxR</i>	Kim et al. (1999)
toxR-R1		ATA-CGA-GTG-GTT-GCT-GTC-ATG		"	" " " "
toxR-F2		AGC-CCG-CTT-TCT-TCA-GAC-TC	390	<i>toxR</i>	" " " "
toxR-R2		AAC-GAG-TCT-TCT-GCA-TGG-TG		"	" " " "
toxR-F3		CGC-TTT-CTT-CAG-ACT-CAA-GC	394	<i>toxR</i>	" " " "
toxR-R2		AAC-GAG-TCT-TCT-GCA-TGG-TG		"	" " " "
284-RAPD		CAG-GCG-CAC-A	–	–	Wong et al. (1999)
1 RAPD		GGT-GCG-GGA-A	–	–	Okuda et al. (1997)
2 RAPD		GTT-TCG-CTC-C			" " " "
VPF2		CGC-TTA-GAT-TTG-GGG-GTG-TG	327	–	Khan et al. (2002)
VPR2		GTT-GGT-TGA-GGC-ATA-GGT-AGC			" " " "
L-tl		AAA-GCG-GAT-TAT-GCA-GAA-GCA-CTG	450	<i>tl</i>	Brasher et al. (1998)
R-tl		GCT-ACT-TTC-TAG-CAT-TTT-CTC-TGC			" " " "

P-tl	ACG-GAC-GCA-GGT-GCG-AAG-AAC-TTC-ATG-TTG			" " " "
L-tdh	GTA-AAG-GTC-TCT-GAC-TTT-TGG-AC	269	<i>tdh</i>	Bej et al. (1999)
R-tdh	TGG-AAT-AGA-ACC-TTC-ATC-TTC-ACC		"	" " " "
L-trh	TTG-GCT-TCG-ATA-TTT-TCA-GTA-TCT	500	<i>trh</i>	" " " "
R-trh	CAT-AAC-AAA-CAT-ATG-CCC-ATT-TCC-G		"	" " " "
ERIC 1R	ATG-TAA-GCT-CCT-GGG-GAT-TCA-C	-	-	Marshall et al. (1999)
GS-VP.1	TAA-TGA-GGT-AGA-AAC-A	651	<i>toxRS</i>	Matsumoto et al. (2000)
GS-VP.2	ACG-TAA-CGG-GCC-TAC-A		"	" " " "
F-03MM824	AGG-ACG-CAG-TTA-CGC-TTG-ATG	369	<i>ORF8</i>	Myers et al. (2003)
R-03MM1192	CTA-ACG-CAT-TGT-CCC-TTT-GTA-G		"	" " " "
FP	AAA-CAT-CTG-CTT-TTG-AGC-TTC-CA	75	<i>tdh</i>	Blackstone et al. (2003)
RP	CTC-GAA-CAA-CAA-ACA-ATA-TCT-CAT-CAG		"	" " " "
P probe	FAM-TGT-CCC-TTT-TCC-TGC-CCC-CGG-TAMRA			" " " "

A. hydrophila

AP1	CAA-GGA-GGT-CTG-TGG-TGG-CGA-CA	208	β -hemolysin	Xia et al. (2004)
AP2	TTT-CAC-CGG-CGG-TAG-CAG-GAT-TG		"	" " " "
EUB f933	GCA-CAA-GCG-GTG-GAG-CAT-GTG-G	500	16S rDNA	Ji et al.(2004)
EUB r1387	GCC-CGG-GAA-CGT-ATT-CAC-CG		"	" " " "
<i>Aero-F</i>	TGT-CGG-SGA-TGA-CAT-GGA-YGT-G ^a	720	<i>aero</i>	Kong et al. (2002)
<i>Aero-R</i>	CCA-GTT-CCA-GTC-CCA-CCA-CTT-CA		"	" " " "
AERO1	CCA-AGG-GGT-CTG-TGG-CGA-CA	-	<i>aero</i>	Tombelli et al. (2000)
AERO2	TTC-CAC-CGG-TAA-CAG-GAT-TG		"	" " " "
AERO probe	CAC-CAG-GTA-TTG-GAC-GCT-GTC-CC	-	"	" " " "
Aerola	CCA-AGG-GGT-CTG-TGG-CGA-CA	209	<i>aero</i>	Pollard et al. (1990)

(Continued)

Table 8.1 (Continued)

Species or genus	Primer or probe	Sequence (5' → 3')	Size of amplified sequence (bp's)	Gene or DNA target sequence	References
<i>P. shigelloides</i>	Aerolb	TTT-CAC-CGG-TAA-CAG-GAT-TG		"	" " " "
	AHCF1	GAG-AAG-GTG-ACC-ACC-AAG-AAC-A	232	AHCYTOEN	Kingombe et al. (1999)
	AHCR1	AAC-TGA-CAT-CGG-CCT-TGA-ACT-C		"	" " " "
	PS23FW3	CTC-CGA-ATA-CCG-TAG-AGT-GCT-ATC-C	84	23S rDNA	González-Rey et al. (2000)
	PS23RV3	CTC-CCC-TAG-CCC-AAT-AAC-ACC-TAA-A		"	" " " " "
	PSG237-F	TTC-CAG-TAC-GAG-ATC-CTG-GCT-AA	68	<i>gyrB</i>	Fuchushima & Tsunomori (2005)
	PAG110R	ACC-GTC-ACG-GCG-GAT-TAC-T		"	" " " " "
	Forward	AGC-GCC-TCG-GAC-GAA-CAC-CTA	112	23S rDNA	Loh & Yap (2002)
	Reverse	GTG-TCT-CCC-GGA-TAG-CAG		"	" " " "
	Probe	LCRed640-GGT-AGA-GCA-CTG-TTA-AGG-CTA-GGG-GGT-CAT-C-P			" " " "
<i>L. monocytogenes</i>	RAPD: LMPB1	GGA-ACT-GCT-A	–	–	Gu et al.(2006) from Boerlin et al. (1995)
	RAPD: LMPB4	AAG-GAT-CAG-C	–	–	" " " " " " " " "
	Forward	GCC-AGC-GGG-AAG-GGA-AGA-ACC	435	<i>hugA</i>	Herrera et al. (2006)
	Reverse	GTC-GCC-CCA-AAC-GCT-AAC-TCA		"	" " " "
	LM1	CCT-AAG-ACG-CCA-ATC-GAA	702	<i>hly</i>	Mengaud et al. (1988)
	LM2	AAG-CGC-TTG-CAA-CTG-CTC		"	" " " "
	prfA-A	CTG-TTG-GAG-CTC-TTC-TTG-GTG-AAG-CAA-TCG	1,060	<i>prfA</i>	Wernars et al. (1992)
	prfA-B	AGC-AAC-CTC-GGT-ACC-ATA-TAC-TAA-CTC		"	" " " "

a1-hlyA	CCT-AAG-ACG-CCA-ATC-GAA-AAG-AAA	858	<i>hly</i>	Norton et al. (2001) from Bsai & Batt (1993)
β1-hlyA	TAG-TTC-TAC-ATC-ACC-TGA-GAC-AGA		"	" " " " " " " " "
LMrt3F	CAA-AGC-GAG-AAT-GTG-GCT-ATA-AAT-GA	-	<i>actA</i>	Oravcová et al. (2007)
LMrt3R	TAA-TTT-CCG-CTG-CGC-TAT-CCG		"	" " " "
ListP	ACC-CTG-GAT-GAC-GAC-GCT-CCA-CT		"	" " " "
RAPD UBC 155	CTG-GCG-GCT-G	-	-	Destro et al. (1996)
RAPD UBC 127	ATC-TGG-CAG-C	-	-	" " " "
RAPD UBC155	CTG-GCG-GCT-G	-	-	Vogel et al. (2001a) from Farber & Addison (1994)
RAPD HLWL85	ACA-ACT-GCT-C	-	-	" " " " " Wernars et al.(1996)
RAPD DAF4	CGG-CAG-CGC-C	-	-	" " " " " Wiedman-al-Ahmad et al.(1994)
RAPD OPM-01	GTT-GGT-GGC-T	-	-	" " " " " Lawrence et al. (1993)
RAPD PB1	GGA-ACT-GCT-A	-	-	Cao et al. (2005) from Boerlin et al. (1995)
RAPD PB4	AAG-GAT-CAG-C	-	-	" " " " " " " " "
RAPD HLWL74	ACG-TAT-CTG-C	-	-	" " " " " Mazurier & Wernars(1992)
C. botulinum type E				
BoTE1	GTG-AGT-TAT-TTT-TTG-TGG-CTT-CCG-AGA	307	<i>bont/E</i>	Alsallami & Kotlowski (2001)
BoTE2	TTA-TTT-TCA-CCT-TCG-GGC-ACT-TTC-TG		"	" " " " "
RAPD OPJ-6	TCG-TTC-CGC-A	-	-	Leclair et al. (2006)
RAPD OPJ-13	CCA-CAC-TAC-C	-	-	" " " "
BE1430F	GTG-AAT-CAG-CAC-CTG-GAC-TTT-CAG	269	<i>bont/E</i>	Kimura et al. (2001)
BE1709R	GCT-GCT-TGC-ACA-GGT-TTA-TTG-A		"	" " " "
BE1571FP	6-FAM-ATG-CAC-AGA-AAG-TGC-CCG-AAG-GTG-A-TAMRA		"	" " " "

(Continued)

Table 8.1 (Continued)

Species or genus	Primer or probe	Sequence (5' → 3')	Size of amplified sequence (bp's)	Gene or DNA target sequence	References
CBMLE1		CCA-AGA-TTT-TCA-TCC-GCC-TA	389	<i>bont/E</i>	Lindström et al. (2001)
CBMLE2		GCT-ATT-GAT-CCA-AAA-CGG-TGA		"	" " " "
GF-1		AAA-AGT-CAT-ATC-TAT-GGA-TA	762	<i>bont/E</i>	Franciosa et al. (1994)
GF-3		GTG-TTA-TAG-TAT-ACA-TTG-TAG-TAA-TCC		"	" " " "
BAC-3		ACG-GCC-CAG-ACT-CCT-ACG-GGA-GGC	763	16S rRNA	" " " "
BAC-4		GGG--TTG-CGC-TCG-TTG-CGG-CAC-TTA		"	" " " "
E1		TAT-ATA-TTA-AAC-CAG-GCG-G	745	<i>bont/E</i>	Szabo et al. (1993)
E2		TAG-AGA-AAT-ATT-GGA-ACT-G		"	" " " "

*S = G or C; Y = C or T.

Jaturapahu et al. (2005) developed a PCR-reverse cross-blot hybridization (PCR-RCBH) assay system for detection of all members of *Pseudomonas* and for specific identification of *P. fluorescens*, *P. putida*, *P. diminuta*, and *P. aeruginosa*. All four of these species are commonly isolated from tropical fish. PCR primers from Sawada et al. (1997) P16sf and P23sr (for all information on primers mentioned in the text, see Table 8.1) amplified an ~650 bp sequence of the 16S rRNA-23S rRNA spacer regions and were inclusive of both the 16S and 23S rDNA gene sequences and were labeled with biotin. *P. putida* also yielded two other PCR products, of 700 and 350 bp, due to multiple copies of the 16S-23S spacer region. The 650 bp amplicons were sequenced and used for specific species probe designs. The 16S-23S rRNA spacer sequences of *P. diminuta* and the fluorescent pseudomonads were found to be identical, which greatly facilitated their use for identification of the members of the genus *Pseudomonas*, with respect to the 650 bp amplicon. The specific probes for the four *Pseudomonas* species were presumably derived from the 16S-23S rDNA sequences of the individual species.

8.3 Seafood-borne Bacterial Pathogens

8.3.1 The Genus *Vibrio*

Members of the genus *Vibrio* are Gram-negative short asporogenous curved or straight rods, that are motile by means of polar flagella. They are all facultative anaerobes exhibiting fermentative metabolism in the absence of oxygen-producing acid but no gas (H₂ or CO₂). They are cytochrome oxidase positive and are usually non-pigmented. All members of the genus are considered sensitive to the vibriostatic agent O/129 which is considered a diagnostic criterion for the genus. The molar G + C value for the genus ranges from 40% to 50%. Members of the genus are not considered spoilage organisms and are usually not found among the dominant flora of stale fish. The genus has several species that are notable human pathogens such as *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* associated with the consumption of raw seafood.

8.3.1.1 *Vibrio cholerae*

Vibrio cholerae is considered a heterogeneous species with 206 serotypes presently recognized. However, only two serotypes are associated with epidemic infections, O1 and O139. The O139 strains have been shown to be genetically similar to O1 strains and are hypothesized as having evolved from strains of the early seventh pandemic by a mechanism involving insertion of an exogenous DNA fragment encoding the O139 LPS (Bik et al. 1995, 1996; Dumontier & Berche 1998; Karaolis et al. 1995). *V. cholerae* O1 is divided into two biotypes: classical and El Tor. The classical biotype has been gradually replaced by the El Tor biotype since 1961 and currently the classical is considered extinct having not been seen since 1983. The El Tor biotype is therefore currently considered the most significant. In the USA, crabs, shrimp, and oysters have been the most frequently implicated vectors of the El Tor biotype.

Seven pandemics of cholera have been recorded since 1817, with the ongoing seventh pandemic starting in 1961 in Asia. There have been two major upsurges of the seventh pandemic: one in the 1970s spread to Africa and the other in 1991 spread cholera to South America. Both continents had been free of pandemic cholera for over a century. In January 1991, an outbreak of cholera started in Peru and rapidly spread throughout

most of Latin America. In Peru alone, over 1 million cases occurred from 1991 to 1992 with about 10 000 deaths due presumably to an Asian ship blowing out its ballast tanks along the Peruvian coast and contaminating the fish. Sun-dried fish were then consumed without cooking, according to long established customs, in coastal and Peruvian mountain regions. Several major factors contributed to the rapid transmission of cholera in this Peruvian outbreak. One was the lack of medical care in remote villages of the Andes Mountains, and a second contributing factor was the lack of chlorination for the drinking water in Lima, the capital city of Peru. The outbreak resulted in 3–4 million cases of human infections in Latin America and ~30 000 deaths, indicating that not only contaminated water but fish eaten uncooked can result in severe outbreaks of cholera.

Fields et al. (1992) reported on the use of primers CTX2/CTX3 that amplified a 564 bp sequence of the *ctxA* gene for its detection in 150 *V. cholerae* isolates derived from patients, food, and water from the 1991–1992 outbreak. One hundred forty isolates were found to be toxigenic by both PCR and immunoassay.

Koch et al. (1993) reported on the development of a PCR assay for detection of *V. cholerae* with seeded oysters, crab meat, shrimp, and lettuce. The primers P1/P3 amplified a 778 bp sequence of the *ctxB* gene from a *V. cholerae* O1 strain. A detection limit of 1 CFU/10 g of food was obtained. Hoshino et al. (1998) developed a multiplex PCR assay for rapid detection of toxigenic *V. cholerae* O1 and O139. The assay targeted the *rfb* sequence specific for the O1 and O139 serogroups and the *ctxA* gene. Primers O139-F2/O139-R2 amplified a 449 bp sequence of the *rfb* gene from O139 strains. The primers O1F2-1/O1F2-2 amplified a 192 bp sequence of the *rfb* gene from O1 strains. The primers VCT1/VCT2 amplified a 308 bp sequence of the *ctxA* gene.

Chow et al. (2001) developed PCR assays for detection of the *rtxA*, *rtxC*, (encoding the RTX repeat in toxin), and *ctxB* toxin genes among 166 clinical and environmental isolates of *V. cholerae*. All 166 isolates were O1 El Tor, O139 or non-O1 serotypes and all harbored the *rtxA* and *rtxC* genes which are considered specific for all *V. cholerae* isolates. Only the non-O1 serogroups failed to harbor the *ctxB* gene. Lalitha et al. (2008) developed a PCR assay specific for all strains of *V. cholerae*, including O1, O139, and non-O1/non-O139 serogroups and biotypes. The primers VHMF/VHA-AS5 amplified a 519 bp sequence of the *lolB* gene that encodes an outer membrane lipoprotein. The diagnostic sensitivity and specificity with 633 clinical rectal swab samples were 98.5% and 100% respectively. Mendes et al. (2008) developed a multiplex single-tube PCR assay for detection of the *V. cholerae* serotype. The *ctxA* gene was targeted with a pair of external primers and a pair of internally nested primers that yielded a final amplicon of 302 bp. In addition, a pair of primers was added that amplified a 198 bp sequence of the *rfbN* gene that encodes the O1 serotype.

Khuntia et al. (2008) developed a quadruplex PCR for simultaneous detection of serotype, biotype, toxigenic potential, and control regulating factors of *V. cholerae*. The assay specifically targeted the *rfb* genes for O1 and O139 serotypes (primers from Hoshino et al. 1998; see Table 1.8), *ctxA* (primers from Keasler & Hall 1993; see Table 8.1), *tcpA* (El tor primers from Keasler & Hall 1993), *tcpA* (classical primers from Rivera et al. 2001; see Table 8.1), and *toxR* (primers from Miller et al. 1987) genes.

Molecular Typing of *V. cholerae* Isolates

Coelho et al. (1995) described five (1–5) random primers used for distinguishing 50 El Tor, four classical, and two Gulf coast strains of *V. cholerae*. An additional five (6–10) random primers were described for distinguishing more closely related El Tor and five

Bengal strains. In addition, primers NA1/NB2 were developed to amplify a 16S/23s rRNA spacer region of the strains. Under conditions of low stringency, a series of bands were obtained. The Bengal strains yielded a more prominent band of 0.35 kbp and weaker bands at the top of the pattern compared to the other biotypes. A 0.5 kbp band was produced from the El Tor, Bengal, and Gulf Coast strains and was absent from classical strains. A 0.8 kbp strong band was produced by all four groups.

In 1994, a cholera epidemic occurred in Italy and Albania after more than a decade of absence. Pazzani et al. (2006) examined 110 *V. cholerae* El Tor isolates from this epidemic using random amplified polymorphic DNA RAPD with six random primers, *Bgl*I ribotyping, and pulsed-field gel electrophoresis (PFGE) with *Sfi*I and *Not*I. All strains were of biotype 6 and the respective RAPD and PFGE patterns were identical as well. These findings indicated that the 1994 isolates belonged to the same clone and that the clone was part of the larger global spread of epidemic ribotype 6, which started in southern Asia in 1990.

Scrascia et al. (2006) examined 80 *V. cholerae* O1 strains, selected to represent the 1998–1999 history of the largest cholera epidemic in Kenya, with respect to ribotyping antimicrobial susceptibility, and RAPD using the six random primers from Pazzani et al. (2006). Sixty-one of the 80 isolates fell into a single ribotype designated B27 and were resistant to chloramphenicol, spectinomycin, streptomycin, sulfamethoxazole, and trimethoprim. The 61 ribotype B27 strains fell into a single RAPD cluster. Six of the 61 ribotype B27 isolates were also resistant to doxycycline and tetracycline resulting from the presence of a conjugative plasmid. These observations were interpreted to indicate that the predominant B27 ribotype strains had a common clonal origin which rapidly spread from West Africa to eastern Africa.

In order to investigate the origin of *V. cholerae* O1 biotype El Tor isolates in Japan in 1997, Arakawa et al. (2000) subjected 67 strains to PFGE after digestion with *Not*I and *Sfi*I. Thirty-six strains were from patients who had gone abroad and 31 strains were isolates from patients just returning from India and various geographic regions in Asia. Sixty-six of the 67 O1 El Tor isolates belonged to serotype *Ogawa* and only one belonged to serotype *Inaba*. Among the 365 domestic isolates, 25 belonged to a single PFGE subtype. In contrast, PFGE analysis separated the 31 imported strains into 13 subtypes, with only one of these strains exhibiting the same PFGE pattern common to the major domestic PFGE subtype.

The reader is referred to Levin (2010) where there is a more detailed presentation of molecular techniques applied to isolates of *V. cholerae* along with a listing of all genes used for PCR identification of the organism and a more extensive tabulation of primers and probes.

8.3.1.2 *Vibrio vulnificus*

Vibrio vulnificus is considered the most invasive of all human pathogenic vibrios in the US, accounting for 95% of all seafood-related deaths in this country. The organism requires at least 0.5% NaCl for growth and has been found to be a natural inhabitant of marine coastal waters and to be globally ubiquitous. The most frequent symptoms include fever, chills, nausea, hypotension, and endotoxic shock which are usually associated with endotoxicity derived from Gram-negative lipopolysaccharides (LPS). Cirrhosis of the liver due to chronic alcoholism is considered a high risk factor for infection by this organism, presumably due to increased levels of serum iron released by damaged hepatocytes. Additional factors regarding susceptibility to infection include

chronic renal disease, diabetes, and immunocompromising diseases which are thought to be responsible for the observation that outbreaks involving the consumption of oysters from a specific lot usually involve only a single susceptible individual developing symptoms (McPherson et al. 1991). Septicemic infections by the organism usually result from the consumption of raw shellfish and can result in fatality rates as high as 60% (Oliver 1989). Secondary necrotic lesions of the extremities frequently occur (69%), often necessitating surgical debridement or limb amputation (Oliver & Kaper 1997).

The organism was originally referred to in 1976 as the “lactose fermenting vibrio” (Hollis et al. 1976) and is of particular concern along the warm coastal waters of the US Gulf Coast. *V. vulnificus* possesses a unique species-specific H-antigen allowing flagella antiserum in slide agglutination assays to distinguish the organism from all other vibrios (Simonson & Siebeling 1986; Tassin et al. 1983).

Vibrio vulnificus Biotypes

Tison et al. (1982) were the first to allocate *Vibrio* strains pathogenic for eels to the species *V. vulnificus*. They performed a comparative study of human clinical, environmental, and eel pathogenic isolates of *V. vulnificus* using phenotypic comparison, eel and mouse pathogenicity, and DNA-DNA hybridization studies and concluded that human clinical isolates should be allocated to biotype 1 and that eel pathogen isolates be allocated to biotype 2. Biotype 2 was phenotypically defined as differing biochemically from biotype 1 in being negative for indole production, ornithine decarboxylase activity, acid production from mannitol, and sorbitol and growth at 42 °C. Interestingly, they also reported that neither human clinical or environmental isolates exhibited pathogenicity for eels, whereas all human clinical, environmental, and eel isolates tested resulted in mortality in suckling mice, with eel isolates yielding the highest level of mortality.

During 1996–1997, 62 cases of wound infections and bacteremia due to *V. vulnificus* were found to result from contact with purchased inland pond-raised tilapia in Israel (Bisharat et al. 1999). The outbreak was due to a new marketing policy of selling live fish instead of marketing them packed in ice postmortem. The isolates were atypical biochemically, were non-typeable by PFGE and all had the same PCR-restriction fragment length polymorphism (PCR-RFLP) pattern derived from a 388 bp DNA fragment of the *cth* gene. These isolates were distinguishable from biotypes 1 and 2 biochemically and the authors allocated these isolates to a newly established biotype 3.

Dalsgaard et al. (1999) agreed with Arias et al. (1997) that the division of *V. vulnificus* into two biotypes based on phenotypic criteria originally established by Tison et al. (1982) is no longer tenable and leads to taxonomic confusion.

PCR for Detection and Identification of *V. vulnificus*

Hill et al. (1991) were the first to develop a PCR procedure for detection of *V. vulnificus*. They seeded the organism into oyster homogenates and found that among several DNA extraction procedures, DNA recovered from cells in homogenates by lysing with guanidine isothiocyanate (GITC) followed by extraction with chloroform and precipitation with ethanol was most suitable for use as a PCR template. In contrast, extraction of the homogenates with GITC alone notably inhibited the PCR. Primers VVp1 and VVp2 targeted a 519 bp sequence of the *cth* gene. With the GITC-chloroform DNA extraction method, 20 µL of oyster extract was found to be non-inhibitory when added directly to

PCR reactions. The limit of detection was 10^2 CFU/g of oyster tissue following a 24-hour enrichment at 33–35°C in a 1:10 homogenate of oyster tissue in alkaline peptone water (APW).

Aono et al. (1997) used the two primers VVp1 and VVp2 developed by Hill et al. (1991) for evaluating the effectiveness of the PCR in identifying isolates of *V. vulnificus* from marine environments. A total of 13325 bacterial isolates from seawater, sediments, oysters, and goby specimens collected along the coastal regions of Tokyo Bay were metabolically screened. Among these, 713 grew at 40°C, required NaCl for growth, formed greenish colonies on thiosulfate-citrate-bile-salts sucrose (TCBS) agar, and were presumptively identified as *V. vulnificus*. The PCR amplified the targeted 519bp sequence of the *cth* gene with 61 of these isolates. DNA-DNA hybridization with the type strain of *V. vulnificus* and the API 20E system confirmed the PCR results. The authors concluded that the PCR method is useful for rapid and accurate identification of *V. vulnificus* from marine sediments.

Brasher et al. (1998) developed a multiplex PCR method for simultaneous amplification of targeted gene segments of five Gram-negative pathogens, including *V. vulnificus*, in shellfish tissue homogenized in APW. The primers L-CTH and R-CTH targeted a 205bp sequence of the *cth* gene of *V. vulnificus*. A 6-hour enrichment at 35°C was used prior to DNA purification and the PCR. The sensitivity of detection was 10 – 10^2 CFU following a double multiplex PCR. Amplicons were detected by agarose gel electrophoresis and ethidium bromide staining of DNA bands.

Hervio-Heath et al. (2002) examined French coastal water and mussels for the presence of several pathogenic vibrios including *V. vulnificus*. The primers VV-1 and VV-2R of Lee et al. (1997) targeting the 704bp sequence of the *vvhA* cytolysin gene were used to confirm the identity of the presumptive *V. vulnificus* isolates, as well as the primers Vv oligo 1 and Vv oligo 3 of Brauns et al. (1991) delineating a 386bp fragment of the 704 *cth* sequence. Among a total of 190 *Vibrio* isolates, 20 were identified as *V. vulnificus*, with 16 derived from estuarine water samples and four from mussels.

Lee et al. (1997) made use of a primer pair designated Choi-1 and Choi-2 for PCR amplification of a 704bp sequence of the *vvhA* gene following enrichment of seeded homogenates of octopus tissue. Sensitivity of detection was 10 CFU/mL of homogenates.

Panicker et al. (2004) described a SYBR Green I-based real-time (Rti) PCR assay for detection of *V. vulnificus* in oyster tissue homogenate. A pair of primers designated L-*vvh* and R-*vvh* were used to amplify a 205bp sequence of the *vvh* gene. The minimum level of detection was 100 CFU per PCR tube. A 5-hour enrichment allowed detection of 1 CFU per mL of tissue homogenate which is equivalent to 10 CFU/g of tissue. The assay required 8 hours for completion.

Ribotyping and RAPD Analysis of *V. vulnificus* Isolates

Aznar et al. (1993) subjected strains of *V. vulnificus* to ribotyping which resulted in discrimination of biotypes 1 and 2 in addition to individual strains. These authors also found that strains of *V. vulnificus* biotypes 1 and 2 could be differentiated by RAPD with the universal primer M13, T3, or T7. Compared with ribotyping, RAPD appeared to be a faster method for diagnosing the identity of *V. vulnificus* biotypes. Høi et al. (1997) reported that ribotype profiles can be used to distinguish biotype 1 and biotype 2 isolates. Arias et al. (1997) determined the intraspecies genomic relatedness of 44 biotype 1 and 36 biotype 2 isolates from different geographic origins by ribotyping and with the

use of amplified fragment length polymorphism (AFLP). Ribopatterns of DNAs digested with *KpnI* and hybridized with labeled oligonucleotide probe 1038 revealed up to 19 ribotypes which were different for the two biotypes. Ribotyping clearly separated the eel pathogenic strains from the clinical and environmental isolates, whereas AFLP distinguished individual strains and therefore constitutes one of the most discriminative methods for epidemiological and ecological studies.

Nucleic acid probes have been found unable to differentiate between biotypes 1 and 2 (Aznar et al. 1994; Wright et al. 1993). Radu et al. (1998) subjected 26 biotype 1 and 10 biotype 2 isolates to RAPD analysis using two random primers designated Gen 1-50-03 and Gen 1-50-09. A total of six RAPD types were distinguished with primer Gen1-50-03, with all six RAPD types represented by one or more strains of biotype 1. With biotype 2 strains, only three of these RAPD types were distinguished. With primer Gen 1-5-009, a total of five RAPD types were distinguished, with all five types represented by one or more strains of biotype 1. With biotype 2 strains, only four of these RAPD types were generated. Results also indicated that certain biotype 1 and biotype 2 strains yielded identical RAPD profiles with both RAPD primers, indicating a high degree of DNA sequence similarity between such strains of the two biotypes.

Arias et al. (1998) determined the genetic relationships among 132 strains of *V. vulnificus* derived from human infections, diseased eels, seawater, and shellfish with the use of RAPD and ribotyping. RAPD was performed with the universal primers M13 and T3. Both ribotyping and RAPD revealed a high level of homogeneity of diseased eel isolates in contrast to the genetic heterogeneity of seawater-shellfish isolates of the Mediterranean. Although differentiation within diseased eel isolates was only possible by ribotyping, the authors proposed that RAPD is a better technique than ribotyping for less laborious and rapid typing of new *V. vulnificus* isolates.

Warner and Oliver (1999) developed an RAPD protocol for detecting *V. vulnificus* and for distinguishing this organism from other members of the genus *Vibrio*. A 10-mer primer previously described by Warner and Oliver (1998) was used. Each of 70 *V. vulnificus* strains examined produced a unique banding pattern, indicating that members of this species are highly heterogeneous. All of the clinical isolates yielded a unique band (178–200bp) that was only occasionally found with environmental strains. The authors concluded that this band may be correlated with human pathogenicity. Subsequent observations by DePaola et al. (2003) with this primer indicated that only 70% of clinical isolates possessed this amplicon and that a band of ca. 460bp was present in 86% of these same strains. Vickery et al. (1998) made use of a random primer designated R-PSE420 for generating RAPD profiles of *V. vulnificus* strains. The primer yielded 15 different DNA banding profiles with 16 strains. Extensive genomic heterogeneity was observed with strains derived from different oyster samples and even from strains derived from the same patient with wound infections. In a subsequent RAPD study, Vickery et al. (2000) used the same primer for RAPD profiling of 10 *V. vulnificus* isolates from patients who succumbed to infections derived from consuming raw oysters. Analysis of the DNA band profiles revealed significant genetic heterogeneity among these strains also.

Høi et al. (1997) screened 10 RAPD primers for analysis of *V. vulnificus* isolates and found that one primer was superior and yielded 10–15 bands. Use of this primer for RAPD analysis of isolates failed to distinguish between Danish and US strains and to separate biotype 1 and 2 strains due to excessive heterogeneity of the RAPD profiles. In contrast, ribotyping differentiated Danish and US strains and distinguished between biotypes 1 and 2.

DePaola et al. (2003) examined strains of *V. vulnificus* from market oysters and oyster-associated primary septicemia cases (25 strains from each group) for potential virulence markers that could possibly distinguish strains from these two sources. The isolates were analyzed for plasmid content, the presence of the 460bp amplicon by RAPD using the primer of Warner and Oliver (1998), and for virulence in inoculated mice with serum iron overload. Both groups of isolates yielded strains with similar results. About half of both oyster and clinical isolates produced the 460bp band. The authors concluded that nearly all *V. vulnificus* strains in oysters are virulent and that these assay methods cannot distinguish between fully virulent and less virulent strains or between clinical and environmental isolates.

Gutacker et al. (2003) applied RAPD primer M13 to 62 strains of *V. vulnificus*, yielding a total of 28 different RAPD profiles with isolates falling into two divisions I and II. One cluster within division II included all 11 strains from diseased eels derived from several different geographic areas plus isolates not associated with eel pathogenicity and exhibiting a positive indole reaction. Another cluster within division II comprised all the four human clinical isolates from Israel (biotype 3) with identical RAPD profiles. From multilocus enzyme electrophoresis (MLEE), RAPD, and sequence typing, indole-negative eel pathogenic strains from different geographic origins tended to cluster as a separate genotype, in contrast to a variable phylogeny with the indole-positive eel pathogenic isolates.

Molecular Detection of Viable But Non-culturable (VBNC) *V. vulnificus*

Detection of mRNA is thought to be a reliable marker for viability due to its short half-life. With this in mind, Fischer-Le Saux et al. (2002) detected VBNC cells of *V. vulnificus* by applying semi-nested reverse transcription-PCR (RT-PCR) targeting the *vvhA* gene of VBNC populations induced by holding cells at 4°C in artificial seawater. The VvhA nested system of Lee et al. (1998) was used. Following RT, two external primers designated VV1 and VV2R delineating a 704bp sequence within the open reading frame of the *vvhA* gene (Lee et al. 1997) were used in conjunction with an internal primer designated VV3 delineating a 604bp fragment in conjunction with the VV2R in the semi-nested PCR. Transcripts were shown to persist in non-culturable populations for over 4.5 months, with a progressive decline of the signal over time. The methodology not only detected VBNC cells but also ensured that only viable cells were detected.

In a PCR technique developed for *V. vulnificus*, higher levels of DNA were required to detect VBNC than growing cells (Brauns et al. 1991; Coleman & Oliver 1996). With an RAPD method to detect grown cells, the loss of a signal of RAPD amplification products was observed with starved and VBNC cells (Warner & Oliver 1998).

8.3.1.3 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is an enteropathogenic marine *Vibrio*, capable of causing mild gastroenteritis to severe debilitating dysentery. *V. parahaemolyticus* is widespread globally and appears to be limited to inshore coastal and estuarine areas. The organism is halophilic with an optimum NaCl concentration of about 3.0% and is considered to have a minimum growth temperature of 9–10°C and a maximum growth temperature of about 44°C (Horie et al. 1966; Jackson 1974). Beuchat (1973), however, reported moderate growth at 5°C. Infections of the GI tract are usually due to consumption of raw shellfish. The incubation period can range from 4 to 96 hours. The symptoms include acute abdominal pain, cramps, nausea, vomiting, low-grade fever, and chills

with watery and in some cases bloody diarrhea. Gastrointestinal infections due to *V. parahaemolyticus* resulting from the ingestion of raw seafood are usually mild, with a duration of 2–3 days. A more severe and debilitating dysenteric form of gastrointestinal infection with bloody stools and marked leukocytosis requiring hospitalization has been observed due particularly to strains of the serotype O3:K6. Extraintestinal infections due to *V. parahaemolyticus* are also recognized involving wounds of the extremities, eye infections, and bacteremia.

All strains of *V. parahaemolyticus* possess a thermo-labile hemolysin encoded by the *lht* gene which is not directly related to virulence. PCR primer pairs have been developed utilizing the resulting amplicons for identification of all isolates of *V. parahaemolyticus*. Virulence is associated with two principal genes that code for (1) a thermally stable direct-acting hemolysin (*tdh*) and (2) a thermally stable direct acting-related hemolysin (*trh*). However, not all clinical strains have been found to possess the *trh* gene. Primer pairs targeting sequences of the *tdh* gene are therefore used to distinguish virulent from non-virulent strains. Virulent strains are usually characterized as Kanagawa phenomenon (KP) positive which refers to beta-hemolysis on a special blood agar known as Wagatsuma blood agar (Wagatsuma 1968). Epidemiological studies have indicated that specific clones of certain serotypes having enhanced virulence, notably O3:K6, have become endemically established in certain global locales. Hemolysis on Wagatsuma's agar has been found to correlate well with human pathogenicity. Sakazaki et al. (1968) reported that 2655/2720 (96.6%) of human clinical isolates were KP+ and that only 7/650 (1%) of environmental isolates were KP+. Thompson et al. (1976) found only 4/2218 environmental isolates to be KP+.

The purified thermo-stabile direct hemolysin (TDH) is a dimeric protein of 44 000 da (Miyamoto et al. 1980), is responsible for the Kanagawa phenomenon, causes fluid accumulation in the ligated mouse ileum, is cytolytic against cultured mammalian cells, and lyses erythrocytes of various animal species (Douet et al. 1992). It is stable to heating at 100 °C for 10 minutes (Douet et al. 1992; Sakurai et al. 1973) and is activated by Ca⁺⁺ (Chun et al. 1974). The heat-labile hemolysin is usually found in KP– strains, but not consistently (Miyamoto et al. 1980).

Molecular epidemiological studies have revealed that not only strains carrying the *tdh* gene but strains carrying a *trh* gene or both genes are strongly associated with gastroenteritis (Okuda et al. 1997; Shirai et al. 1990). Shirai et al. (1990) used *tdh* and *trh* gene probes to detect the TDH- and TRH-producing genes in strains of *V. parahaemolyticus* and found that TRH as well as TDH are important virulence factors for *V. parahaemolyticus*.

Kelly and Stroh (1989) found that the expression of the Kanagawa hemolysin was not absolutely essential for pathogenesis of *V. parahaemolyticus* and that gastroenteritis in the Pacific Northwest may be due to strains that are KP–.

Application of PCR for Detection of *V. parahaemolyticus*

Tada et al. (1992) established PCR protocols for the specific detection of the *tdh* and *trh* genes of *V. parahaemolyticus*. The selection of primers took into consideration that the *tdh* and *trh* genes are known to have sequence divergence of up to 3.3% and 16% respectively. An annealing temperature of 55 °C was required with the three primer pairs D1/D2, D5/D2, and D5/D3 for detection of the *tdh* gene. An annealing temperature of 60 °C was required with the primer pair R3/R5 for high-specificity detection of the *trh1* gene. The R2/R6 primer pair detected both *trh1* and *trh2* genes with an annealing

temperature of 55°C. The limit of sensitivity was 400 fg of cellular DNA in each PCR reaction tube derived from 100 cells.

Brasher et al. (1998) developed a PCR assay for detection of *V. parahaemolyticus* utilizing a 450 bp sequence of the *tlh* gene. The sensitivity of detection was 10–10² cells.

Bej et al. (1999) developed a multiplex PCR assay for total and virulent strains of *V. parahaemolyticus* based on the amplification of a 450 bp sequence (Brasher et al. 1998) of the thermo-labile hemolysin gene (*tlh*), a 269 bp sequence of the thermostable direct hemolysin gene (*tdh*), and a 500 bp sequence of the thermostable direct-related hemolysin-related (*trh*) gene. All 111 *V. parahaemolyticus* isolates studied yielded the *tlh* amplicons. However, only 60 isolates yielded the *tdh* amplicon, and 43 yielded the *trh* amplicon. Sensitivity of detection for all three amplicons was 10–10² CFU/g of oyster tissue following homogenization in alkaline peptone water and incubation at 35°C for 6 hours.

Venkateswaran et al. (1998) developed a PCR procedure targeting a 285 bp sequence of the *gyrB* gene for specific detection of *V. parahaemolyticus*. The *toxR* gene is well conserved among species of *Vibrio*. Kim et al. (1999) developed a DNA colony hybridization test with the use of a 678 bp polynucleotide probe (Lin et al. 1993) for the *toxR* gene of *V. parahaemolyticus*, to confirm the identity of isolates. Kim et al. (1999) also developed a specific PCR assay for the identification of *V. parahaemolyticus* based on amplicons of the *toxR* gene. Three effective primer pairs were identified. A total of 373 strains of *V. parahaemolyticus* were all found to carry the *toxR* gene.

Blackstone et al. (2003) developed a real-time PCR assay for detection of *V. parahaemolyticus* in oysters with the use of a pair of primers amplifying a 75 bp sequence of the *tdh* gene (see Table 8.1) in conjunction with a dual-labeled fluorogenic probe.

Kaufman et al. (2002) examined eight clinical and nine oyster isolates of *V. parahaemolyticus* isolated during the Pacific Northwest outbreak in 1997 and an additional three clinical isolates from the 1994 outbreak. A multiplex PCR assay for simultaneous detection of the *tdh*, *trh*, and *tlh* genes was used. All isolates of *V. parahaemolyticus* studied possessed *tlh* + which is considered a specific marker for all isolates of *V. parahaemolyticus* (Bej et al. 1999). All 11 clinical isolates harbored both the *tdh* and *trh* genes. The authors suggested that the *tdh*, *trh*, and urease test can be used to identify and track potentially virulent strains in oysters.

Molecular Typing of *V. parahaemolyticus* Below the Species Level

Wong et al. (1996) screened 16 restriction nucleases for use in conjunction with PFGE analysis of *V. parahaemolyticus* strains. The restriction nuclease *Sfi* I was found to yield 17 clear and discernible bands and was applied to 130 clinical strains from Thailand. These 130 isolates were grouped into 14 PFGE types and each type was subdivided into 1–6 patterns resulting in a total of 39 discernible PFGE patterns

Wong et al. (1999) subjected 308 clinical isolates of *V. parahaemolyticus* derived from food outbreaks in Taiwan between 1993 and 1995 to RAPD analysis. The 10-mer primer designated 384 was used and generated 41 RAPD patterns. The patterns were grouped into 16 RAPD types, the first four of which were the major patterns and accounted for 91.25% of the domestic clinical isolates. The RAPD typing patterns were correlated with previously reported PFGE typing patterns (Wong et al. 1996) of these isolates.

Marshall et al. (1999) compared various molecular typing methods for distinguishing 38 clinical and 16 environmental strains of *V. parahaemolyticus*. Enterobacterial repetitive intergeneric consensus (ERIC) PCR and ribotyping were the most informative and discriminating methods, especially when used together.

The O3:K6 Pandemic Clone of *V. Parahaemolyticus*

Hondo et al. (1987) were the first to report on the isolation of KP-clinical isolates of *V. parahaemolyticus* belonging to the serovar O3:K6. All 11 strains of O3:K6 caused fluid accumulation in the rabbit ileal loop assay. Nasu et al. (2000) found that a phage designated f237 was specifically and exclusively associated with O3:K6 serovar strains isolated since 1996.

Myers et al. (2003) described the development and use of a set of primers specific for a 369 bp sequence of ORF8 for the O3:K6 serovar designated F-03MM824 and R-03MM1192.

Khan et al. (2002) reported that O3:K6 strains possessed a specific 850 bp sequence that was absent in other *Vibrio* species and related organisms. A set of primers was then developed that amplifies a 327 bp segment of this unique sequence.

Matsumoto et al. (2000) showed with RAPD analysis that O3:K6 strains from six countries including the US isolated from 1997 and later belong to the same clone.

8.3.2 *Aeromonas hydrophila*

Aeromonads are widespread, being encountered in natural aquatic habitats and numerous marine foods. Among the five recognized human infectious species of *Aeromonas*, *A. hydrophila* is the species most frequently implicated in gastroenteritis. *A. hydrophila* is a Gram-negative, facultatively anaerobic non-spore-forming rod, motile by a single polar flagellum, DNase positive, protease positive, catalase positive, cytochrome oxidase positive, and ferments glucose with acid and gas production. The optimum growth temperature is considered to be ~28 °C and most isolates are capable of psychrotrophic growth at refrigerator temperatures, with some isolates exhibiting growth at -1 °C (Daskalov 2006).

Several genes have been utilized for the specific PCR detection of potentially virulent aeromonads in foods and environmental samples. These include the gene encoding beta-hemolysin, the *aero* gene encoding aerolysin that exhibits hemolytic and cytolytic properties, and the effector gene *aexU* involved in a type III secretion system. Human clinical isolates of *A. hydrophila* presently fall into three DNA hybridization groups: HG1, HG2, and HG3 (Kirov 2003). Pathogenicity is associated with the ability to produce exotoxins (agglutinins and hemolysins), cytotoxins, endotoxins, siderophores, invasins, adhesins (pili), S-layer (surface array protein layer), and flagella (Daskalov 2006).

Two types of gastroenteritis have been attributed to *A. hydrophila*. The first and most common is a cholera-like illness characterized by watery stools and a mild fever. The second is a dysentery-like illness characterized by the presence of blood and mucus in the stools. The organism has also been implicated in extraintestinal infections involving septicemia and meningitis, as well as respiratory and wound infections. In more recent years, the involvement of *A. hydrophila* has been increasingly detected in infections of severe burn patients (Barillo et al. 1996; Chim & Song 2007).

8.3.2.1 PCR Detection of *Aeromonas hydrophila*

Pollard et al. (1990) developed a PCR for the rapid and specific detection of the aerolysin gene in hemolytic strains of *A. hydrophila* associated with human infections. The sequence of the *aero* gene from *A. sobria* was found to have only 77% homology to the *aero* gene from *A. hydrophila* which allowed the design of a species-specific pair of

primers. The primers Aerola/Aerolb amplified a 209bp sequence of the *aero* gene derived only from strains of *A. hydrophila*.

Kingombe et al. (1999) developed a PCR for detecting enterotoxin and aerolysin genes in *Aeromonas* spp. The *A. hydrophila* cytolytic enterotoxin gene (*AHCYTOEN*) served as the reference gene in that it has been described as a multivirulence gene resulting in lethality in mice, hemolysis, cytotoxicity, and enterotoxigenicity (Chakraborty et al. 1986). Some of these activities are part of the virulence factors of other *Aeromonas* species. The primers AHCF1/AHCR1 amplified a 232bp sequence of the *AHCYTOEN* gene and were developed because of 100% homology between the *AHCYTOEN* gene and an extracellular hemolysin gene which represented the two main groups of virulence factors in the genus *Aeromonas* (enterotoxin and hemolysin). Among 220 *Aeromonas* isolates from raw food samples (beef, fish, vegetables), 157 (71%) were PCR positive; among 59 *Aeromonas* isolates from environmental water samples, 34 (74%) were PCR positive; and among 71 human clinical *Aeromonas* isolates, 36 (51%) were PCR positive. Characterization of the PCR products by PCR-RFLP using the endonuclease *HpaII* and PCR-amplicon sequence analysis (PCR-ASA) revealed three types of amplicons, indicating that the virulence genes classified into three main groups: (1) aerolysins-hemolysins, (2) cytolytic enterotoxins, and (3) cytotoxic enterotoxins.

The pathogenicity of *A. hydrophila* depends in part on the production of aerolysin encoded by the *aero* gene. Aerolysin is a hydrophilic protein which exhibits both hemolytic and cytolytic properties. Tombelli et al. (2000) developed a unique DNA PCR piezoelectric biosensor for identification of *A. hydrophila* based on the *aero* gene. The primers AERO1/AERO2 amplified a 205bp sequence of the *aero* gene. A 233-mer biotinylated probe was immobilized onto a streptavidin-coated gold disk on the surface of a quartz crystal to achieve piezoelectric detection.

Kong et al. (2002) amplified a 720bp sequence of the *aero* gene that encodes the cytolytic autolysin utilizing the primers *Aero-F/Aero-R* for detecting *Aeromonas* in marine waters. Restriction digestion with *TaqI* yielded fragments of 44, 310, and 366bp which were found to be specific for *A. hydrophila*.

Xia et al. (2004) developed a species-specific PCR for *A. hydrophila*. A pair of primers AP1/AP2 amplified a 208bp sequence of the beta-hemolysin gene. Ji et al. (2004) utilized a universal primer PCR (UPPCR) involving a pair of universal primers EUB f933/EUB r1387 to amplify a 500bp sequence of the conserved 16S rDNA in conjunction with denaturing gradient gel electrophoresis (DGGE) and single-stranded conformation polymorphism (SSCP) for rapid PCR detection of *A. hydrophila*, among other pathogens. When the 500bp amplicon was subjected to DGGE, the 500bp amplicon from *A. hydrophila* exhibited a distinctly different migration location compared to other organisms. Single-strand conformational polymorphism (SSCP) yielded three major bands with *A. hydrophila* which were distinct from the banding profiles of other organism. The combination of UPPCR-DGGE was found to yield a highly sensitive method for identification of *A. hydrophila* that was somewhat superior to UPPCR.

8.3.3 *Plesiomonas shigelloides*

The genus *Plesiomonas* in the family Vibrionaceae consists of one species, *P. shigelloides*, which is a Gram-negative, polarly flagellated rod native to aquatic animals and environments. Its metabolism is similar to that of the genus *Vibrio*, being facultatively

anaerobic, cytochrome oxidase positive, fermenting sugars to acid without gas production, and sensitive to the vibriostatic agent O/129. Mild to severe self-limiting diarrhea is the most frequent symptom derived primarily from uncooked shellfish although extraintestinal infections of high mortality are known to occur, particularly among children and immunocompromised individuals. The organism has been ranked third as a cause of traveler's diarrhea in Asia. Uncooked oysters are the major food incriminated in outbreaks in the US. The minimum temperature range for growth is 8–10 °C and the maximum is 42–45 °C. Most isolates grow from a pH of 4.0–9.0. The utilization of inositol with acid production is a unique characteristic of the organism that is used for its selective and differential isolation. The organism produces a cholera-like (CL) enterotoxin, a thermostable (TS) and a thermolabile (TL) enterotoxin.

8.3.3.1 Application of PCR and Rti-PCR to *P. shigelloides*

González-Rey et al. (2000) were the first to develop a PCR assay specific for *P. shigelloides*. The assay was used to confirm the identity of 25 isolates from aquatic environments, 10 isolates from human clinical cases of diarrhea, and five isolates from animals. The forward primers PS23FW3/PS23RV3 amplify a 284 bp sequence of the 23S rRNA gene. Gu et al. (2006a) subjected 26 isolates of *P. shigelloides* from Sweden (10 freshwater, six fish, 10 human clinical) to RAPD analysis with the use of two random primers (LMPB1 and LMPB4; see Table 8.1). Prior to RAPD analysis, the identity of all isolates was confirmed via PCR utilizing the primer pair of González-Rey et al. (2000). There was notable genetic variability among most of the isolates, and none of the isolates had the same composite RAPD profile.

A rapid and efficient procedure for quantitative detection of *P. shigelloides* in pure culture was developed by Gu et al. (2006b). A quantitative assay for *P. shigelloides* in clams and oysters based on the conventional PCR was developed by Gu and Levin (2006). The primers used were those of González-Rey et al. (2000). The assay involved the treatment of homogenized tissue samples with 4.0% formaldehyde that presumably denatured DNases and proteases present in the tissue which would otherwise inactivate the PCR reaction. The level of detection of *P. shigelloides* in clam tissue without enrichment was 60 CFU/g. The level of detection of *P. shigelloides* in oyster tissue without enrichment was 6×10^5 CFU/g. The addition of 4.0% formaldehyde to oyster tissue homogenates plus 0.1% BSA reduced the level of detection to 2×10^2 CFU/g.

Herrera et al. (2006) developed a PCR assay for detection of *P. shigelloides* from marine fish tissue. The procedure utilized a non-selective enrichment in tryptic soy broth plus 0.6% yeast extract (TSBY) for 24 hours at 37 °C. The primers used amplified a 435 bp sequence of the *hugA* gene that encodes an outer membrane receptor *HugA*, required for heme iron utilization, highly specific for *P. shigelloides*.

Loh and Yap (2002) were the first to develop a Rti-PCR assay for *P. shigelloides*. The primer pair used amplifies a 112 bp sequence of the 23 s rRNA gene. Their assay involved the use of SYBR Green I in conjunction with a probe labeled at the 5'-end with LCRed640 for the establishment of a fluorescent resonance energy transfer (FRET) system.

Fuchushima and Tsunomori (2005) developed a Rti-PCR assay using SYBR Green for detecting *P. shigelloides* in stool samples. The forward primer PSG237-F and the reverse primer PAG110R amplify a 68 bp sequence of the *gyrB* (DNA gyrase B) gene. A commercial stool extraction kit was used for DNA extraction and purification. The assay was completed within 2 hours.

A quantitative assay for *P. shigelloides* in pure culture and oysters based on Rti-PCR and utilizing SYBR Green was developed by Gu and Levin (2008). The primers PS23FW3/PS23RV3 from González-Rey et al. (2000) were used. With seeded oyster tissue homogenates, without formaldehyde or coated charcoal treatments, the lowest level of detection for *P. shigelloides* was 1×10^7 CFU per gram of tissue, equivalent to the DNA from 2.5×10^5 CFU per Rti-PCR. The combination of adding 4.0% formaldehyde to oyster tissue homogenates and treatment with coated charcoal reduced the level of detection of *P. shigelloides* to 1×10^3 CFU per gram, equivalent to the DNA from 25 CFU per Rti-PCR.

8.3.4 *Listeria monocytogenes*

Listeria monocytogenes is a peritrichously flagellated Gram-positive intracellular bacterial pathogen that has been isolated from a wide variety of foods, including fish, and is capable of growth at refrigerator temperatures as low as 1°C. Most healthy adults infected with *L. monocytogenes* experience only mild flu-like symptoms. Listeriosis, however, is an infectious disease, which is characterized by monocytosis, growth of the organism in macrophages, septicemia, and the formation of multiple focal abscesses in the viscera. Infection of pregnant women may lead to invasion of the fetus, resulting in stillbirth or abortion. The most common form of listeriosis is meningitis, which develops predominantly in newborns and the aged, resulting in approximately 70% mortality if untreated (Killinger 1970; Seeliger & Finger 1976).

Among the seven species of *Listeria*, only *L. monocytogenes* and *L. ivanovii* are pathogens for humans and animals. A major virulence factor that contributes to the pathogenicity of *L. monocytogenes* and its ability to enter, survive, and grow within mammalian cells is the SH-activated alpha-hemolysin (listeriolysin O). The listeriolysin O gene (*hlyA*) from *L. monocytogenes* has been shown to be absent in other *Listeria* species (Mengaud et al. 1988). The high level of species specificity of this gene has allowed it to be used as a valuable target for detection of *L. monocytogenes* by PCR. The USDA specifies a zero tolerance for *L. monocytogenes* in ready-to-eat meat products and certain other foods.

8.3.4.1 Application of PCR and Rti-PCR to *L. monocytogenes*

Border et al. (1990) confirmed that the LM1/LM2 set of primers of Mengaud et al. (1988) for amplification of a 702 bp sequence of the listeriolysin O gene were species specific for *L. monocytogenes*.

Wernars et al. (1992) made use of the transcriptional activator gene (*prfA*) to develop a highly specific PCR for recognition of pathogenic *L. monocytogenes* strains. The primers used, *prfA*-A/*prfA*-B, flank a 1060 bp sequence encompassing the entire *prfA* gene, were specific for all virulent strains of *L. monocytogenes* tested, and readily distinguished them from an avirulent strain of *L. monocytogenes* and representatives of the other six *Listeria* species.

Simon et al. (1996) described four different protocols for extraction of total DNA from cold-smoked salmon for PCR detection of *L. monocytogenes* without enrichment. Each of the protocols used proteinase K to facilitate cell lysis and the precipitation of DNA with Cetyl trimethylammonium bromide (CTAB) as described by Murray and Thompson (1980). A nested PCR detection protocol was used. The first PCR

amplification used primers PRFA and PRFB which amplified a 1060 sequence of the *prfA* gene (Wernars et al. 1992). The second PCR amplification employed primers LIP1 and LIP2 that amplified a 274bp sequence of the *prfA* gene. Protocol 4, which incorporated CTAB extraction with filter membrane separation to remove particles greater than 0.2 μ after cell lysis followed by silica column purification, allowed detection of 0.8×10^3 CFU/g of tissue.

Aznar and Alarcón (2002) undertook an extensive examination of nine sets of primers for detection of *L. monocytogenes* and found that the *hlyA* primer pair LM1/LM2 developed by Mengaud et al. (1988) was superior to the other eight primer pairs in terms of specificity for *L. monocytogenes*.

Norton et al. (2001) made use of the commercial BAX PCR system for detecting *L. monocytogenes* in three smoked fish processing plants. A total of 531 samples including raw fish, smoked fish, and environmental samples yielded 95 (17.9) positive samples. Ribotyping indicated that specific ribotype strains exhibited long-term persistence and were part of the resident microflora of these plants.

Oravcová et al. (2007) developed a Rti-PCR assay for the detection of *L. monocytogenes* in foods following enrichment. The primers LMrt3F/LMrt3R amplified an unspecified base-pair sequence length of the *actA* gene. A dual-labeled probe listP with FAM at the 5'-end and TAMRA at the 3'-end was used for detection of amplification. The limit of detection with seeded samples of fish was 10 CFU/25 g.

8.3.4.2 Application of RAPD and PFGE to *L. monocytogenes* Isolates

Destro et al. (1996) subjected 115 strains of *L. monocytogenes* collected from different areas of a shrimp processing plant in Brazil over a 5-month period to RAPD and PFGE analysis. Two random primers were used for RAPD analysis designated UBC 155 and UBC 127 that generated 11 and 16 different RAPD profiles respectively. The use of composite profiles derived from both RAPD and PFGE resulted in an increase in strain discrimination.

Vogel et al. (2001a) compared the RAPD profiles of 148 isolates of *L. monocytogenes* from vacuum-packed cold-smoked salmon derived from 10 different Danish smokehouses. A total of 16 different RAPD profiles were obtained using four separate primers. The grouping of all 148 strains was exactly the same with each of the four primers used. Isolates, which were indistinguishable using a single primer, were on no occasion found to be dissimilar with the other three primers. The authors noted that the same RAPD types were found in products produced after 6 and 8 months for two of the smokehouses, indicating long-term establishment of specific strains in smokehouses. Different RAPD types dominated products from different smokehouses. Some identical RAPD types were isolated from several smokehouses. Each smokehouse carried its own specific RAPD type, suggesting a possible persistence of closely related strains of *L. monocytogenes*.

Cao et al. (2005a) subjected 99 randomly selected isolates of *Listeria monocytogenes* from several processing environment locations in a shrimp processing plant, obtained during a 5-month sampling period, to RAPD analysis with the use of four primers: UBC155, PB1, PB4, and HLWL74. Preliminary studies indicated that the number of DNA bands and their intensity differed greatly with respect to the commercial source of the *Taq* polymerase used with individual isolates. Eighteen composite RAPD types were discerned with the use of the four primers. Among these 18 composite RAPD types,

type 1 was composed of 14 indistinguishable isolates, and type 9 was composed of 49 indistinguishable isolates. These results indicated that the shrimp processing plant was dominated by these two RAPD types that comprised 63.6% of the 99 randomly selected isolates.

Cao et al. (2005b) sampled fresh fish fillets over a 24-month period from two major supermarket retail outlets in Amherst, Massachusetts, USA, designated A and B, for the incidence of *L. monocytogenes* and numbers of the organism present per 100 g of tissue. Fifteen species of fish were represented and 74 samples out of a total of 320 were confirmed by PCR as yielding *L. monocytogenes*. From retail source A, a total of 171 samples yielded 59 (34.5%) that were positive for the presence of *L. monocytogenes*. In contrast, from retail source B, a total of 149 samples yielded 15 (10.0%) that were positive. A total of 221 strains of *L. monocytogenes* were derived from the MPN cultures, 164 from retail source A and 57 from retail source B. All 221 strains were subjected to RAPD analysis using three random primers. Primer PB1 yielded 21 RAPD profiles, primer PB4 yielded 19 profiles, and primer HLWL74 yielded 26 profiles. A total of 55 composite profiles were identified by combining the profiles derived from the three primers. Source A yielded 50 composite RAPD profiles, whereas source B yielded only 10 composite profiles. In addition, 27 of the 55 composite profiles were derived from individual isolates and RAPD types 11 and 18 included 49 and 27 isolates respectively. Fish from retail source A clearly harbored far more RAPD types than did source B. The results clearly indicated that two major retail sources in close geographic proximity can vary considerably with respect to the incidence and numbers of *L. monocytogenes* present on the fish tissue. It was not possible to determine whether the processor furnishing fish to retail outlet A or the supermarket itself was responsible for the notably higher incidence and numbers of *L. monocytogenes* on fish from retail source A compared to fish from retail source B.

There are relatively few reports of listeriosis derived from seafood but one such outbreak involving two cases that occurred in New Zealand in 1992 was studied in detail by Brett et al. (1998). PFGE profiles derived from the two restriction endonucleases *ApaI* and *SmaI* indicated that the isolates from both patients were identical to that obtained from refrigerated mussels of a specific brand still possessed by one patient. Isolates from refrigerated retail packets of the same brand and the processing environment from which they were derived yielded isolates of the same PFGE profile as that of the two patients. This strain was found to persist in the processing environment from 1990 to 1993.

Listeria monocytogenes is not considered a natural contaminant of fish (Autio et al. 1999). Contamination of fishery products is considered to be a result of processing contamination. The processing of cold-smoked rainbow trout does not inactivate *L. monocytogenes* (Autio et al. 1999). In addition, most such products are vacuum packaged and consumed without cooking which can pose a potential listeriosis threat. Only one sample of rainbow trout among a total of 60 was found positive before processing in a cold smoked processing plant (Autio et al. 1999). None of 49 fillets sampled were positive. The frequency of fish contaminated with *L. monocytogenes* was found to clearly rise after brining and the most contaminated processing sites were the brining and post-brining areas. A total of 303 isolates of *L. monocytogenes* from the raw fish, processing environment, and final product were characterized by Autio et al. (1999) using PFGE. *AscI* and *SmaI* yielded nine and six profiles respectively and in combination resulted in

a total of nine types. The predominating types of the final product were associated with brining and slicing. The use of hot steam, hot air, and hot water was effective in eliminating the organism from the plant and final product.

The routes of contamination of two Danish cold-smoked salmon processing plants by *L. monocytogenes* were investigated by Vogel et al. (2001b) by analyzing 3585 samples from products (1995–1999) and processing environments (1998 and 1999). The level of product contamination in plant I varied from 31% to 85% and no *L. monocytogenes* was found on raw fish. In plant II, the levels of both raw fish and product contamination varied from 0% to 25%. A total of 429 isolates of *L. monocytogenes* were subjected to RAPD analysis with a single 10-mer primer HLWL85 and 55 different profiles resulted. The RAPD types detected on the product were identical to types found on the processing equipment and the processing environment, suggesting that contamination in both plants was from the processing environment and not from the raw fish. In plant I, the same predominant RAPD type was found over a 4-year period. In plant II, which had a lower prevalence of *L. monocytogenes*, no RAPD type persisted over long periods of time. Persistent strains (125) were also typed by PFGE and AFLP analysis which confirmed the results obtained by RAPD profiling. The authors concluded that persistent strains may be avoided by vigorous cleaning and sanitation.

8.3.5 *Clostridium botulinum*

Clostridium botulinum is a Gram-positive obligately anaerobic spore-forming rod of which there are seven types, A–G, based on serological distinction of the respective neurotoxins produced. Human botulism is caused by types A, B, E, and rarely type F. Types C and D cause botulism in animals. Type G is not associated with neurotoxicity in humans or animals.

8.3.5.1 Relationship Between Botulism and Seafood

In recent years, an average of 450 botulism outbreaks have been reported annually in the international literature, 12% of the outbreaks being caused by type E (Hatheway 1995). Coastal marine environments usually exhibit serotype E as predominant. Isolates of type E are truly psychrotrophic and exhibit the ability to grow in seafood tissue under refrigerated conditions ($\sim 4^{\circ}\text{C}$). Type E is therefore the most frequent cause of botulism derived from seafood.

There is a well-established history of salted fish causing type E botulism. Uneviscerated, salt-cured fish have been implicated in a number of additional botulism outbreaks (Badhey et al. 1986; Kotev et al. 1987; Telzak et al. 1990). The intestines of uneviscerated, salted fish are thought to result in a low-salt environment allowing spores of *C. botulinum* to germinate, grow, and produce toxin. Weber et al. (1993) reported on a massive outbreak of type E botulism associated with the consumption of traditional salted fish in Cairo. Low levels of type E toxin are known to result in primarily GI symptoms. Sobel et al. (2007) reported on an outbreak of clinically mild botulism type E illness among five individuals resulting in predominantly GI symptoms consisting of nausea, vomiting, abdominal pain, dry mouth, shortness of breath, and in one individual diplopia (double vision). Fresh, uneviscerated whitefish with salt had been placed in a sealed ziplock bag and stored for ~ 1 month at ambient temperature prior to consumption. Remnant fish tested positive for botulinum type E toxin.

Commercially produced vacuum-packaged hot-smoked fish is presently considered one of the most important botulism food vehicles. Hot-smoked Canadian whitefish was reported by Korkeala et al. (1998) to be the cause of a single family outbreak of type E botulism in 1997. The fish was smoked only 5 days before consumption, indicating that toxin production had been rapid and that there had been marked temperature abuse during storage or transport of the fish. Type E toxin was confirmed by toxin neutralization and the mouse bioassay and by PCR.

In the Baltic Sea area, where non-proteolytic Group II *C. botulinum* is known to predominate, a particularly high prevalence of type E has been reported (Hielm et al. 1998a; Hyytiä et al. 1998). Hyytiä et al. (1998) described contamination levels of type E in Finland of 10–40% in raw fish and fish intestines, with the highest prevalence being Baltic herring, and 4–14% in fish roe. In addition, 30% of German raw fish have been found to contain type E spores (Hyytiä-Trees et al. 1999).

8.3.5.2 PCR Detection of *C. botulinum*

Szabo et al. (1993) developed PCR assays for detection of BoNT genes A, B, C, D, and E. The primer pair E1/E2 amplified a 745 bp sequence of the *bontE* gene.

Franciosa et al. (1994) studied the effectiveness of PCR in detecting type A, B, and E BoNT genes among 209 strain of *C. botulinum* and 29 strains of other *Clostridium* species. A pair of primers GF-1/GF-3 yielded a 772 bp amplicon of the *bontE* gene. A universal bacterial primer pair was also used to yield a 763 bp amplicon derived from the 16S rRNA which served as a positive amplification control.

Hielm et al. (1996) developed PCR methodology for the MPN-PCR detection and enumeration of BoNT types A, B, E, and F in fish and sediment samples. The general BoNT primers BonT1/BonT2 of Campbell et al. (1993) were used for detection of all BoNT types and yielded a 1184 bp amplicon from all types except A, which yielded an amplicon of 1038 bp. The 16S rRNA universal bacterial primers BAC-3/BAC-4 from Franciosa et al. (1994) yielding a 760 bp amplicon were incorporated into each PCR reaction to ensure against PCR inhibition and false-negative results. The primers GF-1/GF-3 from Franciosa et al. (1994) yielded a 762 bp amplicon for type E strains. Rainbow trout were seeded with spores of *C. botulinum* type E at 102–106 spores/kg of tissue in addition to the inoculation of fish intestines. Each sample was subjected to a 5-day enrichment in TPGY broth followed by transfer of 0.5 mL into 10 mL with overnight incubation prior to PCR reactions. Washed vegetative cells from such enrichment broth cultures were boiled for 10 minutes and 1 mL incorporated into PCR reactions. All seeded samples were detected as positive. Among 10 sediment samples tested, eight (80%) were positive for *C. botulinum* type E spores with spore counts of *C. botulinum* type E ranging from 95 to 2710/kg of sample.

Lindström et al. (2001) described the development of a multiplex PCR (mPCR) for detection of the BoNT A, B, E, and F genes in food and fecal samples. The primer pairs CBMLE1/CBMLE2 yielded an amplicon of 389 bp for type E strains. With a two-step enrichment, the detection limit in food and fecal samples was one spore per 10 g sample or less.

Alsallami and Kotlowski (2001) developed improved primer pairs for detection of the BoNT/B and BoNT/E genes. The detection limit was increased from 1 to 0.1 ng of DNA by increasing the annealing temperature from 50°C to 62°C. The primers BoTE1/BoTE2 amplified a 307 bp sequence of the *bontE* gene.

Kimura et al. (2001) developed a Rti-PCR assay for quantifying *C. botulinum* type E in modified-atmosphere packaged fish samples (jack mackerel). The primers BE1430F/BE1709R amplified a 269 bp sequence of the *bontE* gene. The dual-labeled probe BE1571FP was labeled at the 5'-end with 6-FAM and at the 3'-end with TAMRA. The quantifiable range was 10^2 to 10^8 CFU/g which allowed detection much earlier than toxin could be detected with the mouse bioassay. The prevalence of *C. botulinum* types A, B, E, and F in river lamprey caught in Finnish rivers was determined by Merivirta et al. (2006) using a quantitative PCR-MPN (most probable number) analysis. The multiplex PCR assay and primers of Lindström et al. (2001) were utilized. Among 67 raw whole lampreys, one (1.5%) was positive for the *bontE* gene with an estimated *C. botulinum* spore count of 100 spores/kg. Two type E strains were isolated from the positive sample and confirmed as different genotypes by PFGE using *SmaI* and *XhoI*. The authors concluded that vacuum packaging with refrigerated storage may constitute a safety hazard in processed lamprey from the Baltic Sea area and recommended a storage temperature of 3°C or below for such products.

8.3.5.3 Molecular Typing of *Clostridium botulinum* Type E Strains

The distribution of *C. botulinum* serotypes A, B, E, and F in Finnish trout farms was assessed using PCR by Hielm et al. (1998b). The PCR primers from Hielm et al. (1996), including those for *bontE*, were used. A total of 333 samples were tested with neurotoxin gene-specific PCR assays. *C. botulinum* type E was found in 68% of farm sediment samples, in 15% of fish intestinal samples, and in 5% of fish skin samples. No other serotypes were found. The average spore count in sediments, fish intestines, and skin were 2×10^3 , 1.7×10^2 , and 3×10^2 per kg respectively. PFGE with *SmaI* of 42 Finnish isolates plus 12 North American reference strains generated 28 PFGE profiles indicating extensive genetic diversity.

The genetic diversity of 92 type E strains of *C. botulinum* was assessed by Hyttiä et al. (1999). Sixty-seven were of Finnish seafood and fishery origin, 15 were from German farmed fish, and 10 from North American seafoods. PFGE performed with *SmaI-XmaI* resulted in 75 typeable strains which yielded 33 profiles. PFGE performed with *XhoI* allowed 91 strains to be typed, yielding 51 profiles. All 92 strains were typeable with RAPD primers OPJ-6 and OPJ-13 which yielded 27 and 19 banding patterns respectively. The frequent occurrence of small fragments and faint bands made RAPD interpretation difficult. A high level of genetic diversity among the isolates was observed regardless of their source, presumably because of the absence of strong evolutionary selection factors.

Leclair et al. (2006) undertook a comparative typing study involving the PFGE, RAPD, and automated ribotyping of *C. botulinum* type E strains derived from clinical and food sources associated with four botulinum outbreaks that occurred in the Canadian Arctic. All type E strains previously untypeable by PFGE, even with the use of a formaldehyde fixation step, could be typed by the addition of 50 mM thiourea to the electrophoresis running buffer. Digestion with *SmaI* and *XhoI* followed by PFGE was used to link food and clinical isolates from the four different type E *C. botulinum* outbreaks and to differentiate them from among 31 recently isolated Arctic environmental group II *C. botulinum* strains. *SmaI* PFGE typing yielded 18 profiles while *XhoI* PFGE typing yielded 23 profiles. Strain differentiation was unsuccessful with the automated ribotyping system which yielded only two profiles. RAPD analysis of the group II strains was not consistently reproducible with primers OPJ-6 and OPJ-013. Primer OPJ-13 did, however, yield 28 profiles.

8.4 Conclusions and Future Perspectives

The major genera and species of bacteria responsible for various aspects of seafood spoilage are well recognized. Molecular techniques have recently been developed for rapid detection and enumeration of these major spoilage bacteria. Genomic studies will continue to clarify the taxonomic status of these microorganisms and their relationship to one another. Outbreaks of certain human pathogenic bacteria such as members of the genus *Vibrio* are frequently associated with the consumption of raw and undercooked shellfish. The ability to detect and enumerate such pathogens in the viable state by the direct use of molecular techniques has allowed enhanced public health surveillance of such products and has the potential to facilitate the reduction of such pathogens in shellfish.

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9

Assessment of the Microbial Ecology of Meat and Meat Products at the Molecular Level: Current Status and Future Perspectives

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9.1 Introduction

Assessment of the microbial ecology of meat and meat products has been traditionally performed by classic microbiological techniques. However, such an approach may only detect the culturable proportion of the microbiota and lacks sufficient discrimination power for epidemiological and biodiversity studies. Molecular microbiology techniques, when properly applied, may result in significant improvements that enable such types of studies. Moreover, it is possible to obtain results in significantly less time, which is crucial in the case of pathogen detection.

Through the wealth of information that has been generated after years of application, referring to both the qualitative and quantitative study of ecosystem composition as well as pathogen detection, the critical steps that determine the quality of the outcome using molecular techniques have been identified. These include the extraction of amplifiable nucleic acids that is largely influenced by the food matrix itself, and the polymerase chain reaction (PCR), referring mostly to the selection of the appropriate genomic target as well as post-PCR amplicon visualization and the possibility of quantitative detection through qPCR. Improvements for each step have been experimentally evaluated and effective interventions have been proposed.

The molecular microbiology techniques may be divided into those that allow the assessment of the microcommunity structure, such as temperature gradient gel electrophoresis (TGGE), denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), and terminal restriction fragment length polymorphism (T-RFLP), and those that only allow the detection of selected target(s) such as specific PCR, reverse transcription-quantitative PCR (RT-qPCR) and fluorescent *in situ* hybridization (FISH). Moreover, several techniques such as pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), and repetitive element palindromic PCR (rep-PCR) when applied to isolates may be used for epidemiological as well as biodiversity purposes. In the following pages, an update of the knowledge regarding the study of the microbial ecology of meat and meat products at the molecular level is presented.

9.2 Extraction of Nucleic Acids

The first step towards the molecular assessment of microbial ecology is biomass separation from the food matrix. Meat products are generally rich in protein and fat that may interfere with biomass preparation as well as the subsequent nucleic acid extraction and concomitantly compromise PCR.

Several protocols have been effectively applied. The simplest approach was to use a portion of the first serial decimal dilution after allowing the debris to set for a couple of minutes (Pennacchia et al. 2011; Villani et al. 2007). In order to obtain more biomass, a dilution of 1:5 up to 1:1 with saline-peptone water has been applied (Ercolini et al. 2006). In that case, a centrifuge at 95–1000 × g is very often employed to assist debris removal and the supernatant is used for biomass pelleting (Hu et al. 2008; Nguyen et al. 2013; Vasilopoulos et al. 2008). More sophisticated approaches have been applied by Fontana et al. (2005) and Rantsiou et al. (2005). In the former case, a washing step with petrol ether-hexane (1:1) was applied for lipid removal whereas in the latter study, a first washing step with a solution containing ammonium hydroxide, ethanol, petroleum ether, and 10% SDS was followed by the resuspension of the resulting pellet in a solution containing urea, ethanol, petrol ether, SDS and sodium acetate.

9.3 Microbial Communities Assessment

The technique almost exclusively employed for the assessment of the composition and dynamics of the microbial communities of meat and meat products is PCR-DGGE. On the other hand, PCR-SSCP as well as T-RFLP have been presented as promising alternatives but have met only limited application.

9.3.1 PCR-DGGE

PCR-DGGE is based on the electrophoretic separation of PCR-generated amplicons in a polyacrylamide gel containing a gradient of chemical denaturants (urea and formamide). Each amplicon is partially chemically denatured at different concentrations of the denaturants in a sequence-dependent manner; this partial denaturation results in the arrest of the electrophoretic migration that is characteristic of the taxonomic unit that the PCR was designed to detect.

Assessment of the prokaryotic content of a microecosystem has been performed by analysis of the V1 (Cocolin et al. 2001a), V1–V3 (Dewettinck et al. 2001), V2–V3 (Walter et al. 2000), V3 (Ampe et al. 1999; Muyzer et al. 1993), V3–V4 (Meroth et al. 2003), V4–V5 (Ercolini et al. 2003), V6 (van Beek & Priest 2002), V6–V8 (Zoetendal et al. 1998), and V9 (Ferris et al. 1996) regions of the 16S-rRNA gene. In addition, the eukaryotic portion has been studied through the 18S (Ampe et al. 2001), 28S rRNA (Silvestri et al. 2007), and 26S rRNA genes (Cocolin et al. 2002; Rantsiou et al. 2005). The specific genomic regions as well as the primers used in the case of meat and meat products are presented in Table 9.1. Analysis of either the V3 or the V6–V8 regions of the 16S-rRNA gene is most frequently performed. The former was originally described by Muyzer et al. (1993) amplifying a 194bp fragment and a touchdown thermocycling regime to minimize non-specific by-products. Amplification of the V6–V8 region of about 450 bp

Table 9.1 Genomic regions and primers used in assessing the prokaryotic and eukaryotic content of the microecosystem of meat and meat products by PCR-DGGE.

Region	Name	Sequence (5'–3')	Reference
V1	P1	GC-clamp- GCGGCGTGCCTAATACATGC	Fontana et al. 2005; Silvestri et al. 2007; Villani et al. 2007
	P2	TTCCCCACGCGTTACTCACC	
V1-V3	P1	GC-clamp- GCGGCGTGCCTAATACATGC	Russo et al. 2006
	518r	ATTACCGCGGCTGCTGG	
V2-V3	Bact-0124GCf	GC-clamp- CACGGATCCGGACGGGTGAGT AACACG	Fontana et al. 2005
	Uni-0515r	ATCGTATTACCGCGGCTGCTGCTGGCA	
V3	V3f (gc338f)	GC-clamp- CCTACGGGAGGCAGCAG	Cocolin et al. 2001b; Hu et al. 2008; Nguyen et al. 2013; Vasilopoulos et al. 2008; Villani et al. 2007
	V3r (518r)	ATTACCGCGGCTGCTGG	
	V3f	GC-clamp- CCTACGGGAGGCAGCAG	
	Uni-0515r	ATCGTATTACCGCGGCTGCTGCTGGCA	
V6-V8	U968	GC-clamp- AACGCGAAGAACCTTAC	Ercolini et al. 2006; Pennacchia et al. 2011
	L1401	GCGTGTGTACAAGACCC	
28S-rDNA	U1	GC-clamp- GTGAAATTGTTGAAAGGGAA	Silvestri et al. 2007
	U2	GACTCCTTGGTCCGTGTT	
26S-rDNA	NL1	GC-clamp- GCCATATCAATAAGCGGAGGAAAAG	Villani et al. 2007
	LS2	ATTCCCAAACAACCTCGACTC	

was described by Zoetendal et al. (1998). In that study, an annealing temperature of 56°C was originally applied but a touchdown approach was also later proposed (Ercolini et al. 2006). Minimization of artifacts was also attempted by nested protocols, such as the ones described by Hu et al. (2008) and Fontana et al. (2005). In both cases, a first round of amplification resulted in an approximately 800 bp amplicon spanning the V2 and V3 regions. Then, a second round of amplification was utilized to amplify specifically parts of the V2–V3 region in the first case and the V3 region in both cases.

Amplicon visualization is achieved by polyacrylamide gel electrophoresis (7–8% v/v polyacrylamide) containing a variable degree of denaturing gradient, in TAE buffer. The denaturing gradient may be as wide as 30–60% (with 100% defined as the solution containing 7 M urea and 40% formamide) that was used by Cocolin et al. (2001b) to differentiate members of the Micrococcaceae family through the V3 region and Villani et al. (2007) to differentiate yeasts through the 26S-rRNA gene or as narrow as the 30–50% and the 40–60% that are very often used.

Identification of single constituents of a microecosystem takes place by co-migration with reference amplicons corresponding to the desired identification unit or by excision of each band and further amplification by PCR and sequencing.

PCR-DGGE has been extensively applied as a culture-independent approach in both food and environmental microecosystems. This extensive application has revealed the limitations. The major ones are associated with the selection of the genomic region. As far as bacterial systematics is concerned, the 16S-rRNA gene has been rapidly adopted for the assessment of the bacterial phylogenetic affiliation. As a result, current databases contain a plethora of 16S-rRNA gene sequences from every described bacterial species. However, there are certain limitations that need to be taken into consideration in such studies: closely related species may not be differentiated, horizontal transfer and recombination has been suggested as possible, and multiple but not identical copies of the gene may be present (Klappenbach et al. 2000; Schouls et al. 2003). These may create artifacts and problems very often encountered in the application, such as co-migration and identical denaturation of closely related species, multiple banding of single species, heteroduplexes, etc.

Another issue that refers to the PCR step of the experimental procedure is the limit of detection. It has been demonstrated that populations below 10^3 – 10^4 CFU/g may not be detected (Cocolin et al. 2001a). Moreover, the detection of minor populations compared to a dominant one may be problematic. Indeed, the DNA of a prevailing population may dominate the PCR reaction while microorganisms whose population is 1 or 2 logarithms below may not be reproducibly detected. Moreover, it has been reported that differences in the sequence of even the conserved regions of small subunit rRNA genes may exist, preventing the hybridization of the universal primers that are very often used (Baker et al. 2003). This may result in underestimation of the sample biodiversity. Another issue that has been raised regarding this technique is the inclusion of DNA from dead cells in the analysis due to the persistence of this nucleic acid after cell death (Josephson et al. 1993). Failure to differentiate between dead and living cells may insert a bias in the description of a microecosystem. Therefore, treatment with ethidium monoazide bromide (EMA) is necessary in the respective protocols in order to obtain this differentiation. Another alternative that may ensure discrimination between living and dead cells is to use RNA after reverse transcription as a template for PCR-DGGE. This approach has been repeatedly used in the case of dairy products but so far not so much with meat and meat products (Dolci et al. 2010, 2013; Masoud et al. 2011).

Application of PCR-DGGE for the assessment of the microbial ecology of meat and meat products has failed to reveal hidden or inactive populations, simply because of the limited level of complexity of these microecosystems and the fact that they have been extensively studied by the culture-dependent approach. However, from a technical point of view, much advancement has occurred providing specific protocols depending on the microbial target of the analysis and of course within the framework defined by the inherent limitations discussed above.

9.3.2 PCR-SSCP

PCR-SSCP is a technique based on the differences of the electrophoretic mobility of single-stranded DNA resulting from differences of secondary structures adopted under non-denaturing conditions due to dissimilarities of the DNA sequence. It is a technique extensively used in mutation and polymorphism detection (Gui et al. 2014; Han et al. 2014; Yang et al. 2014; Zhang et al. 2015; Zhou et al. 2014). In the assessment

of microcommunity composition, it has not been widely used, at least compared to T/DGGE (King et al. 2005; Larentis et al. 2015; Peters et al. 2000; Schmalenberger et al. 2008). In the case of meat and meat products, it has only been applied for the reconstruction of the original fungal flora of heat-processed meat products by Dorn-In et al. (2013). In that study, an attempt was made to explore the fungal species contaminating the raw materials used for the manufacture of heat-processed meat products. The recovery of DNA from a variety of species such as *Aureobasidium pullulans*, *Eurotium amstelodami*, *Candida* spp., and *Pichia membranifaciens* possibly originating from meat and its processing facilities as well as plant pathogens such as *Lewia infectoria*, *Botrytis aclada*, and *Iterosonia perplexans* possibly originating from the spices were reported. The limitations of SSCP are similar to those already discussed for DGGE, especially the ones referring to the genomic region (ribosomal RNA genes) used for the study.

A comparison between SSCP and DGGE for analysis of the bacterial and archaeal communities obtained from a methanogenic bioreactor on the basis of V3–V4 16S-rRNA gene was performed by Hori et al. (2006) and revealed significant differences in the bacterial banding profiles. More accurately, SSCP resulted in a larger number of sharp and easily differentiated bands whereas DGGE resulted in fewer and smeared ones, leading to the basic conclusion that SSCP was superior in detecting the dynamics of this specific bacterial community. Both techniques performed equally in assessing the dynamics of the archaeal community that has been assigned to the simple structure of that community. Similar results were obtained by Hong et al. (2007) during profiling of bioremediation microcommunities with the V3 region of the 16S-rRNA gene. Generally, more phylogenetic groups were detected with capillary electrophoresis (CE)-SSCP although some of the dominating microorganisms were detected by both methods. In both studies, the time required for sample preparation and analysis was less in SSCP than DGGE.

9.3.3 Terminal Restriction Fragment Length Polymorphism

T-RFLP analysis is based on the differences between the length and the sequence of terminal restriction fragments (TRFs) generated by the application of restriction endonucleases on fluorescently labeled amplicons of a particular gene target. It is a method widely applied in the microbial characterization of soil ecosystems (Dunbar et al. 2000; Hartmann 2005; Lukow et al. 2000; Pesaro et al. 2004) as well as dairy products (Rademaker et al. 2005, 2006; Sánchez et al. 2006), wine and beer fermentations (Bokulich & Mills 2012) and seafood (Reynisson et al. 2009; Wang et al. 2009; Wilson et al. 2008; Yuichiro et al. 2010). In the case of meat products, it has been applied by Nieminen et al. (2011) to characterize the psychrotrophic bacterial communities in modified atmosphere-packaged meat. The results obtained were in agreement with the culture-dependent approach executed in parallel and revealed the capability of this approach for rapid and high-throughput characterization of microbial consortia as well as a significant limitation that originates from the need of the database; the quality of the result of techniques relying on databases is only as high as the quality of the database itself.

A comparison between PCR-DGGE, PCR-SSCP, and T-RFLP in the analysis of the bacterial community of arable soils was performed by Smalla et al. (2007). It was concluded that only limited variability to each micro-ecosystem was inserted from the technique applied.

Several studies have been performed comparing the effectiveness of culture-independent techniques in studying the microbial ecology of complex environmental samples and the comparative advantages and disadvantages have been discussed. DGGE and SSCP are distinguished for their ability to generate bands accessible for further analysis as they can be reamplified and sequenced (Costa et al. 2006; Mohr & Tebbe 2006). However, gel-to-gel variation may exist, which is not the case for T-RFLP that has been characterized by greater resolution power and reported as more suitable for routine analysis (Nunan et al. 2005). However, it should be mentioned that in nearly all cases, the genomic target is the 16S-rRNA gene and therefore all approaches are prone to the limitations that this target confers and which have been previously discussed.

9.3.4 Next-generation Sequencing

Advancements in the field of DNA sequencing technologies have led to the development of next-generation sequencing (NGS) that is increasingly used for the characterization of microecosystems. 16S-rRNA targeted 454 pyrosequencing has been used to study the dynamics of the bacterial microcommunity during cold storage of minced meat as well as Zhenjiang Yao meat (a traditional Chinese pork meat product that has been cooked, marinated, and jellied) under modified atmosphere packaging and supplemented with different preservatives (Stoops et al. 2015; Xiao et al. 2013). In both cases, a wealth of genomic reads was obtained and used for the subsequent analysis of the dynamics, thus providing a more integrated image of the respective microcommunities.

This metagenomic approach was also used by Nieminen et al. (2012) to compare the microcommunities between marinated and unmarinated broiler meat, and by de Filippis et al. (2013) to investigate the potential sources of bacterial spoilers in beef-steaks. In the first case, it was concluded that the acetic acid-containing marinade favored leuconostocs and diminished carnobacteria. Therefore, the former prevailed when the marinade was used whereas the latter prevailed in the unmarinated product. Moreover, it was indicated for the first time that *Vibrio* spp. belonged to the predominant microbial consortium of unmarinated meat; this genus was not associated with this type of product before. Additionally, many operational taxonomic units (OTUs) belonged to families that have not been associated with meat before, suggesting the extended diversity of bacteria that the product was exposed to before the packages were sealed. In the latter study, a remarkable carcass microecosystem was revealed including OTUs from 15 different phyla. Storage conditions resulted in a significant decrease in this complexity. It was suggested that the microbial species that have been associated with meat spoilage originate from the carcass itself and are simply carried out along the processing line until the population of the most efficient of them reaches a level that is perceived as spoilage.

9.4 Detection of Selected Bacterial Target

In many studies, the presence of a particular pathogen is the exception rather than representative of the normal composition of the microecosystem. Either DNA-based or protein-based detection is suitable here.

9.4.1 DNA-based Detection

Existence of sequences specific to the microbial target at the desired level of detection (i.e., genus/species/serotype/strain), their amplification through PCR and concomitant detection are the essence of DNA-based detection. This type of detection offers, in most cases, improved selectivity, sensitivity, specificity, and reliability compared to the classic microbiological techniques.

This approach, although not yet included widely in standardized protocols, is very often applied merely as an indication of pathogen existence that should be verified by the respective standardized protocols.

The issues that limit the application and greatly influence the result are the following.

- Pathogens are usually present at low numbers and the detection should be performed in the presence of a dominant microbial population at a much higher population level.
- Meat and meat products are rich in protein and fat that may interact with the PCR and compromise the detection step. Therefore, protocols capable of ensuring the isolation of DNA of adequate quality and quantity are required.

In the following paragraphs, the most effective interventions employed to provide solutions to the above mentioned challenges in detecting foodborne pathogens in meat and meat products are discussed.

It has been generally accepted that PCR-based techniques fail to ensure reproducible detection of microorganisms below a population of 10^3 CFU/g. Given the fact that pathogenic microorganisms are in the majority of the cases present in much lower populations and are accompanied by a dominant microbial consortium, there is an imperative need for a selective enrichment or concentration of the target. The former is the intervention most frequently employed and may be achieved by incubation in selective broths at temperature and time depending on the target pathogen.

The conditions allowing the simultaneous enrichment of more than one pathogen have been the subject of intensive study. Murphy et al. (2007) used tryptic soy broth supplemented with 20 g/L novobiocin and incubation at 42°C for 16 hours for the simultaneous propagation of *E. coli* O26, O111, and O157 from retail minced beef. The same broth without novobiocin was proved inadequate for effective enrichment of *L. monocytogenes* according to Kawasaki et al. (2005). In that study, the simultaneous enrichment of *L. monocytogenes*, *S. Enteritidis*, and *E. coli* O157:H7 from meat samples was attempted. It was concluded that only a broth termed No. 17 could enable growth of all pathogens at equal populations. Similarly, Li et al. (2005) successfully used brain–heart infusion broth and incubation at 37°C for 24 hours for the simultaneous propagation of *E. coli* O157:H7, *S. Typhimurium*, and *Shigella flexneri* from ready-to-eat meat products. However, such an approach requires extensive experimentation and therefore is not applied very often.

Alternatively, concentration of the target pathogen may be achieved with techniques such as immunomagnetic separation and buoyant density gradient centrifugation. The former is based on the use of antibodies immobilized on the surface of magnetic beads and their specific interaction with antigens present on the surface of the target cells. Then, the bead-target complex is separated from the food matrix, resulting in both concentration and separation from the food sample. This approach has been successfully applied in the detection of *S. Typhimurium* in raw meats (Moreira et al. 2008), *Campylobacter jejuni* in spiked chicken wash samples (Waller & Ogata 2000), *E. coli*

O157:H7, *Salmonella*, and *Shigella* present in ground beef (Wang et al. 2007), *Salmonella* in poultry (Fluit et al. 1993) and ground beef (Mercanoglu & Griffiths 2005), as well as *L. monocytogenes* in turkey meat (Bilir Ormanci et al. 2008).

Buoyant density gradient centrifugation is based on the separation of the sample constituents when a solution of density gradient is applied. This approach has been successfully applied for the detection of *S. flexneri* (Lindqvist et al. 1997), *E. coli* O157 (Lindqvist 1997), *Y. enterocolitica* (Lambertz et al. 2000; Lantz et al. 1998; Wolffs et al. 2004), and *C. jejuni* (Wolffs et al. 2005) from various meat products. As far as effective DNA extraction is concerned, all factors already discussed apply in this case as well.

Several genomic targets have been employed for the detection of pathogens through PCR. Multiplex format is preferred due to the simultaneous detection of more than one target. In Table 9.2, an example is presented of target genes, primers used, and amplicon sizes that have been effectively used in pathogen detection through multiple PCR discipline in meat and meat products. However, this approach, whether applied after an enrichment or concentration step or not, does not allow quantification of the pathogen population. The latter can be achieved by application of qPCR schemes. As in the previous case, a large amount of data exists regarding the detection of single pathogens whereas the challenge remains the multiplex format. In Table 9.3, an example of target genes along with the primers and probes effectively applied for the quantification of pathogen presence in meat and meat products is given.

Fluorescent *in situ* hybridization (FISH) is a very promising approach to the culture-independent detection of pathogens. It is based on the cytometric observation of cells in which fluorescently labeled probes have been hybridized to the target nucleic acid sequence, depending on the desired taxonomic level. It has been routinely used in medical applications (Jehan et al. 2012); however, the effect of the food matrix and the low population of the target microorganisms have limited widespread application. Apart from them, the limitations of this approach are derived from the specificity offered by the molecule on which hybridization is designed to occur. In order to alleviate these limitations, several interventions have been proposed (Rohde et al. 2015).

The potential of this approach has been revealed from the very first application in meat products. In a study by Regnault et al. (2000), a 24-mer probe complementary to 16S-rRNA was tested for the *in situ* detection of *E. coli* in raw ground beef. The hybridization of the probe to *Shigella* spp. as well as *E. fergusonii* exposed some of the limitations of this approach. Similar results were obtained by Fang et al. (2003) during detection of salmonellae in naturally contaminated food samples of various types. More accurately, the amount of false-negative results obtained depended upon the probe as well as the presumptive false positives, compared to the conventional method that was used in parallel.

Detection of campylobacters in chicken products was studied by Moreno et al. (2001) and Schmid et al. (2005). More accurately, in the first study 16S-rRNA probes were designed to detect thermotolerant campylobacters in both spiked and naturally contaminated samples. It was concluded that FISH was less sensitive than PCR regarding the detection, although the sensitivity was improved after selective enrichment. In the latter study, 16S-rRNA probes specific for *Campylobacter* spp. and 23S-rRNA probes specific for thermotolerant campylobacters were developed and effectively applied in poultry fecal samples spiked with *C. jejuni* and in naturally contaminated chickens.

An improvement of the FISH method was presented by Almeida et al. (2010), according to which peptide nucleic acid probes (PNAs), i.e. synthetic DNA analogues capable of hybridizing to an RNA sequence, were effectively used for the detection of *Salmonella*

Table 9.2 Primer sequences, target genes, and amplicon sizes used in multiplex format for pathogen detection in meat and meat products.

Target pathogen	Target gene	Name and sequence (5'–3')	Amplicon size	Reference
<i>Salmonella</i> spp.	<i>invA</i>	139 F: GTGAAATTATCGCCACGTTCTGGGCAA 141R: TCATCGCACCGTCAAAGGAACC	284	Jofré et al. 2005
<i>L. monocytogenes</i>	<i>prfA</i>	Lip1F: GATACAGAAACATCGGTTGGC Lip2R: GTGTAACCTTGATGCCATCAGG Lip3R: TGACCGCAAATAGAGCCAAG	274 (Lip1–Lip2) 215 (Lip1–Lip3)	
<i>S. Enteritidis</i>	<i>fimI</i>	SF: CCTTT CTCATCGTC CTGA A SR: TGGTG TTATC TGCCT GACC	85	Wang et al. 2004
<i>L. monocytogenes</i>	<i>hly</i>	LF: TCCGC AAAAG ATGAAGTTC LR: ACTCCTGGTG TTTCT CGATT	98	
<i>S. enterica</i>	<i>invA</i>	SAL-F: AATTATCGCCACGTTCTGGGCAA SAL-R: TCGCACCGTCAAAGGAACC	278	Germini et al. 2009
<i>L. monocytogenes</i>	<i>prfA</i>	LIS-F: TCATCGACGGCAACCTCGG LIS-R: TGAGCAACGTATCCTCCAGAGT	217	
<i>E. coli</i> O157:H7	<i>eaeA</i>	ESC-F: GGCGGATAAGACTTCGGCTA ESC-R: CGTTTTGGCACTATTTGCC	151	
<i>Salmonella</i> spp.	NA	TS-11: GTCACGGAAGAAGAGAAATCCGTACG TS-5: GGGAGTCCAGGTTGACGGAAAATTT	375	Kawasaki et al. 2005
<i>L. monocytogenes</i>	NA	LM1: CGGAGGTTCCGCAAAGATG LM2: CCTCCAGAGTGATCGATGTT	234	
<i>E. coli</i> O157:H7	NA	VS8: GGCGGATTAGACTTCGGCTA VS9: CGTTTTGGCACTATTTGCC	120	
<i>C. jejuni</i>		C1: CAAATAAAGTTAGAGGTAGAATGT C4: GGATAAGCACTAGCTAGCTGAT	159	Wang & Slavik 2005
<i>E. coli</i> O157:H7		UidAa: GCGAAAAGTGTGGAATTGGG UidAb: TGATGCTCCATAACTTCCTG	252	
<i>S. Typhimurium</i>		S29: CAGTATCAGGGCAAAAACGGC S30: TTCAAAGTTCTGCGCTTTGTT	360	
<i>L. monocytogenes</i>		FP: AGCTCTTAGCTCCATGAGTT RP: ACATTGTAGCTAAGGCGACT	450	
<i>E. coli</i> O157:H7	<i>uidA</i>	PT-2: GCGAAAAGTGTGGAATTGGG PT-3: TGATGCTCCATCACTTCCTG	252	Li et al. 2005
<i>S. Typhimurium</i>	NA	ST-11: AGCCAACCATTGCTAAATTGGCGCA ST-15: GGTAGAAATCCCAGCGGGTACTG	429	
<i>Sh. flexneri</i>	<i>ipaH</i>	ipaH-1: GTTCCTTGACCGCCTTTCCGATACCGTC ipaH-2: GCCGGTCAGCCACCCTCTGAGAGTAC	620	

Table 9.3 Sequences of primers and probes used for the simultaneous and quantitative detection of bacterial pathogens in meat and meat products.

Target pathogen	Target gene	Primers	Reference
<i>E. coli</i>	<i>uidA</i>	<i>E. coli</i> -1 TTGACCCACACTTTGCCGTAA <i>E. coli</i> -2 GCGAAAACCTGTGGAATTGGG <i>E. coli</i> -p VIC-TGACCGCATCGAAAACGCAGCTTAMRA	Wang et al. 2007
<i>Salmonella</i> spp.	NA	<i>Sal</i> -1 GCTATTTTCGTCCGGCATGA <i>Sal</i> -2 GCGACTATCAGGTTACCGTGGA <i>Salmonella</i> -p FAMTAGCCAGCGAGGTGAAAACGACAAAGGTAMRA	
<i>Shigella</i> spp.	<i>ipaH</i>	<i>Shig</i> -1 CTTGACCGCCTTTCCGATA <i>Shig</i> -2 AGCGAAAGACTGCTGTCAAG <i>Shigella</i> -p TET-AACAGGTCGCTGCATGGCTGGAATAMRA	
<i>S. Typhimurium</i>	<i>fliC</i>	SfC-F: TGCAGAAAATTGATGCTGCT SfC-R: TTGCCAGGTTGGTAATAGC ST-JOE: JOE-ACCTGGGTGCGGTACAGAACCGTBHQ1a	Lee et al. 2009
<i>S. Enteritidis</i>	<i>sefA</i>	SsA-F: GGTAAGGGGCTTCGGTATC SsA-R: TATTGGCTCCCTGAATACGC SE-Cy5: Cy5-TGGTGGTGTAGCCACTGTCCCGBHQ1a	

spp. and compared to real-time PCR, an immunocapture assay and a culture-dependent approach to detect *S. Enteritidis* and *E. coli* O157 in various food types (Almeida et al. 2013a, b). It was reported that all methods presented a similar detection level and the PNA-FISH and immunocapture assay were in 100% agreement with the ISO methods (ISO 6579:2002; ISO 16654:2001) and additionally very rapid.

Another improvement was presented by Ootsubo et al. (2003). The technique, termed FISHFC (for Filter Cultivation), included a short (6h) cultivation step that drastically improved the detection efficacy of Enterobacteriaceae in, among others, minced chicken. This approach was also used by Fuchizawa et al. (2008) and Shimizu et al. (2009) for the specific detection and enumeration of *Listeria* spp. and *Clostridium perfringens*, respectively. In both cases it resulted in an equally accurate but significantly faster outcome compared to the conventional plating technique.

The possible limitations of this approach were revealed during a study by Vieira-Pinto et al. (2008) in which the effectiveness of FISH in the detection of *Salmonella* with and without pre-enrichment was assessed in comparison to the respective ISO method (ISO 6579:2002). It was reported that the FISH application resulted in many false-negative results and possibly even more false-positive ones.

9.4.2 Immunological Detection

Immunological detection is an alternative to DNA-based detection that has not been as widely applied despite the significant number of protocols described. This consists of two discrete and subsequent steps: (1) capture and (2) detection of the target organism.

When the required limit of detection is as low as 1 CFU/g, then selective enrichment, as already described, is necessary.

The first step, namely capturing of the target cells, is most often performed by immunomagnetic separation and immunocapture. In the former case, small superparamagnetic beads coated with antibodies against surface antigens of the target cells are used to separate the target microorganisms from the other members of a microbial consortium. Then, the cells–beads complex can be removed with, for example, a magnetic particle separator and detected (Olsvik et al. 1994). On the other hand, the typical sandwich ELISA scheme is used for immunocapture; an antibacterium antibody is bound to a solid support and the bacterium is then “sandwiched” between the primary antibody and a reporter enzyme-labeled antibody conjugate.

A variety of approaches have been described as a detection step, with the colorimetric one being the most commonly applied. Alternatively, reflectance measurement, chemiluminescence, electrochemiluminescence, immunoelectrochemical detection, bioluminescence, and time-resolved fluorescence are among the techniques that have been successfully applied.

Many studies for the detection of foodborne pathogens in meat and meat products have been performed. Padhye and Doyle (1991) used the typical sandwich-ELISA protocol for the detection of *E. coli* O157:H7 in ground beef. Chemiluminescent detection coupled with sandwich immunoassay and enzymatic signal amplification for the detection of *E. coli* O157:H7 inoculated in ground beef has been demonstrated by Gehring et al. (2004). Brewster & Mazenko (1998) described a rather simple and comparatively rapid assay for the detection of *E. coli* O157:H7. According to this, an enzyme–antibody conjugate was used to label the target cells that were concomitantly captured by filtration of the sample through a 0.2 µm filter. Then, the enzyme-labeled cells were detected by placing the filter on the surface of an electrode, which measured the current produced by oxidation of the electroactive enzyme product.

Immunomagnetic separation has proven very powerful and compatible with a variety of detection methods. It was combined with electrochemical detection for the detection of *E. coli* O157:H7 in porcine rinse water (Gehring et al. 1999) and electrochemiluminescence for the detection of *E. coli* O157:H7 in ground beef (Crawford et al. 2000; Yu & Bruno 1996). Immunomagnetic separation followed by incubation with an ATP detection reagent has been used to detect *E. coli* O157:H7 in ground beef (Tu et al. 2000). The simultaneous detection of *E. coli* O157:H7 and *Salmonella* inoculated into ground beef was performed by immunomagnetic separation combined with time-resolved fluorescence (Tu et al. 2002).

9.5 Biodiversity Assessment

Microbial biodiversity has been the subject of intensive study. This type of study may serve multiple purposes: support for epidemiological studies, accurate depiction of strain diversity within a microecosystem, dynamics of particular isolates, etc.

Pulsed-field gel electrophoresis has been extensively used, especially for the subtyping of pathogenic microorganisms, due to its high discriminating power as well as good epidemiological concordance. Regarding the latter, the clonality of the microorganism should always be taken into consideration in the interpretation of the results. Therefore,

PFGE typing may not be effective in all cases and assistance from other techniques, such as multi-locus variable-number tandem repeat analysis (MLVA), multi-locus sequence typing (MLST), single nucleotide polymorphisms (SNPs) and clustered regularly interspaced short palindromic repeats (CRISPRs), is sometimes required. In all cases, suitable protocols for the subtyping of foodborne pathogens such as *E. coli* O157:H7 (Bai et al. 2015; Lindstedt et al. 2003b; Manning et al. 2008; Noller et al. 2003; Zhang et al. 2006), *Salmonella* (Bachmann et al. 2014; Bergamini et al. 2011; Boxrud et al. 2007; Davis et al. 2009; Lindstedt et al. 2003a, 2004; Liu et al. 2011; Octavia & Lan 2007; Sukhnanand et al. 2005; Tien et al. 2011; Torpdahl et al. 2005), *Listeria monocytogenes* (Chen et al. 2011; Ducey et al. 2007; Parisi et al. 2010; Revazishvili et al. 2004; Wang et al. 2015; Ward et al. 2008), *Staphylococcus aureus* (Feil et al. 2003; Morandi et al. 2010; Song et al. 2015), *Campylobacter jejuni* (Guyard-Nicodeme et al. 2015), *Clostridium difficile* (Marsh et al. 2011), *Yersinia enterocolitica* (Virtanen et al. 2013), etc. currently exist. In all cases, further study and certain improvements are required in order to identify the proper genetic markers that will provide sufficient epidemiological concordance.

Apart from its use in epidemiological studies, PFGE has also been extensively used for the assessment of the biodiversity level of several microecosystem members (Doulgeraki et al. 2010, 2011; Paramithiotis et al. 2008). The advantage that this technique offers in relation to biodiversity assessment studies is a high level of intra- and interlaboratory reproducibility that may be further improved with the use of reference strains. However, highly trained personnel are required for both execution and interpretation of the results and the cost of the analysis is very high compared to other techniques with the same purpose. Alternatively, biodiversity assessment may also be performed by PCR-based arbitrary primed profile-based comparisons, such as RAPD-PCR and rep-PCR. This approach is prone to reproducibility issues that arise from nearly every experimental parameter implicated with the analysis (Tyler et al. 1997). Although some of them may be controlled and therefore improve the intralaboratory reproducibility to acceptable levels (Hadjilouka et al. 2014), there are many issues that are difficult to control and seriously compromise interlaboratory congruence (Tyler et al. 1997).

9.6 Conclusion and Future Perspectives

Recent advances in the field of molecular biology have allowed the development and application of the next-generation sequencing approach that seems to offer an improved alternative provided that it is accompanied by significant computational power and proper bioinformatic tools. The latter, along with the effect of the NGS platform used, have been identified as the major disadvantages of this approach. However, given that meat and meat product microecosystems have been extensively studied, it is not very likely that significant new members will be revealed. On the contrary, the trophic relationships that lead to the development of these microcommunities are currently understudied and therefore the possibility of significant improvements of our comprehension regarding that subject is very high.

Regarding epidemiological studies, a very high level of knowledge has been reached allowing epidemiological surveys to take place. However, many inconsistencies still

exist resulting from a variety of reasons. Currently, the most important factor that significantly restricts epidemiological concordance of even the most sophisticated approaches is the clonality of the microorganisms under study. This issue may be tackled with the use of the proper molecular markers that in most cases are yet to be discovered.

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Section IV

Fermented Foods and Beverages

10

Revolution in Fermented Foods: From Artisan Household Technology to the Era of Biotechnology

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10.1 Introduction

Sadness and good food are incompatible.

(Charles Simic, poet)

Since time immemorial, human civilization had a close relationship which brought together humans and microorganisms which appeared in several forms, most notably fermented foods. Fermented foods are defined as those products that use the direct and/or indirect effect of microorganisms to cause desirable biochemical changes. The microbial flora responsible for the fermentation may be the microbiota indigenously present on the raw materials, or they may be added as starter cultures (Katongole 2008; Ray & Joshi 2014).

Originally, artisan technology was a way to prepare fermented foods, of course without any knowledge of the microorganisms' role. However, by the middle of the 19th century, two turning points changed the way of conducting and understanding the fermentation process. First, the Industrial Revolution in Europe resulted in the concentration of large masses of people in towns and cities. Consequently, food had to be prepared in large quantities, requiring commercialization of the manufacturing process. Secondly, understanding of the biological basis of the fermentation process spread as a result of a boom in microbiology as a science in the 1850s (Caplice & Fitzgerald 1999). Since then, work has been ongoing in the area of industrial production of fermented products all over the world (Katongole 2008).

Raw materials are converted to fermented products using microorganisms or enzymes. In plant science, breeding is conducted to reduce toxic or antinutritional components of plant or plant part(s), or to increase the protein or vitamin content. Additionally, it has been valuable to identify microorganisms that can synthesize necessary ingredients (e.g., essential amino acids, vitamins) that can be added to food for biofortification for populations where malnutrition is a problem

(National Research Council 1992). Desirable traits for these microorganisms can be summarized as follows:

- ability to produce flavor components that enrich these foods for traditional and new markets
- capability to break down antinutritional factors (e.g., phytic acid) present in some substrates
- production of enzymes to utilize/degrade wastes as substrates
- ability to detoxify toxins and other undesirable secondary products
- thermotolerance and osmotolerance in solid substrate fermentation processes, which are two essential features.

Food fermentations frequently result in the production of alcohol, antibiotics, or other substances that can improve the safety and shelf-life of fermented products by inhibiting the growth of undesirable microorganisms. As a result, modern societies felt a need to transform the traditional art into technological processes to incorporate objective methods of process control and optimization, and to standardize the quality of the final products without losing their desirable traits. Only when conditions such as time, temperature, pH, substrate pretreatment, inoculum-substrate ratio, and so forth are controlled can fermentation can be optimized; because of the surface/volume relationships, the scale-up of solid-state fermentation is particularly challenging. Solid-state reactions can be valuable to reduce raw material losses. Equipment with specific surface characteristics such as semi-porous clay, charred wood, gourds, etc, is a critical challenge to the improvement of some traditional fermentation processes. Research is also needed on the development of continuous fermentations using bioreactors (Sasikumar 2014).

This chapter highlights the answers to many questions, such as:

- Where and when did fermentation begin?
- Is the fermented food from the past different from the food of the present era?
- What is the relation between fermented foods and probiotics?
- With the escalation of food safety crises, is it possible to trace the origin of fermented foods?
- What is the future of fermented foods?

10.2 Historical View: Where and When Did Fermentation Start?

In the period 3000–4000 BC, ancient Egyptians and Babylonians produced bread and beer. It is worth mentioning that the rise of civilizations in the Mediterranean region coincided with the production and consumption of some fermented products such as wine. Remarkably, the fermented foods consumed in China, Japan, and the Far East were different from those in the Middle East although fermentation appeared around the same time (Haaland 2007; McGovern et al. 2004).

The practice of fermentation was widespread during the Roman period, where new technologies as well as new raw materials were used throughout the Empire. The importance of fermented foods arose from the army's need for foods stored for extended periods of time. In addition, it is not surprising that consumption of beer and wine was preferred over water, but the question is why? At that time, the water was probably

contaminated with fecal or other wastes. Also in this era, an evolution occurred in the level of trade in many food industries, including cheese, wine, and bread, especially in the Mediterranean, Europe, and the British Isles (Spangenberg et al. 2008). Religious establishments have played a prominent role in the fermented food industry for hundreds of years, carrying such foods from one continent to another. The most important foods were bread, beer, and cheeses. Monks were the first to develop industrial techniques of fermented foods. At present, the economic policies of the food industry are shifting from government monopoly to privatization followed by imposed taxes as a form of control (Hutkins 2008).

10.3 Fermented Foods: From the Past to the Current Era

Fermentation is one of the oldest food preservative technologies, a process dependent on the biological activity of microorganisms for the production of metabolites which can suppress the growth and survival of undesirable microflora in foodstuffs (Paul Ross et al. 2002). Fermentation technology experienced many important developments throughout its history which can be traced back thousands of years (Figure 10.1).

Slowly, people have realized the nutritional and curative value of fermented foods and drinks, and this has increased in modern times with interest in probiotic microbes (Getz 2012; Paramithiotis et al. 2016).

10.3.1 Fermented Foods as Ambassadors of Various World Civilizations

It is well known that fermented foods have been an integral part of the human diet throughout history, without much appreciation or understanding of their underlying microbial flora, until recently (Chilton et al. 2015; Farnworth 2008). Fermented foods are the best example of human innovation in the preparation of delicious food using microbes (some examples are given in Table 10.1).

Canada is a striking example of this, as in many cases immigrants introduced these foods which then grow in popularity, and consumption became widespread (Chilton et al. 2015). The final result is that fermented food products are consumed on a massive scale all over the world (Table 10.2).

10.3.2 Fermented Foods in the 21st Century

It is interesting to find that more than one-third of the food consumed in the United States is fermented; first among those most consumed foods is beer followed by bread, cheese, and wine. In Asia, the production and consumption of soy sauce topped the list of fermented food products. Although global statistics regarding the consumption of fermented foods are not available in most cases, it is no surprise that alcoholic products are the world's most popular fermented foods (Hutkins 2008). In Africa, fermented foods play an important socioeconomic role as well as making a major contribution to the protein requirements of indigenous populations (Achi 2005; El Sheikha & Montet 2014; El Sheikha et al. 2014). Globally, sales of fermented foods exceed 1 trillion dollars, with an even greater overall economic impact.

The role that fermented food plays is related not only to the development of civilizations and cultural relationship between countries but also to the nutritional importance



Figure 10.1 The main developments of fermented foods through the history of mankind. Adapted from Paul Ross et al. (2002). Reproduced with permission of Elsevier.

of fermented foods. Examples of fermented products that play this important nutritive role include kimchi (from Korea), miso (from Japan), and kefir (from Eastern Europe) in addition to the new generation of fermented foods which have begun to emerge worldwide (Katongole 2008; Steinkraus 1996, 2002).

Table 10.1 Examples of fermented foods in various worldwide regions.

Product name	Substrates	Fermenting microorganisms	Country of origin
Cheddar and Stilton cheeses	Milk and rennet/ chymosin/rennin	<i>Penicillium roqueforti</i> , <i>Yarrowia lipolytica</i> , <i>Debaryomyces hansenii</i> , <i>Trichosporon ovoides</i>	United Kingdom
Crème fraîche	Soured dessert cream	<i>Lactobacillus cremoris</i> , <i>L. lactis</i>	France
Fermented sausage	Meat, salt, nitrite, sugars, and spices	<i>Lactobacillus</i> , <i>Pediococcus</i> , or <i>Micrococcus</i>	Greece and Italy
Sauerkraut	Green cabbage	<i>L. plantarum</i>	Germany
Surströmming	Fermented herring and brine	<i>Haloanaerobium praevalens</i> , <i>Haloanaerobium alcaliphilum</i>	Sweden
Yogurt	Milk	<i>L. bulgaricus</i> , <i>Streptococcus thermophilus</i>	Greece, Turkey
Kefir	Milk and kefir grains	<i>Saccharomyces cerevisiae</i> , <i>L. plantarum</i>	Russia
Kombucha	Black, green, white, pekoe, oolong, or darjeeling tea, water and sugar	<i>Gluconacetobacter</i> , <i>Zygosaccharomyces</i>	Russia and China
Kvass	Beverage from black or rye bread	<i>Lactobacillus</i>	Russia
Dosa	Fermented rice batter and lentils	<i>L. plantarum</i>	India
Kimchi	Cabbage	<i>Leuconostoc mesenteroides</i>	South Korea
Miso	Soybeans	<i>Aspergillus oryzae</i> , <i>Zygosaccharomyces</i> , <i>Pediococcus</i> sp.	Japan
Adjuevan/ Adjonfa	Atlantic bumper and salt	<i>Lactobacillus</i> sp.	Ivory Coast
Busa	Rice or millet	<i>Lactobacillus</i> , <i>Saccharomyces</i>	Egypt
Hussuwa	Sorghum	<i>Lactobacillus saccharolyticum</i> , <i>Gluconobacter oxydans</i> , <i>Acetobacter xylinum</i> , <i>Saccharomyces cerevisiae</i>	Sudan
Kaffir beer	Beverage from kaffir maize	<i>Lactobacillus</i> sp.	South Africa
Pulque	Beverage from agave plant sap	<i>Zymomonas mobilis</i>	Mexico

Sources: El Sheikha & Montet (2014), Chilton et al. (2015). www.mdpi.com/2072-6643/7/1/390/html. Licensed under CC BY 4.0.

10.3.3 Industrialization of Fermented Foods

Industrialization of fermented foods started in the early part of the 19th century during the Industrial Revolution in Europe. There was a migration of people from villages to urban areas that demanded bulk production of bread, beer, butter, and other foods and beverages.

Table 10.2 Average consumption of fermented foods.

Fermented food	Country	Annual consumption (per person)
Beer	Germany	106L
Cheese	UK	10kg
Kimchi	Korea	22kg
Miso	Japan	7kg
Soy sauce	Japan	10L
Tempeh	Indonesia	18kg
Wine	Italy, Portugal	90L
	Argentina	70L
	Finland	40L
Yogurt	Netherlands	25L

Source: Chilton et al. (2015). www.mdpi.com/2072-6643/7/1/390/htm. Licensed under CC BY 4.0.

10.3.3.1 Factors Hampering the Development of Fermented Foods

The production of fermented foods in developing nations is still mostly a home-made enterprise done in a simple manner. As a result, production has not increased compared with other cottage industries. Over time, several acquired the label of food for the poor or were associated with low incomes. Several factors worked against the uptake of traditional fermented foods (Achi 2005).

- Inadequate raw material, grading, and cleaning contributing to the presence of foreign matter (such as insects, stones) in the final product
- Crude handling and processing techniques
- Lack of durability (shelf-life)
- Lack of homogeneity
- Unattractive presentation

Presentation of a product is considered one of the main factors having a significant effect on the purchasing attitudes of consumers. Plastic containers have replaced banana leaves as a package for food. One of the biggest challenges that may face traditional fermented foods is the increasing popularity of food introduced from developed countries (Hesseltine 1983). However, the demand for locally processed foods is increasing due to their price, which is lower compared with imported counterparts. All these factors collectively can be considered a guideline to improve the status of fermented foods (Achi 2005).

10.4 Fermented Foods and Health Effects

10.4.1 Fermented Foods as Delivery Vehicle for Probiotics

Over the past 40 years, the definition of probiotics has evolved. Salminen et al. (1998) defined probiotics as “foods containing live bacteria which are beneficial to health” but this has now been superseded by an Expert Panel convened in 2001 by the Food and

Agriculture Organization and World Health Organization. The FAO/WHO (2006) defines probiotics as “live microorganisms which when administered in adequate amounts, confer a health benefit on the host.”

Felis and Dellaglio (2007) demonstrated that the main genera used to make probiotic products are *Lactobacillus* and *Bifidobacterium*. Moreover, both form part of the normal human intestinal and vaginal microbiota (Korshonov et al. 1999). The last 20 years have seen the growth of probiotic products, mostly as yogurts and fermented milk (Mattila-Sandholm & Saarela 2000).

Not all probiotic products have been proven to confer health benefits. Therefore, it is imperative to conduct clinical trials to determine the appropriate amount that should be consumed for a maximum probiotic effect. The FAO/WHO (2002) has published a guideline on the steps necessary to label a product as probiotic. Such a product should be based on DNA speciation of strains, verification of safety and non-pathogenic and non-toxicogenic properties (Ishibashi & Yamazaki 2001), and ability to provide tangible physiological/health benefits as shown in randomized clinical trials. Additionally, in vitro studies are useful to gain knowledge of strains and mechanisms of the probiotic effect. The benefits of probiotics appear in the host (human body) through metabolic processes carried out by probiotics (resulting from growth). However, it was noted that in vitro data available for particular strains are not sufficient to describe them as probiotic (Collins et al. 1998; Morelli 2000; Reid 2005).

A probiotic must be adequately tested to prove its safety and health benefits. The use of unproven probiotics can lead to confusion about the real impact of probiotics on a particular health situation. For example, “Ecologic 641,” an unproven product, does not meet the WHO definition of probiotics based on published data by Besselink et al. (2008). Thus, if a product is not probiotic, its use can harm the reputation of the whole field (Anukam & Reid 2009).

10.4.1.1 Historical Link Between Fermented Foods and Probiotics

It is not surprising that the consumption of fermented foods has long been associated with good health, given that fermented foods can contain probiotics, prebiotics or both. It is worth mentioning that the Roman historian Pliny advocated the use of fermented milk for treating gastrointestinal infections as far back as 76 AD. The French pediatrician Tissier proposed in the early 1900s that bifidobacteria could be useful in controlling infections in infants, as they were the predominant component of the intestinal microflora of breast-fed infants (Tissier 1906). Metchnikoff (1907) helped to develop the probiotic concept and also observed that the consumption of fermented milk could reverse putrefactive effects of gut microflora. This concept has matured, particularly over the past two decades, based on placebo-controlled clinical trials showing that particular strains have associated health benefits. In addition, there is now more consumer awareness of the basis for ingesting such foods for health promotion and disease prevention (Stanton et al. 2005).

10.4.1.2 Fermented Foods Containing Probiotics – Legislation and Marketing

In Japan, a standard developed by the Fermented Milks and Lactic Acid Bacteria Beverages Association demands a minimum of 10^7 colony-forming units (CFU)/mL of probiotic microorganisms at the end of shelf-life (Ishibashi & Shimamura 1993).

The Codex Alimentarius Standard No. A-11 (a) (1975) defines yogurt thus.

Yogurt is a dairy coagulated product obtained by the action of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* from fresh milk and pasteurized milk (or concentrated partly skimmed milk enriched in solids) with or without addition (milk powder, skim milk powder, etc.). Microorganisms of the final product must be viable and abundant.

The legislation in many countries requires that yogurt bacteria are alive in the product put on sale. Other countries recognize that following a heat treatment to improve the shelf-life, the product will no longer contain live bacteria. This practice is not recommended because it modifies the properties of yogurt (Fellows & Hampton 1992; Sfakianakis & Tzia 2014).

In France, the names “yoghurt” and “yogurt” are strictly limited to milk fermented with the lactic acid bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. These bacteria must be inoculated simultaneously and be live in the finished product at a rate of at least 10^6 bacteria/g until the use-by date (Elli et al. 2006).

Consequently, probiotic bacteria have to be suitable for large-scale industrial production and processing, with an additional requirement that they maintain good viability during storage. These requirements pose a significant challenge from a technological standpoint, as many probiotic bacteria, being of intestinal origin, are sensitive to stresses such as oxygen, heat, and acid exposure. Therefore, these bacteria perform poorly in many food environments and particularly in fermented foods, which can be highly acidic. Therefore, food products with a short shelf-life (2–3 weeks), such as yogurt and fermented milks, are the most common probiotic foods available, although products with a longer shelf-life, such as probiotic Cheddar cheeses, have been developed more recently (Ross et al. 2002).

Probiotics can be carried in food or supplements, such as pills and capsules. Consumers' probiotics purchases reveal an interesting dynamic. The global retail market value of probiotics was \$41 billion in 2015, which included sales of probiotics supplements (9%), sour milk products (16%) and probiotic yogurt (Feldman 2016). According to Euromonitor International, the global market for probiotic dietary supplements was valued at \$4.3 billion in 2016 and this market is growing at a compound annual growth rate (CAGR) of 11% and will be worth \$5 billion globally by 2021. The US is the leading market for probiotic dietary supplements, worth \$1.9 billion in 2016. Additionally, the total probiotic market in food and yogurt is growing at a CAGR of 8%, according to Euromonitor data, and will be worth \$50 billion by 2021.

10.4.1.3 Health Benefits of Fermented Foods as Containers for Probiotics

Mounting scientific evidence indicates that ingestion of certain microbial cultures exerts health benefits not only in the gastrointestinal tract but also in the urogenital and respiratory tracts. Although there is limited information about the effective dose of particular strains, the presence of high numbers of viable bacteria is usually recommended for the efficacy of probiotic foods (Ray et al. 2014).

The reported beneficial health effects of probiotic consumption include improvement of constipation, diarrhea, and intestinal inflammatory conditions, and prevention of allergic disease in infants (Hattori et al. 2003; Kirjavainen et al. 2002; Vitetta et al. 2014). Additionally,

supplementation with foods including probiotics, such as fermented products, has been shown to enhance the immune system and can prevent infection or disease caused by pathogenic microorganisms (Lee & Puong 2002). Cancer and genitourinary tract infections have also been reported to benefit from probiotics (Brown & Valiere 2004). Recently, a promising study showed possible alleviation of peanut allergy in children by co-administering probiotics with a peanut oral immunotherapy (Tang et al. 2015).

The positive health effects of foods containing probiotics can be summarized as follows:

- combating pathogenic microorganisms or their effects
- strengthening the mucosal barrier
- release of immune cell-stimulating and antiinflammatory molecules
- production of antimicrobial substances, including organic acids, hydrogen peroxide, and bacteriocins.

10.4.2 Adverse Effects of Fermented Foods

Taking the fermentation process out of the hands of food manufacturers, who have experience and specialization, may pose health threats (Cocolin et al. 2016). Fermented fish sauce is widely consumed in parts of Asia. A study looking at habitual consumers of fermented fish sauce in China showed an increased risk of esophageal cancer (Ke et al. 2002). Furthermore, *N*-nitroso compounds (NOCs) in pickled vegetables and fish may contribute to gastric carcinogenesis (Chen et al. 1992; Kim et al. 2010). Egyptian researchers also detected the presence of high levels of histamine in fermented fish (Rabie et al. 2011). Interactions between diet “fermented foods” and microbial infections may increase the risk of some cancers (Lampe 2013). Also, mycotoxin production has been found to be associated with certain fermented foods (Abalunan et al. 2013; Chávez et al. 2011; El Sheikha & Mahmoud 2015; Reddy et al. 2010; Samsudin & Abdullah 2013; Talaam 2015). These toxins are produced when fermentation conditions are compromised, and poor hygiene of food sources for fermentation is allowed to persist during production (Kinosita et al. 1968; Nout 1994; Westby et al. 1997).

10.5 Is it Possible to Trace the Geographical Origin of Fermented Foods?

10.5.1 The Geographical Origin of Foods is a Great Concern ... Why?

Food traceability and authenticity have attracted considerable attention during the past decade (El Sheikha et al. 2009; Kelly et al. 2005; Montet et al. 2010). Financial incentives continue to drive retailers/resellers to misidentify the geographic origin of goods and food products (Anderson et al. 1999; El Sheikha 2015). Therefore, the verification of geographic origin is a serious concern not only to consumers but also to producers (e.g., farmers), retailers, and governmental authorities (El Sheikha 2010a; Zhao et al. 2013). Determination of geographic origin has become the most important option to meet the challenges of the food industry, protection of the consumer from overpayment and deception, and the variability in the raw materials used by manufacturers (Anderson et al. 1999; El Sheikha 2017).

10.5.2 Multiple Elements as the Potential Tracing Tool for Fermented Foods

One of the several studies has addressed the determination of geographical origin of fermented foods (i.e., kimchi) by multiple elements in combination with strontium (Sr) isotope ratio (Bong et al. 2012).

Kimchi is a multifunctional health food which has attracted global attention for being rich in vitamins and inorganic materials. It is considered a representative fermented Korean food. Cabbage is the main component of kimchi and during 2010, a sudden increase in cabbage prices in Korea resulted in substantially increased imports of cabbage and kimchi from China. Cheap kimchi from China is being sold as authentic Korean kimchi in Korean markets. Therefore, developing an analytical technique to distinguish the origin of kimchi become an important issue in Korea (Zhang & Park 2009). Bong et al. (2012) concluded from their study that combining multi-elemental and statistical analyses is a promising tool for determining the geographical origins of kimchi. However, there are limitations for this method such as the probable influence of environmental factors, plus the implementation of this strategy is quite difficult in terms of routine analysis, and it is also costly. However, the abundant data collected can be efficiently processed by statistic analysis methods (e.g., linear discriminate analysis, principal component analysis, etc.) (Li et al. 2016).

In the field of food authenticity, besides reliable but time-consuming analytical techniques, there is a need for simple, fast, robust, and cheap methods of proven efficacy and reliability. In fact, a universal scientific method for the determination of the geographical origin of a foodstuff does not exist. The methods which permit analysis of the microenvironment of food are very promising and must be better studied by research teams all over the world (El Sheikha 2015).

10.5.3 Is PCR-DGGE a Promising Traceability Approach for Fermented Foods?

10.5.3.1 The Applicability of PCR-DGGE as a Traceability Tool for Foodstuffs

Polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) is considered as one of the best approaches for monitoring the microbial communities related to food samples in a comprehensive, rapid, and reproducible manner. Also, it has been demonstrated that there is a link between the microbial populations (bacteria, yeast, mold) and the geographical origins of the foodstuffs (El Sheikha 2011). This method is therefore proposed to be a promising analytical traceability tool for foodstuffs such as fish (El Sheikha & Montet 2016; Leasing 2005; Le Nguyen et al. 2008b; Montet et al. 2012; Tatsadjieu et al. 2010), fruits (El Sheikha 2010b; El Sheikha & Montet 2011; El Sheikha et al. 2009, 2011a, b, c, 2012; Le Nguyen et al. 2008a), salt (Dufossé et al. 2013), coffee (Durand et al. 2013; Hamdouche et al. 2016; Nganou et al. 2012), and organic and conventional foods (Bigot et al. 2015). Many microbial species have been identified as biological barcodes, whose detection could be used to trace the origin as well as the production mode of foodstuffs (El Sheikha 2015).

10.5.3.2 Cheese as a Case Study for Fermented Foods

Some studies that used PCR-DGGE to characterize the microbial communities in traditional cheese raised the possibility of its use to identify a product's origin. Ercolini et al. (2008) found that the microbial flora of natural whey cultures used for Italian



Figure 10.2 Locations of the regions traditionally producing Minas cheese. *Source:* Arcuri et al. (2013). Reproduced with permission of Elsevier.

Protected Designation of Origin (PDO) cheese was related to their geographic area of production. Recently, Rychlik et al. (2017) demonstrated that PCR-DGGE could be used as a distinguishing technique for authentic Protected Geographical Indication (PGI) status and geographical origin of Wielkopolska fried ripened curd cheese.

Traditional or “artisanal” Minas cheese includes varieties that are classified according to their region of origin within the state of Minas Gerais, Brazil. The most famous are produced in the areas of Serro, Canastra, Cerrado, and Araxá (Borelli et al. 2006; Lima et al. 2009). Arcuri et al. (2013) used PCR-DGGE to study the genetic diversity of the dominant bacteria in traditional Minas cheese produced in four regions of Minas Gerais state, namely Serro, Cerrado, Araxá, and Canastra (Figure 10.2). The DNA extraction from the cheese samples was based on the method of Ampe et al. (1999) modified by Arcuri et al. (2013). The results of DGGE analysis and sequencing are shown in Figure 10.3 and Table 10.3.

The DGGE revealed only one band of bacteria in cheese samples made from pasteurized milk that was used as a control (see Figure 10.3, lanes Q1 and Q2), and 7–13 bands for traditional Minas cheeses; each region presented distinct DGGE fingerprinting. Cluster analysis of the DGGE gel patterns for two traditional Minas cheese samples from the four different areas of Minas Gerais state showed the community similarity among the different geographic regions (Figure 10.4).

At 65% similarity level, two main groups were observed: the first group included cheese samples S1 and S2 from Serro region, the second group comprised the cheese samples from Araxá (A1 and A2), and the cheese samples from Canastra (Ca1 and Ca2) and Cerrado (Ce1 and Ce2). These results indicated that the application of PCR-DGGE is a new approach to the issue of geographical traceability of traditional Serro, Canastra, Cerrado, and Araxá cheeses, which may represent the basis for setting up a control and certification system in the future (Arcuri et al. 2013).

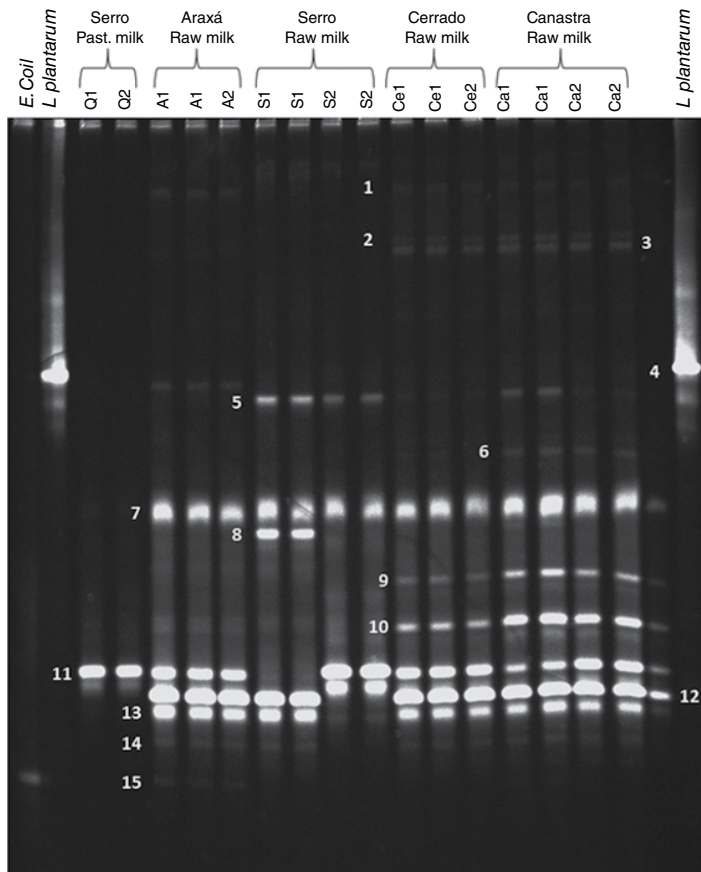


Figure 10.3 PCR-DGGE 16S rDNA profiles of Minas cheese bacteria from four regions (Araxá, Serro, Cerrado, and Canastra), Brazil. *Source:* Arcuri et al. (2013). Reproduced with permission of Elsevier.

10.6 Conclusions and Future Perspectives

Fermentation history has early records in South East Asia (China as the cradle of mold-fermented foods) and Africa (Egyptians developed the concept of the combined brewery-bakery). Egyptian beers were quite similar to some of the traditional opaque beers found in various African countries today (i.e., sorghum, maize, or millet beers). Many countries have now developed the crafts of baking, brewing, wine making, and dairying technologically, and also have established large-scale production of fermented consumer goods such as soy sauce, pickles, cheese, cultured milk, fermented meat products, wines, beers, and spirits.

Early travelers, clergymen, and colonists introduced “high-tech” fermented products to tropical countries. The continuation of fermented food consumption is dependent on two essential aspects: price and quality. These two axes are frequently missing in many local traditional foods as these foods are associated with the poor. Many causes contribute to this impression such as inadequate cleaning and grading of raw materials, crude handling, poor sanitation and processing techniques, and insufficient product protection (food safety) due to lack of hygienic packaging. These causes are easily

Table 10.3 Dominant bacteria species in Minas cheese identified by DGGE.

Band (s)	Closest relative	% Identity*	Source
1	<i>Streptococcus salivarius</i>	98%	GU426030
2	<i>Streptococcus</i> sp. (uncultured)	100%	GU132117
3	<i>Streptococcus</i> sp. (uncultured)	95%	GU132117
4	Uncultured bacterium	94%	HQ176316
5	<i>Lactobacillus plantarum</i>	98%	AY735404
6	Uncultured bacterium	94%	FN782509
7	<i>Streptococcus</i> sp.	98%	GU132117
8	<i>Lactobacillus</i> sp.	98%	HQ697653
9	<i>Streptococcus</i> sp.	98%	GU132117
10	<i>Streptococcus</i> sp.	98%	GU132117
11	<i>Lactococcus lactis</i>	98%	FJ859680
12	<i>Streptococcus thermophilus</i>	98%	EU180609
13	<i>Streptococcus</i> sp.	98%	GU132117
14	NI**	–	–
15	NI	–	–

* % Similarity with the reference strain.

** Not identified.

Source: Arcuri et al. (2013). Reproduced with permission of Elsevier.

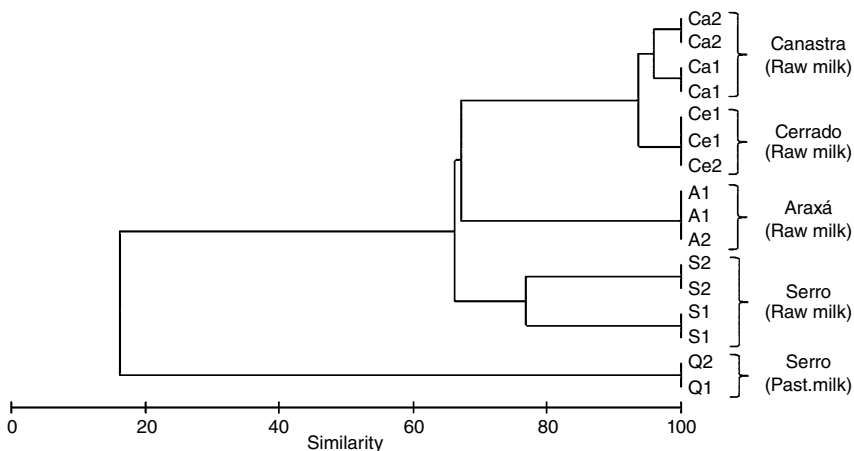


Figure 10.4 Cluster analysis of 16S rDNA profiles of Minas cheese bacteria from four regions (Araxá, Serro, Cerrado, and Canastra), Brazil. Source: Arcuri et al. (2013). Reproduced with permission of Elsevier.

translated into the fear of foodborne diseases and illness. The factors that prevent consumers purchasing such fermented products are variable sensory characteristics (quality), lack of durability (shelf-life), ungraded heterogeneous products, inconvenient unpacked bulk foods, and unattractive presentation of products.

Therefore, a major challenge is how to upgrade the present status of traditional indigenous fermented foods. As these foods are part of the regional cultural heritage, they are accepted by consumers and provide an appropriate basis for the development of a local food industry.

Ribosomal DNA profiles generated by PCR-DGGE may be used to detect variability in microbial populations (bacteria, yeast, fungi) inherent to fermented foods. This is an emerging traceability tool that imprints fermented foods with a unique biological barcode and makes it possible to trace a fermented food product to its original location.

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11

Molecular Techniques for the Identification of LAB in Fermented Cereal and Meat Products

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11.1 Introduction

Fermentation is a process of decomposition of organic substances, which is carried out by microorganisms or enzymes that convert carbohydrates into organic acids or alcohols (FAO 1998). For thousands of years, humans have produced food using the fermentation process. In the modern era, fermentation is expected to play a significant role in preserving fresh vegetables, fruits and other food commodities as the continuous increase in world population requires additional food sources (Marshall & Mejia 2011). Lactic acid bacteria (LAB), a group of bacteria that produces lactic acid as a major end-product, are widely used in food fermentation. Today, most fermented dairy products such as yogurt, cheese, kefir, and buttermilk are manufactured using LAB cultures. These bacteria are also associated with the production of sourdough bread, wine, and fermented vegetable and meat products.

Lactic acid bacteria as a group possess specific microbiological and technological characteristics. Microbiologically, they are Gram-positive, spherical or rod shaped, non-sporulating, non-pathogenic, non-toxicogenic, and with low guanine-cytosine (GC) content. This group of bacteria was first isolated in pure culture in 1873 (Lister 1873). Technologically, LAB are capable of surviving in adverse conditions, such as extreme acidic environments, and microaerobic and nutritionally depleted conditions (Faye et al. 2012; Hussain et al. 2013; Hosseini Nezhad et al. 2015). LAB have a fundamental function of transforming lactose and other carbohydrates into lactic acid. This is the main feature that makes LAB useful for fermentation process in foods.

Identification and characterization of this diverse and widely distributed group of bacteria is a challenging task. The first identification method was published in 1923 by David Hendricks Bergey (Bergey et al. 1923). According to updates on LAB classification by Khalid (2011), the group consists of four core genera (*Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*) and several new associated genera (*Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Globicatella*, *Lactococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, *Weissella*, and *Bifidobacterium*). In this identification system, *Bifidobacterium* is not classified into any of the 12 subdivisions of

LAB due to genetic differences. However, *Bifidobacterium* can be indirectly regarded as LAB because this group also produces lactic acid as an end-product during the fermentation process.

This chapter discusses the latest developments in molecular techniques for the identification of LAB present in different fermented foods, in particular cereal- and meat-based products.

11.2 Fermented Food Products

Fermentation is an ancient method of food preservation that has been in use for approximately 6000 years ago (Campbell-Platt 1994). Over time and based on technological developments, fermentation was later defined by Divya et al. (2012) as a food preservation process which involves a number of microorganism colonies, such as bacteria, yeast, and molds, along with appropriate substrates in accordance with the type of microorganism used and the surrounding conditions. As mentioned by Todorov et al. (2014), the most common bacteria used in the fermentation of food and beverages are LAB. These bacteria play a major role in improving nutritional quality, safety, and shelf-life of food and beverage products.

Liu et al. (2014) stated that food fermentation by LAB could be divided into two distinct phases: homofermentation and heterofermentation. Homofermentative LAB, which are *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Pediococcus*, produce lactic acid (lactate) as the only end-product of glucose fermentation while heterofermentative LAB, which are *Enterococcus*, *Leuconostoc*, and *Weisella*, produce lactic acid, ethanol, and carbon dioxide (CO₂). The ethanol content contributes to the formation of a distinctive aroma and taste in the final products of fermentation.

Food fermentation produces different effects on the end-products, such as enhanced shelf-life and improved nutritional values, sensory features, and safety (Bourdichon et al. 2012). Table 11.1 provides an overview of the diversity of fermented food products used worldwide. For example, in Nigeria, there are many kinds of fermented products such as ogi, using maize, millet, and sorghum as the raw materials. Ogi and kenkey preparations involve soaking the grains until soft, wet grinding, and fermentation. The ogi filtrates are fermented for 1–3 days at room temperature or near a fire. The ogi is usually served by boiling to 10% (w/v) into porridge, primarily as food for infants. Generally, ogi fermentation involves *Lactobacillus plantarum* as the predominant microorganism, while in Benin and Nigeria, beside *L. plantarum*, *L. fermentum* and *L. brevis* are also commonly found. Kenkey fermentation is typically prepared by slurring and removing coarse particles and bran by filtration or sieving, followed by fermentation of the filtrate. Kenkey is commonly consumed in combination with other foods. It has a shelf-life of only a few days. Fermentation of kenkey has used *L. plantarum*, *L. fermentum*, *L. reuteri*, *L. brevis*, and *Pediococcus pentosaceus* and yeasts, mainly *Candida krusei* and *Saccharomyces cerevisiae*.

Kivunde is a fermented food from East Africa, using cassava as the raw material and *L. plantarum* as the dominant microorganism during fermentation. Fermentation extends the shelf-life of cassava which generally lasts only 5 days, with deterioration commencing 24 hours after harvesting (El Sheikha & Montet 2014a; Franz et al. 2014). Fermented cassava products have a longer shelf-life (up to 30 days) in comparison to that of fresh roots (2–3 days).

Table 11.1 An overview of common and traditional fermented food products.

Products	Region/ country	Fermented food product	Microorganisms
Yogurt	Worldwide	Milk	<i>Streptococcus thermophilus</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. acidophilus</i>
Cheese	Worldwide	Milk	<i>L. lactis</i> subsp. <i>cremoris</i> , <i>L. lactis</i> subsp. <i>lactis</i> , <i>L. delbrueckii</i> subsp. <i>delbrueckii</i> , <i>L. delbrueckii</i> subsp. <i>lactis</i> , <i>L. helveticus</i> , <i>L. casei</i> , <i>L. plantarum</i> , <i>L. salivarius</i> , <i>Leuconostoc</i> spp., <i>Strep. thermophilus</i> , <i>Enterococcus durans</i> , <i>Ent. faecium</i> , and <i>Staphylococcus</i> spp., <i>Brevibacterium linens</i> , <i>Propionibacterium freudenreichii</i> , <i>Debaryomyces hansenii</i> , <i>Geotrichum candidum</i> , <i>Penicillium camemberti</i> , <i>P. roqueforti</i>
Bread	Worldwide	Wheat	<i>S. cerevisiae</i>
Mahewu (magou)	Benin, Togo	Maize, sorghum or millet	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. delbrueckii</i> subsp. <i>delbrueckii</i>
Ogi	Nigeria	Maize, sorghum or millet	<i>L. fermentum</i> , <i>L. plantarum</i>
Koko and kenkey	Ghana	Maize, sorghum or millet	<i>L. fermentum</i> , <i>L. salivarius</i> , <i>L. pantheris</i>
Uji	Kenya, Uganda, Tanzania	Maize, sorghum or millet	<i>L. plantarum</i> , <i>L. paracasei</i> <i>L. fermentum</i> , <i>L. buchneri</i>
Potopoto	Congo	Maize	<i>L. gasseri</i> , <i>L. acidophilus</i> , <i>L. delbrueckii</i> , <i>L. reuteri</i> , <i>L. casei</i>
Guedj	Senegal and West Africa	Fish	<i>L. lactis</i>
Agbelima	Ghana	Cassava	<i>L. plantarum</i> , <i>L. brevis</i> , <i>L. fermentum</i> , <i>Leuc. mesenteroides</i> <i>Penicillium</i> sp.
Tapé	Indonesia	Cassava	<i>Streptococcus</i> sp., <i>Rhizopus</i> sp., <i>Saccharomycopsis fibuligera</i>
Gari	West and Central Africa	Cassava	<i>L. plantarum</i> , <i>L. fallax</i> , <i>L. fermentum</i> , <i>W. paramesenteroides</i> , <i>L. brevis</i> , <i>Leuc. pseudomesenteroides</i> , <i>Strep. lactis</i> , <i>Geotrichum candidum</i>
Wara	Nigeria	Milk	<i>L. lactis</i>
Takju	Korea	Rice	<i>S. cerevisiae</i>
Kimchi	Korea	Korean cabbage, radish, vegetables	<i>L. mesenteroides</i> , <i>L. brevis</i> , <i>L. plantarum</i>

(Continued)

Table 11.1 (Continued)

Products	Region/ country	Fermented food product	Microorganisms
Burong mustala	Philippines	Mustard	<i>L. brevis</i> , <i>Ped. cerevisiae</i>
Narezushi	Japan	Sea water fish, cooked millet	<i>L. mesenteroides</i> , <i>L. plantarum</i>
Koumiss	Central Asia, China	Milk	<i>L. plantarum</i> , <i>L. helveticus</i> , <i>L. casei</i> , <i>L. kefir</i>
Suan-tsai	China	Cabbage, mustard	<i>Lactobacillus</i> , <i>Leuconostoc</i> , <i>Ped. pentosaceus</i> , <i>Tetragenococcus halophilus</i>
Sucuk	Turkey	Chopped pork or beef	Species of LAB, <i>Staph. spp.</i> , <i>Micrococcus spp.</i> , Enterobacteriaceae
Kargyong	India	Yak, beef, pork	<i>L. sakei</i> , <i>L. divergens</i> , <i>L. carnis</i> , <i>L. sanfranciscensis</i> , <i>L. curvatus</i> , <i>Leuc. mesenteroides</i> , <i>Ent. faecium</i> , <i>B. subtilis</i> , <i>B. mycoides</i> , <i>B. thuringiensis</i> , <i>Staph. aureus</i> , <i>Micrococcus sp.</i> , <i>Deb. hansenii</i> , <i>Pic. anomala</i>
Suka ko masu	India	Goat, buffalo meat	<i>L. carnis</i> , <i>Ent. faecium</i> , <i>L. plantarum</i> , <i>B. subtilis</i> , <i>B.</i> <i>mycoides</i> , <i>B. thuringiensis</i> , <i>Staph. aureus</i> , <i>Micrococcus</i> <i>sp.</i> , <i>Debaromyces hansenii</i> , <i>Pic. burtonii</i>
Sai-krok- prieo	Thailand	Pork, rice	<i>L. plantarum</i> , <i>L. salivarius</i> , <i>Ped. pentosaceus</i>
Tocino	Philippines	Pork	<i>Ped. cerevisiae</i> , <i>L. brevis</i> , <i>Leuc. mesenteroides</i>

Source: Adapted from El Sheikha (2015); El Sheikha & Montet (2014a, b); Franz et al. (2014); Genççelep et al. (2008); Ray et al. (2014); Tamang et al. (2016).

Traditional rice wine from Korea uses *S. cerevisiae* as the microorganism starter during fermentation. It contains less than 8% alcohol along with insoluble solids and yeast cells. Another well-known fermented product from Korea is kimchi, using cabbage, radish, and other vegetables with *L. mesenteroides*, *L. brevis*, and *L. plantarum* as starters during fermentation. Kimchi preparation starts with cutting or shredding the cabbage, then soaking it in water containing about 10% (w/v) of salt concentration overnight. Subsequently, the cabbage is washed and drained and additional ingredients are mixed in. The mixture is then packed and buried in the ground, fermented during winter for one or two months and consumed for 3–4 months until the spring is over (Rhee et al. 2011).

Koumiss is a fermented milk product from central Asia. To produce koumiss, fresh milk is obtained from a camel or horse and the raw unpasteurized milk is fermented over the course of hours or days with frequent stirring or churning. During fermentation, lactobacilli bacteria produce lactic acid and yeasts turn it into a carbonated and mildly alcoholic drink. Koumiss is ready for consumption when a sour flavor is achieved, with ~2% alcohol and pH less than 4. The end-product is a kind of homogenous liquid with a milky or light yellow color. Species in the *Lactobacillus* genus are the dominant acid bacteria applied during the fermentation process (Ray et al. 2014).

Another kind of fermented food is suan-tsai from China, made from Chinese cabbage or mustard. Its preparation begins with washing and boiling leaves for sterilization, then the leaves are soaked with salt in anaerobic conditions and mechanical pressure is applied to extract the liquid. Fermentation takes place for a month at room temperature and then the end-product can be consumed. Dominant LAB involved are lactobacilli, *Leuconostoc*, and *Pediococcus*. However, if mustard is used as the raw material, *P. pentosaceus* and *Tetragenococcus halophilus* can also be used in fermented suan-tsai (Liu et al. 2014).

Table 11.1 presents examples of foods which use LAB as the starter during fermentation.

11.3 Lactic Acid Bacteria and Fermented Foods

Over many centuries, LAB have been used for the preservation and quality improvement of foods, especially in the dairy industry. The LAB are the major group of microorganisms associated with many fermented food products (Rai et al. 2010; Stiles & Holzapfel 1997; Tamang 2010). Table 11.2 provides examples of fermented food products and the specific LAB identified in them. Major LAB genera such as *Alkalibacterium*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Axelsson et al. 2012; Holzapfel & Wood 2014; Salminen et al. 2004) have been isolated from various fermented food products. The LAB are generally recognized as safe (GRAS) microorganisms and known to exert beneficial effects on consumer health (Choi et al. 2005; Klaenhammer et al. 2005). LAB strains have the potential to compete with pathogenic microorganisms during fermentation and can improve the safety of food products (Arqués et al. 2015). LAB as beneficial microorganisms (probiotics) are attractive to consumers and can lead to the development of novel functional foods (Molina et al. 2012). The balance of LAB in the gastrointestinal (GI) tract is important in reducing the chances of getting an illness. This is because if a sufficient amount of probiotics is maintained in the GI tract, they are believed to reduce the colonization of pathogens (Butel 2014).

Fermented foods that are enriched with LAB may have specific textural and physiological features. For example, LAB can produce important substances such as exopolysaccharides (EPS). EPS can improve fermented food product texture and therapeutic value through beneficial effects on human health, such as an ability to lower cholesterol levels (Pigeon et al. 2002), immunomodulation and antitumor activities (Chabot et al. 2001), and contribution to digestion (Korakli et al. 2002). EPS are especially relevant in yogurt, cheese, sour cream, and other cultured dairy products (Ruas-Madiedo et al. 2002).

In brief, LAB possess many valuable characteristics that can bring versatility, innovation, safety, and therapeutic value to fermented foods. Therefore, exploitation of the full potential of LAB in fermented food products can provide nutritional value and new eating experiences for consumers and will be useful to drive future markets (Mokoena et al. 2016). In recent years, hundreds of LAB strains from different sources, including dairy products, fruits, fresh and fermented vegetables, fermented meats, fermented cereal products and soil, have been isolated and screened for their industrial and functional activities using advanced molecular tools.

Table 11.2 Examples of different fermented foods categories and specific LAB strains.

Fermented food category	Product	LAB strains
Dairy based	Mongolian traditional dairy products	<i>L. casei</i> subsp. <i>pseudoplanatarum</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. paracasei</i> , <i>L. plantarum</i> , <i>L. casei</i> , <i>L. pentosus</i>
	Sour milk	<i>L. plantarum</i>
	Fermented camel milk	<i>L. casei</i> subsp. <i>pseudoplanatarum</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. curvatus</i>
	Xinjiang cheese	<i>L. lactis</i>
	Traditional dairy products	<i>L. plantarum</i> , <i>L. pentosus</i>
	Yogurt	<i>Strep. thermophilus</i> , <i>L. delbrueckii</i> subsp. <i>bulgaricus</i> , <i>L. acidophilus</i> , <i>L. casei</i> , <i>L. rhamnosus</i> , <i>L. gasseri</i> , <i>L. johnsonii</i> , <i>Bifidobacterium</i> spp.
	Fermented probiotic milk	<i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>L. johnsonii</i> , <i>L. casei</i> , <i>B. lactis</i> , <i>B. bifidum</i> , <i>B. breve</i>
	Cheese	<i>L. lactis</i> subsp. <i>lactis</i> , <i>L. lactis</i> subsp. <i>cremoris</i> , <i>Strep. thermophilus</i>
	Butter and buttermilk	<i>L. lactis</i> subsp. <i>lactis</i> biovar <i>diacetyllactis</i> , <i>L. lactis</i> subsp. <i>lactis</i> , <i>L. lactis</i> subsp. <i>cremoris</i> , <i>Leuc. mesenteroides</i>
	Dahi	<i>L. bif fermentans</i> , <i>L. alimentarius</i> , <i>L. paracasei</i> , <i>L. lactis</i> , <i>Strep. cremoris</i> , <i>Strep. lactis</i> , <i>Strep. thermophilus</i> , <i>L. bulgaricus</i> , <i>L. acidophilus</i> , <i>L. helveticus</i> , <i>L. cremoris</i> , <i>Ped. pentosaceus</i> , <i>P. acidilactici</i> , <i>W. cibaria</i> , <i>W. paramesenteroides</i> , <i>L. fermentum</i> , <i>L. delbrueckii</i> subsp. <i>indicus</i> , <i>Saccharomycopsis</i> sp., <i>Candida</i> sp.
Kefir	<i>L. brevis</i> , <i>L. caucasicus</i> , <i>Strep. thermophilus</i> , <i>L. bulgaricus</i> , <i>L. plantarum</i> , <i>L. casei</i> , <i>L. brevis</i> , <i>Tor. holmii</i> , <i>Tor. delbrueckii</i>	
Laban rayeb	<i>L. casei</i> , <i>L. plantarum</i> , <i>L. brevis</i> , <i>L. lactis</i> , <i>Leuconostoc</i> sp., <i>S. kefir</i>	
Meat based	Cantonese sausage	<i>L. plantarum</i>
	Fermented meat products	<i>L. plantarum</i> , <i>L. delbrueckii</i> , <i>L. pentosus</i> , <i>L. casei</i> , <i>L. curvatus</i>
	Taiwanese fermented ham	<i>L. sakei</i>
	Peperoni	Species of LAB, <i>Micrococcus</i> spp.
	Nham (musom)	<i>Ped. cerevisiae</i> , <i>L. plantarum</i> , <i>L. brevis</i>
Fish based	Fermented fish products	<i>L. alimentarius</i> , <i>C. piscicola</i>
	Burong bangus	<i>Leuc. mesenteroides</i> , <i>L. plantarum</i> , <i>W. confusus</i>
	Ngari	<i>L. lactis</i> , <i>L. plantarum</i> , <i>L. pobuzihii</i> , <i>L. fructosus</i> , <i>L. amylophilus</i> , <i>L. coryniformis</i> , <i>Ent. faecium</i> , <i>B. subtilis</i> , <i>B. pumilus</i> , <i>B. indicus</i> , <i>Micrococcus</i> sp., <i>Staph. cohnii</i> subsp. <i>cohnii</i> , <i>Staph. carnosus</i> , <i>T. halophilus</i> subsp. <i>flandriensis</i> , <i>Clostridium irregulare</i> , <i>Azorhizobium caulinodans</i> , <i>Candida</i> sp., <i>Saccharomycopsis</i> sp.

Table 11.2 (Continued)

Fermented food category	Product	LAB strains
Fruit based	Wine	<i>O. oeni</i>
	Citron	<i>L. acidophilus</i>
	Fermented fruit juice	<i>Ent. faecalis</i> , <i>Ent. durans</i>
Vegetable based	Pickles	<i>L. plantarum</i> , <i>L. brevis</i> , <i>L. curvatus</i>
	Fermented vegetable juice	<i>L. plantarum</i> , <i>L. brevis</i>
	Fermented vegetable	<i>L. casei</i> , <i>L. rhamnosus</i>
	Chinese sauerkraut	<i>L. rhamnosus</i>
	Fermented radish	<i>L. brevis</i>
	Fermented Chinese cabbage	<i>P. acidilactici</i>
	Gundruk	<i>L. fermentum</i> , <i>L. plantarum</i> , <i>L. casei</i> , <i>L. casei</i> subsp. <i>pseudopplantarum</i> , <i>Ped. pentosaceus</i>
Kimchi	<i>Leuc. mesenteroides</i> , <i>Leuc. citreum</i> , <i>Leuc. gasicomitatum</i> , <i>Leuc. kimchii</i> , <i>Leuc. inhae</i> , <i>W. koreensis</i> , <i>W. kimchii</i> , <i>W. cibaria</i> , <i>L. plantarum</i> , <i>L. sakei</i> , <i>L. delbrueckii</i> , <i>L. buchneri</i> , <i>L. brevis</i> , <i>L. fermentum</i> , <i>Ped. acidilactici</i> , <i>Ped. pentosaceus</i> , <i>L. lactis</i> , yeast species of <i>Candida</i> , <i>Halococcus</i> , <i>Haloterrigena</i> , <i>Kluyveromyces</i> , <i>Lodderomyces</i> , <i>Natrialba</i> , <i>Natronococcus</i> , <i>Pichia</i> , <i>Saccharomyces</i> , <i>Sporisorium</i> and <i>Trichosporon</i>	
Sunki	<i>L. plantarum</i> , <i>L. fermentum</i> , <i>L. delbrueckii</i> , <i>L. parabuchneri</i> , <i>L. kisonensis</i> , <i>L. otakiensis</i> , <i>L. rapi</i> , <i>L. sunkii</i>	
Soybean based	Stinky tofu	<i>L. buchneri</i> , <i>L. agilis</i> , <i>L. brevis</i> , <i>L. crispatus</i> , <i>L. curvatus</i> , <i>L. delbrueckii</i> , <i>L. farciminis</i>
	Miso	<i>Ped. acidilactici</i> , <i>Leuc. paramesenteroides</i> , <i>Micrococcus halobius</i> , <i>Ped. halophilus</i> , <i>Streptococcus</i> sp., <i>Saccharomyces rouxii</i> , <i>Zygosaccharomyces rouxii</i> , <i>Asp. oryzae</i>
	Tempe	<i>Rhiz. oligisporus</i> , <i>Rhiz. arrhizus</i> , <i>Rhiz. oryzae</i> , <i>Rhiz. stolonifer</i> , <i>Asp. niger</i> , <i>Citrobacter freundii</i> , <i>Enterobacter cloacae</i> , <i>K. pneumoniae</i> , <i>K. pneumoniae</i> subsp. <i>ozaenae</i> , <i>L. fermentum</i> , <i>L. lactis</i> , <i>L. plantarum</i> , <i>L. reuteri</i>
Cereal based	Boza	<i>Lactobacillus</i> sp., <i>Lactococcus</i> sp., <i>Pediococcus</i> sp., <i>Leuconostoc</i> sp., <i>S. cerevisiae</i>
	Hussuwa	<i>L. fermentum</i> , <i>Ped. acidilactici</i> , <i>Ped. pentosaceus</i> , yeasts
	Jalebi	<i>S. bayanus</i> , <i>L. fermentum</i> , <i>L. buchneri</i> , <i>L. lactis</i> , <i>Ent. faecalis</i> , <i>S. cerevisiae</i>

Source: Adapted from Chokesajjawatee et al. (2009); Divya et al. (2012); El Sheikh & Montet (2014a, b); El Sheikh & Bakar (2014); Liu et al. (2011); Marshall & Mejia (2011); Panda et al. 2011; Ray et al. (2014); Tamang et al. (2016).

11.4 Molecular Approaches Used to Study Fermenting Microflora

Rapid development occurred in the application of molecular tools for identifying microorganisms and investigating their physiological characteristics. The classic methodology used to recognize the type of microflora associated with a fermented food product has two major steps: isolation and identification. However, high-throughput approaches based on molecular tools are increasingly applied to study fermenting microflora.

There are several ways to isolate and identify the specific type of bacterial strain, including conventional methods, rapid methods, and high-throughput techniques. Conventional methods are generally referenced in *Bergey's Manual* (Yelnetty et al. 2014) whereas rapid and high-technology methods are mostly DNA based using the polymerase chain reaction (PCR) system (de Medici et al. 2015).

The first step in identifying LAB is isolation, which generally involves growing the bacterial culture from fermented food product samples on a selective medium. MRS (de Man, Rogosa, Sharpe) agar is the medium most widely used to grow and isolate LAB (de Man et al. 1960). Once colonies are grown then a single colony is picked to obtain a pure culture of selected LAB isolate. The selected LAB isolate is further characterized for identification purposes through morphological, physiological, and biochemical analyses. Morphological characterization involves observation for size, color, texture, and formation of colony. Physiological characterization includes Gram staining and growth kinetics studies in different conditions (temperatures, pH, etc.), and biochemical characterization is conducted by testing catalase activity and the production of gas and certain amino acids. However, the identification of LAB strains using the classic or conventional approach is laborious, time consuming, and less convincing (Moraes et al. 2013). These characteristics are less discriminative due to the high similarities among many LAB species and strains in their nutrient and growth requirements. Further developments in the conventional approach to identify LAB strains include the API system (BioMerieux, Marcy l'Etoile, France) and BioLog (Biolog, Hayward, CA) that are based on the fermentation abilities of the microorganisms.

The most recent and more reliable identification approaches employ molecular tools based on 16S ribosomal DNA sequences or PCR techniques or other techniques involving detection of macromolecules (proteins) or other compounds produced by the LAB. Molecular techniques like amplification, hybridization, and electrophoresis are commonly used in microbial analysis of fermented foods (Rodriguez et al. 2006). Generally, molecular tools are divided into those based on nucleic acids and other molecular approaches (primarily used for identification), and approaches used to analyze cell activity (studying the physiological properties).

Molecular tools available to identify LAB strains can be classified into three groups. First, PCR-based molecular techniques that include multiplex PCR, real-time PCR, denaturing gradient gel electrophoresis (PCR-DGGE), PCR single-strand conformation polymorphism (SSCP), random amplified polymorphic DNA-PCR (RAPD-PCR), and temperature gradient gel electrophoresis (TGGE). Second, non-PCR-based molecular methods like DNA-DNA hybridization, ribo-printing, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), matrix-assisted laser

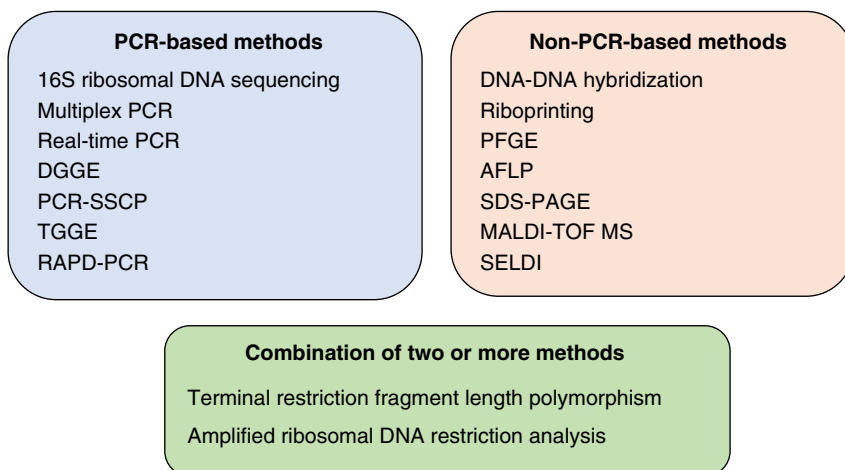


Figure 11.1 Common molecular approaches available for the identification of LAB in fermented food products.

desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), surface-enhanced laser desorption/ionization (SELDI) mass spectrometry, pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), terminal restriction fragment length polymorphism (T-RFLP), amplified ribosomal DNA restriction analysis (ARDRA), and (automated) ribosomal intergenic spacer analysis (ARISA). Third, techniques using the combination of two or more molecular methods (Bagheripoor-Fallah et al. 2015; Justé et al. 2008). Moreover, to identify different microbial groups several other technologies such as microarray have been developed (Patro et al. 2015). Microarray is useful to identify the presence of microbial ingredients and determine microbe differences in dietary supplements.

Figure 11.1 gives an overview of molecular techniques commonly applied to identify LAB in fermented foods. Further details of molecular techniques can be found in other chapters of this book based on the type of food.

11.5 Identification of Lab in Fermented Cereal and Meat Products

Accurate identification of LAB present in a specific fermented food is vital to control food processing and ensure consistent quality of the end-product. Proper LAB strain identification is also required for legal and regulatory purposes (Amor et al. 2007). There are several well-characterized molecular techniques used to identify LAB, as discussed previously. Molecular techniques to identify fermenting microorganisms in fermented fruits and vegetables (see Chapter 12), fermented dairy products (see Chapter 13), and wine (see Chapter 14) are covered in other chapters. This chapter will focus on the application of various molecular methodologies to identify LAB in fermented cereal and meat products.

11.5.1 LAB in Fermented Cereal Products

Cereals are a major component of the human diet and fermented cereal products are consumed all over the world. Maize, wheat, rice, barley, oat, sorghum, and millet are the most common cereals. Globally, many different types of cereal-based fermented foods are produced that are classified on the basis of the raw material or type of fermentation process used (Blandino et al. 2003). Cereals are rich in carbohydrate as an energy source and also contain relatively high levels of minerals, vitamins, sterols, and other growth factors that support microbial growth.

Cereal fermentation is mainly represented by species of yeasts and bacteria dominated by the LAB (Corsetti & Settanni 2007). *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Weissella* are common bacteria associated with cereal fermentation (De Vuyst et al. 2009; Moroni et al. 2011). Cereal fermentations are often initiated by mixed microbial populations. It is a synergetic microbial interaction where the proliferation of yeasts in fermenting material is favored by the acidic environment created by the LAB while the growth of LAB is stimulated by the presence of yeasts, which provide growth factors such as vitamins and soluble nitrogen compounds (Jespersen 2003). The initial increase of acidity eliminates or inactivates non-lactic acid microorganisms during the fermentation. Surviving LAB are known to develop a synergy with some yeasts during the cereal fermentation (Mbata et al. 2009). Among the most common examples, bread fermentations are carried out using native yeast strains of *Saccharomyces cerevisiae* as the principal fermenting microflora (Hammes et al. 2005), but other non-*Saccharomyces* yeasts are also significant in many cereal fermentations, including *Candida*, *Debaryomyces*, *Hansenula*, *Kazachstania*, *Pichia*, *Trichosporon*, and *Yarrowia* (Johnson & Echavarri-Erasun 2011).

Sourdough bread fermentations are dependent on LAB populations. The microbiology of fermented cereal products is complex and the diversity of microflora depends on raw material, fermentation process, and geographic location. Several molecular techniques have been employed to determine the representative microflora of fermented cereal products (Table 11.3).

The microbiology of sourdough is more complex than that in traditional bread. Sourdough contains flour, water, and metabolically active microorganisms, mainly LAB and yeast. Technically, it is an intermediate product between dough and traditional bread preparation. The metabolites produced by LAB during fermentation of the dough play a key role in improving the organoleptic and technological properties, nutritional value, and shelf-life of bread (Hammes & Ganzle 1998). *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Weissella* are the LAB genera most commonly isolated from sourdough whereas the majority of the strains belong to the genus *Lactobacillus* (De Vuyst & Neysens 2005).

Identification of LAB species and strains in sourdough has been reported by several authors. Corsetti et al. (2007) used a polyphasic approach, consisting of 16S rRNA gene sequencing, multiplex PCR assays and physiological features, to identify subdominant sourdough LAB. *Ent. faecium*, *Ped. pentosaceus*, and *L. sanfranciscensis* were found in sourdough from the Abruzzo region (central Italy). Identification of LAB in rye sourdoughs from four bakeries with different propagation parameters was performed using

Table 11.3 Examples of conventional and molecular techniques employed to identify LAB in fermented cereal products.

Technique(s)	Fermented cereal product	Food category/ origin	Identified LAB	Reference
Culture-dependent and culture-independent microbiological analysis	Sourdough (rye, wheat)	Leavened bread (America, Europe, Australia)	<i>L. sanfranciscensis</i> , <i>L. alimentarius</i> , <i>L. buchneri</i> , <i>L. casei</i> , <i>L. delbrueckii</i> , <i>L. fructivorans</i> , <i>L. plantarum</i> , <i>L. reuteri</i> , <i>L. johnsonii</i> , <i>Cand. humilis</i> , <i>Issatchenkia orientalis</i>	De Vuyst et al. 2009; Gänzle et al. 1998
16S rRNA gene sequencing, multiplex PCR	Sourdough (rye, wheat)	Leavened bread (Abruzzo region, central Italy)	<i>L. sanfranciscensis</i> , <i>Ent. faecium</i> , <i>Ped. pentosaceus</i>	Corsetti et al. 2007
DGGE, pyrosequencing of 16S rRNA gene	Sourdough (rye)	Leavened bread (Estonia)	<i>L. amylovorus</i> , <i>L. frumenti</i> , <i>L. helveticus</i> , <i>L. sanfranciscensis</i> , <i>L. pontis</i> , <i>L. zymae</i>	Viiard et al. 2016
Rep-PCR fingerprinting, multiplex PCR, 16S rRNA gene sequencing	Tarhana (sheep milk, wheat)	Soup or biscuit (Cyprus, Greece, Turkey)	<i>L. bulgaricus</i> , <i>Strep. thermophilus</i> , yeasts	Sengun et al. 2009
16S/23S rRNA intergenic spacer region, partial 16S rRNA gene sequencing	Tarhana (wheat)	Tarhana fermenting dough (Adana, Turkey)	<i>Ped. acidilactici</i> , <i>L. plantarum</i> , <i>L. brevis</i>	Settanni et al. 2011
Morphological and biochemical analysis	Mawe (maize)	Multi-purpose, beverage, gels and porridges (Benin, Africa)	<i>L. fermentum</i> , <i>L. cellobiosus</i> , <i>L. brevis</i> , <i>L. curvatus</i> , <i>L. buchneri</i> , <i>Weissella confusa</i> , <i>Candida krusei</i> , <i>Candida kefyr</i> , <i>Candida glabrata</i> and <i>S. cerevisiae</i>	Hounhouigan et al. 1993
GTG(5)-based PCR fingerprinting, 16S rRNA gene sequencing, multiplex PCR	Fura (millet)	Bread (Nigeria, Burkina Faso and Ghana, West Africa)	<i>Ped. acidilactici</i> , <i>W. confusa</i> , <i>L. fermentum</i> , <i>L. reuteri</i> , <i>L. salivarius</i> , <i>L. paraplantarum</i>	Owusu-Kwarteng et al. 2012
Phenotypical and biochemical analysis	Mifen (rice)	Fermented noodles (south China)	<i>S. cerevisiae</i> , <i>Candida rugosa</i> , <i>Candida tropicalis</i> , <i>Lactobacillus</i> , <i>Leuconostoc</i> , <i>Pediococcus</i> , <i>Streptococcus</i> sp., <i>Enterococcus</i> sp., <i>Aerococcus</i> sp.	Lu et al. 2008

(Continued)

Table 11.3 (Continued)

Technique(s)	Fermented cereal product	Food category/ origin	Identified LAB	Reference
16S rRNA gene sequencing, multilocus sequence analysis, M13-PCR gel fingerprints	Ogi and kunu-zaki	Fermented cereal food (Nigeria)	<i>L. fermentum</i> , <i>L. plantarum</i> , <i>Strep. gallolyticus</i> subsp. <i>macedonicus</i> , <i>Ped. pentosaceus</i>	Oguntoyinbo & Narbad 2012
MALDI-TOF MS, automated ribotyping	Baca (millet gruel) and wômi (millet cake)	Fermented cereal food (Africa)	<i>Ped. acidilactici</i> , <i>L. fermentum</i> , <i>Lb. salivarius</i> , <i>Ped. pentosaceus</i> , <i>L. plantarum</i> subsp. <i>plantarum</i>	Soro-Yao et al. 2014

DGGE, denaturing gradient gel electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; PCR, polymerase chain reaction.

plating, DGGE, and pyrosequencing of 16S rRNA gene amplicons (Viiard et al. 2016). Sourdoughs propagated at higher temperature contain *L. amylovorus* and *L. frumenti* or *L. helveticus* and *L. panis* while ambient temperature combined with a short fermentation cycle had *L. sanfranciscensis*, *L. pontis*, and *L. zymae* or *L. helveticus*, *L. pontis* and *L. zymae* as dominating microflora.

Traditional fermented cereal products contain a diverse microflora that is predominantly represented by the members of LAB. For example, a traditional fermented cereal product consumed in South India and in many parts of Sri Lanka, known as idli, has many LAB species (*Leuc. mesenteroides*, *L. fermenti*, *Strep. faecalis*, *L. delbrueckii*, *L. lactis* and *Ped. cerevisiae*) responsible for its fermentation. Ogi is a fermented product from West Africa made of corn, sorghum and millet in which LAB, yeasts, and molds are responsible for the fermentation although *L. plantarum* is the predominant microorganism (Blandino et al. 2003).

Non-PCR-based techniques like MALDI-TOF MS protein analysis have been used to identify LAB from fermented cereal foods. MALDI-TOF MS is a chemotaxonomic method in which LAB species are identified based on mass spectra patterns of ribosomal proteins (Pavlovic et al. 2013). Soro-Yao et al. (2014) used MALDI-TOF MS and identified 23 LAB isolated from fermented cereal foods available in Abidjan, Côte d'Ivoire. *Ped. acidilactici* (56.5%), *L. fermentum* (30.4%), *Lb. salivarius* (4.3%), *Ped. pentosaceus* (4.3%), and *L. plantarum* subsp. *plantarum* (4.3%) were the LAB species and subspecies identified in Abidjan. Results of protein-based identification were confirmed by other molecular tools such as automated ribotyping and by phenotypic characterization. The authors suggested that MALDI-TOF MS protein analysis provided a high level of discrimination among the isolates and could be used for the rapid screening of LAB starter cultures. However, protein-based identification techniques are not commonly used for several reasons (access, cost, and operating expenses).

11.5.2 LAB in Fermented Meat Products

Fermented meat products are divided into two categories: those made from whole meat pieces or slices, such as dried meat and jerky; and those made by chopping or comminuting the meat, usually called sausages (Adams 2010). Traditionally, fermented meat products have been well documented in many countries, such as fermented sausages (Lücke 2015) and salami (Toldra 2007) in Europe, jerky in America and Africa (Baruzzi et al. 2006), *nham* in Thailand (Chokesajjawatee et al. 2009), and *nemchua* in Vietnam (Nguyen et al. 2013).

Identification of microbial strains in fermented meat products has been reported in many studies (Table 11.4), primarily using molecular methods such as 16S DNA sequencing, species-specific PCR, RAPD, plasmid mapping, rRNA hybridization probe, real-time PCR, and denaturing gel electrophoresis (Aymerich et al. 2006). The main microflora in meat fermentation are LAB (Albano et al. 2009; Cocolin et al. 2011; El Sheikha & Bakar 2014; Nguyen et al. 2013) and the second most abundant are coagulase-negative staphylococci, micrococci, and Enterobacteriaceae (Cocolin et al. 2011; Marty et al. 2011). Some species of yeasts (Tamang & Fleet 2009) and molds may also play a role in the ripening of certain fermented meat products (Lücke 2015). The LAB should adapt to the environment of meat fermentation processes, including storage and transportation. It is believed that the microorganisms present in traditional sausages are derived from the raw materials or the environment of manufacturing (Mauriello et al. 2004). However, it must be noted that the microflora isolated from traditional sausages are well documented whereas the resident microflora in meat processing environments are still poorly known.

The most common LAB species in traditional fermented sausages identified using molecular tools were *L. sakei*, *L. curvatus*, and *L. plantarum* (Lebert et al. 2007); *L. alimentarius*, *L. casei*, *L. delbrueckii*, *L. farciminis*, *L. paraplantarum*, *L. pentosus*, and *L. sharpeae* form the minor component of the population. *L. sakei* is reported as a dominant species and can represent more than 42% of the isolates (Papamanoli et al. 2003; Urso et al. 2006). Aymerich et al. (2006) identified 250 LAB isolates from Spanish fermented sausage using RAPD-PCR and the results confirmed 74% of population as *L. sakei*; others were *L. curvatus* (21.2%) and *Leuc. mesenteroides* (4.8%). *L. sakei* was identified in all the Spanish sausages and represented 89% in chorizo and 76% in a traditional Spanish sausage called “fuet” (Aymerich et al. 2006), whereas 100% of the isolates in the final product of a French sausage were *L. sakei* although it was much rarer in the raw materials (Ammor et al. 2005). *L. curvatus* is the second dominant species identified in some Greek or Italian sausages (Comi et al. 2005; Rantsiou et al. 2005) and *L. plantarum* is the third dominant species in a Greek sausage (Drosinos et al. 2005).

Intelligent use of molecular approaches can also help to explore the diversity at strain level in the dominant species in fermented meat products. For example, Aymerich et al. (2006) applied a combination of plasmid profiling and RAPD-PCR that allowed them to distinguish 112 different strains out of 185 isolates of *L. sakei* and 23 different strains out of 53 isolates of *L. curvatus*. RAPD-PCR analysis of 100 strains of *L. curvatus* isolated from Greek, Hungarian, and Italian naturally fermented sausages revealed nine genotype profiles while 168 strains of *L. sakei* from the same samples gave 19 major clusters (Rantsiou et al. 2005). Urso et al. (2006) also used RAPD to determine the diversity and distribution of 353 strains of *L. sakei* and 67 strains of *L. curvatus* associated with three Italian sausages produced using a natural fermentation process.

Table 11.4 Examples of conventional and molecular techniques used to identify lactic acid bacteria (LAB) in fermented meat products.

Technique(s)	Fermented meat product	Food category/origin	Identified LAB	Reference
16S rRNA gene sequencing, RAPD-PCR	Alheira (pork or beef)	Dry/semi-dry sausage (Portugal)	<i>L. plantarum</i> , <i>L. paraplantarum</i> , <i>L. brevis</i> , <i>L. rhamnosus</i> , <i>L. sakei</i> , <i>L. zeae</i> , <i>L. paracasei</i> , <i>Ent. faecalis</i> , <i>Ent. faecium</i> , <i>Leuc. mesenteroides</i> , <i>Ped. pentosaceus</i> , <i>Ped. acidilactici</i> , <i>W. cibaria</i> , <i>W. viridescens</i>	Albano et al. 2009
Microbiological and physico-chemical analysis	Androlla (pork)	Dry pork sausage (Spain)	<i>L. sake</i> , <i>L. curvatus</i> , <i>L. plantarum</i>	García Fontán et al. 2007
16S rRNA and phenylalanyl-tRNA synthase (<i>pheS</i>) gene sequencing	Arjia (large intestine of chevon)	Sausage, curry (India and Nepal)	<i>Ent. faecalis</i> , <i>Ent. faecium</i> , <i>Ent. hirae</i> , <i>Leuc. citreum</i> , <i>Leuc. mesenteroides</i> , <i>Ped. pentosaceus</i> , <i>Weissella cibaria</i>	Oki et al. 2011
16S rRNA, <i>pheS</i> , and <i>rpoA</i> gene sequences	Chartayshya (chevon)	Dried, smoked meat, curry (India)	<i>Ent. faecalis</i> , <i>Ent. faecium</i> , <i>Ent. hirae</i> , <i>Leuc. citreum</i> , <i>Leuc. mesenteroides</i> , <i>Ped. pentosaceus</i> , <i>Weissella cibaria</i>	Oki et al. 2012
Microbiological and physico-chemical analysis	Chorizo (Pork)	Dry sausage (Spain)	<i>L. sake</i> , <i>L. curvatus</i> , <i>L. plantarum</i>	García Fontán et al. 2007
16S rRNA gene sequencing	Nem-chua (pork)	Fermented sausage (Vietnam)	<i>L. pentosus</i> , <i>L. plantarum</i> , <i>L. brevis</i> , <i>L. paracasei</i> , <i>L. fermentum</i> , <i>L. acidipiscis</i> , <i>L. farciminis</i> , <i>L. rossiae</i> , <i>L. fuchuensis</i> , <i>L. namurensis</i> , <i>L. lactis</i> , <i>Leuc. citreum</i> , <i>Leuc. fallax</i> , <i>Ped. acidilactici</i> , <i>Ped. pentosaceus</i> , <i>Ped. stilesii</i> , <i>Weissella cibaria</i> , <i>W. paramesenteroides</i>	Nguyen et al. 2013
Phenotypic methods	Salsiccia (chopped pork meat)	Dry/semi-dry, sausage (Italy)	Species of LAB, <i>Staph.</i> sp., <i>Micrococcus</i> sp., Enterobacteriaceae, yeast	Parente et al. 2001
16S rDNA sequencing	Sudžuk (beef or pork)	Dry sausage (Bosnia and Herzegovina)	<i>Lactococcus</i> sp., <i>Enterococcus</i> sp., <i>Leuconostoc</i> sp., <i>Lactobacillus</i> sp., <i>Pediococcus</i> sp., <i>Weissella</i> sp., <i>L. plantarum</i> , <i>Leuc. mesenteroides</i> , <i>L. lactis</i> , <i>Ent. faecalis</i> and <i>Ent. durans</i>	Čolo et al. 2015
16S rDNA sequencing	Levačka (beef or pork)	Dry sausage (Serbia)	<i>Pediococcus pentosaceus</i> , <i>L. mesenteroides</i> , <i>L. curvatus</i> , <i>L. sakei</i> and <i>L. carnosum</i>	Borovic et al. 2015

Source: Adapted and modified from Tamang et al. (2016).
 PCR, polymerase chain reaction; RAPD, random amplified polymorphic DNA.

LAB strains are used as a starter culture to initiate fermentation and help in controlling the process. Selected and defined starter cultures reduce the pH and time of the fermentation process. Carbohydrate fermentation results in the formation of lactic acid, and gives a sour taste and fermented aroma to food products. *Listeria monocytogenes*, *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus* are some examples of pathogenic microorganisms which affect the quality and safety of fermented meat products (Meloni 2015). LAB strains enhance the safety of food products by inhibiting their growth and extending shelf-life. Viability of LAB strains in the fermentation environment is very important to determine and the production of lactic acid is required to reduce pH and deliver other technological benefits (Rungrassamee et al. 2012). It is a challenging task to maintain processing standards during fermentation due to the complex nature of meat as a fermenting raw material. Future developments in identification, characterization, and design of better LAB starter cultures will be valuable for the meat fermentation industry.

11.6 Advantages of Molecular Techniques

It is important to have an accurate and reliable identification technique for LAB in fermented foods. Unfortunately, there is no universal technique that can be applied to all products and microflora of interest. Traditional methods of identification have serious drawbacks such as poor discrimination power in the case of closely related LAB and being unable to detect non-culturable cells. In general, molecular techniques are more sensitive, more specific, more accurate, and faster than traditional methods. However, they also have some limitations that must be considered prior to choosing a methodology for identification of a target LAB species/strain in a specific fermented food product (Moraes et al. 2013). Table 11.5 summarizes the advantages and limitations of the different molecular techniques available to identify LAB.

11.7 Concluding Remarks

Generally, cereal- and meat-based fermented products are fermented by the indigenous microflora on the raw materials and in the immediate environment. The diversity of microbiota in naturally fermented products such as sourdough and fermented sausages can be exemplified as complex microbial ecosystems. LAB species and strains are frequently identified in fermented products. Recent years have seen tremendous development and innovation in the application of molecular tools (PCR, rep-PCR, 16S DNA sequencing, DGGE, PCR-DGGE, MLSA, RFLP, PFGE, REA-PFGE, MALDI-TOF MS) to identify LAB in fermented cereal and meat products. These advancements have allowed us to understand the microbial diversity of many fermented foods. Future developments will make these molecular tools available to carry out routine analysis of fermented foods for microbiota identification purposes.

Table 11.5 Advantages and limitations of the different molecular techniques available to identify LAB.

Technique	Advantages	Limitations
qPCR	High specificity Better sensitivity Wide quantification range	Requires optimization of primer and probe design Nucleic acid extraction and PCR biases
T-RFLP	High throughput Better sensitivity	Requires optimization of primer, probe design, and restriction digestion Nucleic acid extraction and PCR biases Sensitivity and detection limit of the automated sequencer
FISH	Rapid analysis High specificity	Requires optimization of probe design and hybridization conditions Biases due to ribosomal content of cells Inaccessibility of probe to target site Cell counting/sorting techniques
DNA microarray	High throughput Less labor intensive Better sensitivity	Requires optimization of probe design and hybridization conditions Nucleic acid extraction biases Non-specific hybridization
DGGE/TGGE SSCP	Detect various microorganisms Rapid comparative analysis Very sensitive Potential for high throughput	Low sensitivity Not quantitative Identification only possible with clone library
ARDRA	High level of resolution	Difficult to compare complex patterns Limited sensitivity
ARISA Analysis of clone libraries	Better sensitivity Can identify individual community members Offers a more global view of the community	Insufficient resolution Time-consuming Tedious
DNA array technology	Detects more microorganisms or genes	Unable to identify oligonucleotides taxa Imperfect specificity
PFGE RAPD	High discriminatory power Rapid, sensitive, and inexpensive	High labor intensity Not always comparable
Rep-PCR	High identification ability	Time-consuming and laborious

Source: El Sheikha (2010); Justé et al. (2008); Nagarajan & Loh (2014); Randazzo et al. (2009). ARDRA, amplified ribosomal DNA restriction analysis; DGGE, denaturing gradient gel electrophoresis; FISH, fluorescent *in situ* hybridization; PCR, polymerase chain reaction; PFGE, pulsed-field gel electrophoresis; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; ARISA, automated ribosomal intergenic spacer analysis; SSCP, single strand conformational polymorphism; TGGE, temperature gradient gel electrophoresis.

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12

Molecular Techniques and Lactic Acid-Fermented Fruits and Vegetables

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12.1 Introduction

The Food and Agriculture Organization recommended intake of specific quantities of fruits and vegetables in daily meals to prevent chronic maladies such as heart problems, hypertension, and risk of strokes. The majority of consumers prefer foods which are fresh, highly nutritional, and health promoting (Battcock & Azam-Ali 1998; El Sheikha 2004; El Sheikha 2012; El Sheikha et al. 2008; El Sheikha et al. 2009; El Sheikha et al. 2010a; El Sheikha et al. 2010b; Endrizzi et al. 2009; Ray et al. 2011; Razali et al. 2012). Lactic acid (LA) fermentation of fruits and vegetables is a common practice to improve the nutritional, technological, and sensory properties of food products (di Cagno et al. 2008, 2013; Gardner et al. 2001; Karovičová & Kohajdová 2003; Ong et al. 2012; Paramithiotis et al. 2016).

With the continuous increase in the world's population, LA fermentation plays a significant role in preserving fruits and vegetables and other foodstuffs for feeding humanity, especially in developing countries. Many fermented fruit and vegetable products have a long history in human nutrition and are associated with different communities (El Sheikha & Montet 2014). Fruits and vegetables are easily perishable due to their high moisture content and nutritive values. These conditions, along with high humidity and high temperature prevalent in tropical and subtropical countries, are favorable for microbial growth causing spoilage. Fermented fruits and vegetables can also be used as a potential source of probiotics as they harbor many lactic acid bacteria (LAB) such as *Lactobacillus plantarum*, *L. acidophilus*, *L. brevis*, *L. fermentum*, *Leuconostoc mesenteroides*, etc. (Swain et al. 2014).

Detection, differentiation, and identification of bacteria, including LAB, can be performed by several methods including phenotypic, biochemical and immunological assays, and genotypic techniques such as molecular biology. However, the identification of microorganisms is still intrinsically ambiguous when exclusively based on morphological, physiological, and biochemical characteristics (Settanni & Corsetti 2008). Recently, applications of molecular tools for identifying microbes and analyzing their activity have been explored. These approaches are increasingly applied to LAB, including those used for fermentation as well as those commercialized as probiotics, for identification and analysis of their activity.

This chapter aims to answer many important questions, such as: Why use molecular tools to identify and differentiate LAB present in fermented fruits and vegetables? What are the molecular techniques currently applied to fermented fruits and vegetables? What about the future?

12.2 Fermented Fruits and Vegetables: Between the Past and the Present

One of the oldest food processing techniques is fermentation which results in extension of the shelf-life of perishable foodstuffs and was particularly essential before refrigeration (El Sheikha & Montet 2014). Cabbage fermentation by lactic acid to produce sauerkraut has been studied for many years (Pederson & Albury 1969; Stamer et al. 1971). The basic outline of fruit and vegetable fermentation is given in Figure 12.1. In addition to the success of sauerkraut fermentation, the fermentation of many other vegetables, fruits, and juices has emerged, such as beets, onions, carrots, French beans, caper berries, eggplants, cauliflower, sweet cherries, table olives, sweet potato, carrot juice, and red dragon fruit juice (Abriouel et al. 2008; Botta & Cocolin 2012; di Cagno et al. 2008, 2011; El Sheikha & Ray 2017; Gardner et al. 2001; Ong et al. 2012; Panda et al. 2007; Paramithiotis et al. 2010; Rakin et al. 2007).

12.3 Benefits of Fermented Fruits and Vegetables

Fermented fruits and vegetables (see examples in Table 12.1) are one of the principal sources used to feed the world's population (Battcock & Azam-Ali 1998; Panda et al. 2005; Paramithiotis et al. 2016). They play an essential role in improving the well-being people living in marginalized and vulnerable societies through the provision of "secure and safe" food (Demir et al. 2006; Montet et al. 2006; Paramithiotis & Drosinos 2016).

12.3.1 Nutritional Benefits

Important sources of water-soluble vitamins C and B-complex, provitamin A, phyosterols, dietary fibers, minerals, and phytochemicals in the human diet are fruits and vegetables (Gebbers 2007). Both provide a natural medium for LA fermentation; for example, the content of sugar is small in vegetables but they are rich in minerals and vitamins and have a neutral pH compared to fruits which have a high sugar content and often acidic pH (Buckenhuskes 1997). Lactic acid fermentation of fruits and vegetables enhances their organoleptic and nutritional quality and retains the nutrients and colored pigments (Dahal et al. 2005; Montet et al. 2014; Panda et al. 2009). The consumption of lactic acid fermented fruits and vegetables allows improvement of human nutrition in many ways, such as the achievement of balanced nutrition and the provision of minerals and vitamins.

12.3.2 Health Benefits

Fermented fruits and vegetables are well suited to promoting the positive health image of probiotics, helping to prevent several diseases such as diarrhea and cirrhosis of the

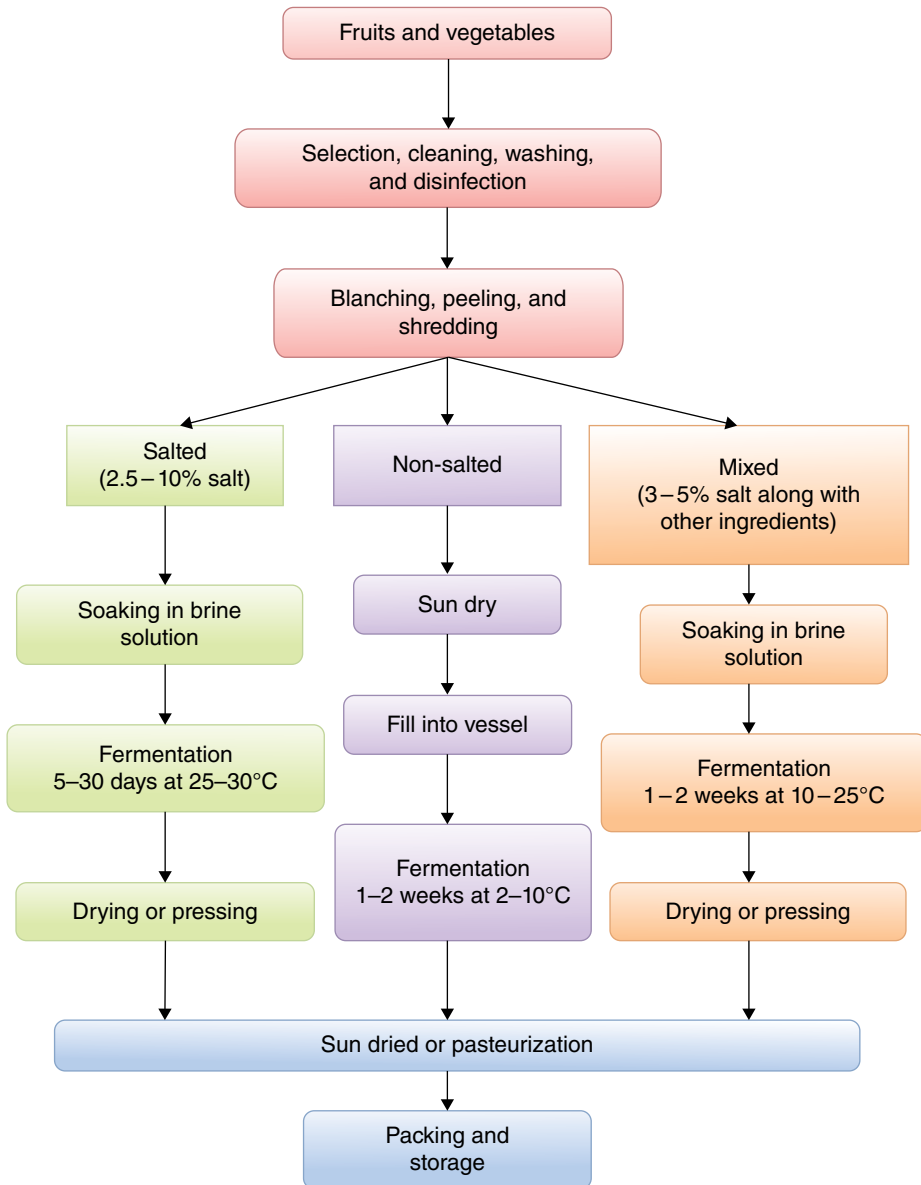


Figure 12.1 How to ferment fruits and vegetables. *Source:* Swain et al. (2014). www.hindawi.com/journals/btri/2014/250424/. Reproduced with permission of corresponding author and licensed under CC BY 3.0.

liver (Ray et al. 2014; Swain & Ray 2016). Colored pigments such as flavonoids, lycopene, anthocyanin, beta-carotene, and glucosinolates can be found in many fermented fruits and vegetables. These pigments act as antioxidants by scavenging free radicals implicated in degenerative diseases such as cancer, arthritis, and aging (Kaur & Kapoor 2001).

Table 12.1 Examples of fermented fruits and vegetables.

	Raw material	Traditional name	Country	Microorganisms
Fruits	Duriyan	Tempoyak	Malaysia	<i>L. brevis</i> , <i>L. mali</i> , <i>L. fermentum</i> , <i>Ln. mesenteroides</i>
	Peaches	Yan-taozih	China and Taiwan	<i>Ln. mesenteroides</i> , <i>W. cibaria</i> , <i>W. paramesenteroides</i> , <i>W. minor</i> , <i>L. lactis</i> subsp. <i>lactis</i> , <i>Ent. faecalis</i> , <i>L. brevis</i>
Vegetables	Cabbage	Sauerkraut	International	<i>Ln. mesenteroides</i> , <i>L. plantarum</i> , <i>L. brevis</i> , <i>L. rhamnosus</i> , <i>L. plantarum</i>
	Cucumber	Khalpi	Nepal	<i>L. plantarum</i> , <i>Ped. pentosaceus</i>
	Broccoli	Yan-tsai-shin	Taiwan	<i>W. paramesenteroides</i> , <i>W. cibaria</i> , <i>W. minor</i> , <i>Ln. mesenteroides</i> , <i>L. plantarum</i> , <i>Ec. sulfureus</i>

Sources: Chen et al. (2013a, b), Dahal et al. (2005), Leisner et al. (2001), Tamang (2009), Viander et al. (2003), Wang et al. (2010), Yang et al. (2010).

12.3.3 Technological Benefits

Lactic acid fermented foods contain biosafety agents able to control storage stability (e.g., organic acids, ethanol, and antifungal compounds) (Montet et al. 2006; Settanni & Corsetti 2008; Swain & Ray 2016).

Binding of bacteriocins to food components and additives is the main reason for not using bacteriocin-producing starter cultures as antimicrobials in food fermentation (Aasen et al. 2003; Settanni & Corsetti 2008; Swain & Ray 2016).

12.3.4 Economic Benefits

Fermentation-based industries are a vital source of income and employment in Asia, Africa, and Latin America (Ogunjobi et al. 2005). Vegetable fermentation is characterized by the type of raw materials in the final fermented product and their commercial importance. Canning and freezing are often too expensive to be affordable by millions of economically deprived people (Paramithiotis et al. 2010).

Lactic acid fermentation of vegetables has industrial significance only for cucumbers, cabbages, and olives (Montet et al. 2006). In Italy, the industrial production of fermented vegetables is limited to sauerkrauts and table olives (di Cagno et al. 2008).

12.4 Techniques of Lab Analysis Used in Fermented Fruits and Vegetables

The properties used for the identification of LAB, such as morphological, physiological, metabolic/biochemical, and chemotaxonomic markers, are given in Figure 12.2. There are many techniques for analyzing the LAB flora of fermented fruits and vegetable products.

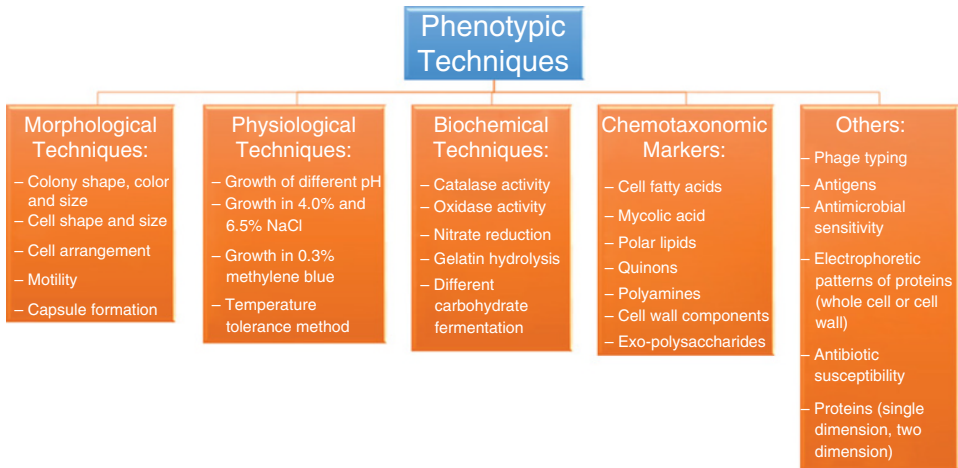


Figure 12.2 Some phenotypic techniques for identification of lactic acid bacteria.

Genotypic methods utilized for this target include the following (Elegado et al. 2004; Rantsiou et al. 2004).

- Polymerase chain reaction (PCR)
- Multiplex PCR
- Species-specific PCR
- Random amplified polymorphic DNA (RAPD)
- Restriction fragment length polymorphism (RFLP)
- Denaturing gradient gel electrophoresis (DGGE)
- Temperature gradient gel electrophoresis (TGGE)
- 16S rDNA sequencing

12.4.1 Benefits of Using Genotypic Methods for Microbial Identification

Traditional biochemical and physiological methods have some limitations in the differentiation of a vast number of isolates showing similar physiological characteristics (Berthier & Ehrlich 1999). In contrast, molecular methods provide a very delicate way to explore microbial diversity and discover the dynamics of microbial communities. The application of molecular tools for the rapid identification and differentiation of LAB has been investigated (Cho et al. 2009). rDNA has been accepted as a potential tool for the identification and phylogenetic analysis of bacteria (Ben Amor et al. 2007; Ong et al. 2012). The properties of genotypic identification methods for LAB are given in Table 12.2.

12.4.2 Overview of Molecular Tools and Microbial Identification

Because of the high-throughput potential provided by nucleic acid-based tools, they are used more frequently. These tools include PCR amplification, exsitu or in situ hybridization with RNA, DNA, peptide nucleic acid probes, and 16S rDNA sequences (Amann & Ludwig 2000). The 16S rDNA-based methodologies are robust and superior

Table 12.2 The pros and cons of genotypic identification methods for lactic acid bacteria.

Method	Pros	Cons	References
Culture-dependent techniques			
Ribotyping	Distinction between various species, allows very sensitive differentiation of different strains of bacteria	The number and types of oligonucleotide probes and restriction enzyme-dependent discrimination power, difficult identification of certain species or strains	Farber (1996)
ARDRA	Numerical analysis of strain relatedness	Less discriminatory power than ribotyping, RAPD, and PFGE	Andrighetto et al. (1998)
AFLP	High repeatability, low cost and high throughput for detection of DNA polymorphism, strain differentiation	Technically demanding	Janssen et al. (1996), Vos et al. (1995)
RFLP	Sensitive and easily applicable method	Selection of enzyme is important	Sato et al. (2000)
PFGE	High discriminatory power and repeatability	Time and labor intensive, strain-dependent specificity	Bush & Nitschko (1999)
MLST	Differentiation of highly related genotypes, excellent discrimination	Requirement for specialized equipment, trained personnel and expensive reagents	Boers et al. (2012)
RAPD	Determination of genetic fingerprinting, identification at intraspecies and interspecies level	Discrimination power was affected negatively by the number of primers using weak bound patterns and repeatability	Cocconcelli et al. (1995), Ehrmann & Vogel (2005)
AP-PCR	Ease of application, not time consuming	No standardization	Welsh & McClelland (1990)
Rep-PCR	Ease of application, good for studying a large number of isolates, high capacity for differentiation	Less discrimination power than PFGE	Olive & Bean (1999), van der Zee et al. (1999)
Culture-independent techniques			
DGGE/ TTGE	Rapid estimation of diversity, simultaneous analysis of multiple samples	Similar migration characteristics to heterologous sequences, overestimation of bacterial diversity because of heteroduplexes	El Sheikha (2010), Shyu et al. (2007)
T-RFLP	Determination of suitable differences in genotypes, comparative community analysis, good sensitivity, high throughput	Sequences must be known for enzyme selection, the same length of fragment for many species, qualitative, requires clone library for identification	Shyu et al. (2007)

Table 12.2 (Continued)

Method	Pros	Cons	References
qPCR	Absolute quantification of LAB	Expensive equipment and chemicals, sensitive to mistakes, inhibition of PCR reactions, complicated data analysis	Furet et al. (2004)
FCM	Investigation of samples quantitatively and qualitatively, estimation of viability of LAB	Labor intensive at species level, requires 16S rRNA gene sequence data	Bunthof et al. (2001)
FISH	Visualization, identification, enumeration, and localization of individual microbial cells	Requires probe design, labor intensive	Ercolini et al. (2003)

AFLP, amplified fragment length polymorphism; AP-PCR, arbitrarily primed-polymerase chain reaction; ARDRA, amplified ribosomal DNA restriction analysis; DGGE, denaturing gradient gel electrophoresis; FCM, flow cytometry; FISH, fluorescent in situ hybridization; MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis; qPCR, quantitative polymerase chain reaction; RAPD, random amplified polymorphic DNA; rep-PCR, repetitive extragenic palindromic polymerase chain reaction; RFLP, restriction fragment length polymorphism; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism.

to conventional methods based on phenotypic criteria, which are often unreliable and lack the resolving power to analyze the microbial content and activity of bacterial populations. However, for probiotic bacteria (e.g., LAB) DNA sequence approaches are more applicable than rDNA approaches. These have been useful for strain differentiation and identification.

Approaches based on complete or partial genomes include DNA arrays that have been introduced in the food industry and can be used in comparative genomics or genome-wide expression profiling (de Vos 2001). Omics techniques are feasible for probiotic bacteria such as *Bifidobacterium longum* (Schell et al. 2002) and *L. plantarum* (Kleerebezem et al. 2003). Other methods are based on the properties of other macromolecules such as proteins. It should be noted that the link with the complete or partial genomes provides the basis to develop proteomics and other omics-related techniques for detecting, identifying, and analyzing the functionality of bifidobacteria and LAB (Klaenhammer et al. 2005).

12.4.3 Molecular Techniques Used for Fermented Fruits and Vegetables

Molecular methods help to resolve identification problems. Nucleic acid probe technology can provide faster and more reliable differentiation. Species-specific PCR, Rep-PCR, multiplex PCR, 16S rDNA sequencing, DGGE, and TTGE are used to analyze the LAB flora of fermented fruits and vegetables (Abriouel et al. 2008; Botta & Cocolin 2012; Cho et al. 2009; de Bellis et al. 2010; di Cagno et al. 2008; Elegado et al. 2004; Kim & Chun 2005; Miyamoto et al. 2005; Panagou et al. 2008; Paramithiotis et al. 2010; Pulido et al. 2005; Sulistiani et al. 2014; Torriani et al. 2001).

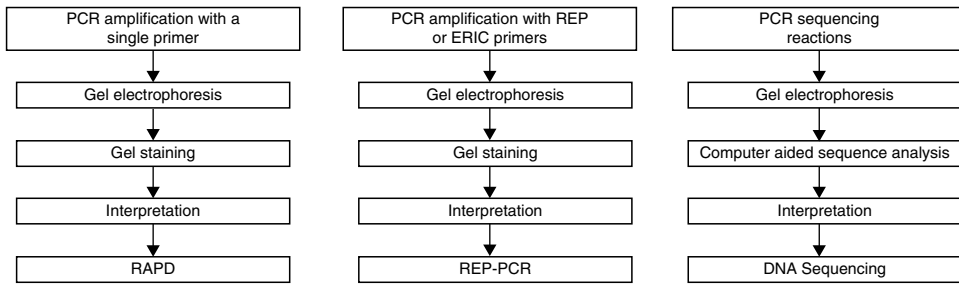


Figure 12.3 Procedural flowcharts for some genotypic methods.

It is now possible to identify different LAB in fermented food without cultivation at the species level within 1 day (Schleifer & Ludwig 1995). Additionally, DNA restriction fragment analysis and ribotyping have been used to distinguish LAB. PCR-based methods (PCR-RFLP, Rep-PCR, PCR ribotyping, RAPD) and pulsed-field gel electrophoresis (PFGE) can be used as primary molecular tools. Comparison of procedural steps provides a better understanding of their principles (Figure 12.3). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a relatively new molecular tool (Nguyen et al. 2013).

12.4.3.1 Ribotyping

Use of the position or structure of genes encoding rRNA to determine the identity of a particular genus or species is called “ribotyping” (Schmidt 1994). Breidt and Fleming (1996) developed a rapid and simple technique to identify LAB from fermented vegetables. They adapted a PCR-based ribotyping method to identify LAB species by using PCR amplification of the intergenic spacer region of rRNA operons. It was found that the spacer region between 16S and 23S genes in bacterial rRNA operons was flanked by highly conserved sequences (Olsen et al. 1992). Intergenic spacer length and the number of rRNA operons can vary between species. PCR amplification of intergenic spacer region can give unique amplified fragments, specific for a particular species (Figure 12.4). One of the advantages of ribotyping technique is minimizing the manipulation of the cellular DNA samples.

12.4.3.2 Restriction Fragment Length Polymorphism (RFLP)

Related sequences of nucleotides can be compared by exposing them to the same restriction endonucleases; this is the principle of RFLP. The characteristic fingerprint can be obtained by electrophoresis and staining of fragments from a given sequence. The comparison between the different sequences can be accomplished by comparing their fingerprints (Bulut 2003; Sato et al. 2000).

Applications of RFLP to Fermented Fruits and Vegetables

Restriction fragment length polymorphism-PCR of 16S rDNA was used to analyze the LAB strains in fermented green olive samples collected from different areas of Sicily (Randazzo et al. 2004). The results obtained showed a remarkable bacterial heterogeneity within the isolates.

Chen et al. (2006) used RFLP and sequence analysis of 16S rDNA to characterize LAB from suan-tsai (fermented mustard), a traditional food in Taiwan.

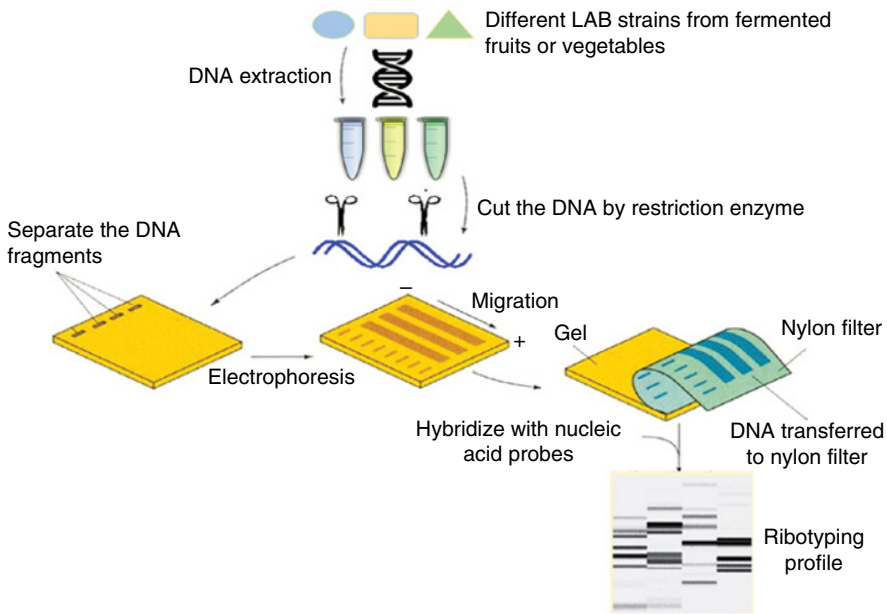


Figure 12.4 Procedural steps for the ribotyping technique.

Arbequina table olives are produced via a conventional process involving spontaneous fermentation in brine. A study was undertaken to evaluate for the first time the microorganism populations in brine during the processing of Arbequina olives. LAB analysis by RFLP-PCR revealed two restriction patterns, assigned to *L. mali* and *L. pentosus/L. plantarum* species. To confirm the identity of these isolates, they were further identified by multiplex PCR assay (Hurtado et al. 2008).

Two different species of LAB, *Enterococcus faecalis* and *Ec. durans*, from red dragon fruit beverages were identified using RFLP and nucleotide sequencing of 16S rRNA gene. Although *Ec. faecalis* is used in food production, its risk to human health cannot be neglected. Furthermore, investigation of *Ec. faecalis* at the strain level is essential to justify its suitability to be incorporated into food and beverages or to eliminate the microbial population in fermented red dragon fruit juice before it can be consumed (Ong et al. 2012).

12.4.3.3 Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA is one of the genotypic methods based on PCR. A single synthetic oligonucleotide primer is needed to initiate synthesis of new DNA strands in this PCR. RAPD is considered a very simple and quick technique (Farber 1996). The PCR that is used for RAPD differs from ordinary PCR because of the following aspects.

- The primers used are very short.
- Sequences are chosen randomly.
- The annealing temperature for RAPD is lower than PCR targeting specific genomic regions.

Identification of LAB by RAPD

Random amplified polymorphic DNA fingerprinting has been used to identify LAB for a long time. PCR-amplified DNA fragments are monitored by agarose gel electrophoresis. When the number of primers is increased, the discrimination power is also raised (Ehrmann & Vogel 2005). RAPD identification of LAB at the interspecies level and, of some species (*Enterococcus*, *Pediococcus*, *Lactobacillus*), at the intraspecies level has been successful (Cocconcelli et al. 1995, 1997).

Applications of RAPD for Fermented Fruits and Vegetables

Random amplified polymorphic DNA-PCR has been used to monitor the progress of starter cultures in vegetable fermentations. The technique does not require prior knowledge of target sequences, which makes it suitable to follow the unmarked starter cultures in commercial fermentations where antibiotic-marked starter cultures are not applicable (Plengvidhya et al. 2004).

Plengvidhya et al. (2004) investigated the use of RAPD to follow the growth, survival, and predominance of a starter culture in sauerkraut fermentations.

The dynamics of the microbial community responsible for the fermentation of caper berries (the fruits of *Capparis* sp.) was investigated using a polyphasic approach combining microbial enumerations with culture media, RAPD, and TGGE fingerprinting of total community DNA and sequencing of partial 16S rDNA. Analysis of RAPD-PCR patterns by cluster showed a high diversity among lactobacilli (with four major groups and five subgroups) while pediococci clustered in two closely related groups (Pulido et al. 2005).

Study of genetic diversity in LAB from the industrial fermentation of “Almagro” eggplants also revealed considerable differences from one enterprise to another (Seseña 2005). Among 127 genotypes detected by RAPD analysis in three enterprises sampled, only three of them belonged to the same strain, suggesting that only a small percentage of strains (1.9%) from this fermentation is cosmopolitan, in spite of the geographic proximity of the analyzed enterprises.

Tamang et al. (2005) and Tamang (2009) isolated 269 LAB strains from gundruk, sinki, inziangsang (leafy fermented vegetable), and khalpi samples and studied their genotypic properties using RAPD-PCR. Differentiation of *Enterococcus* species into three clusters isolated from fermented red dragon fruit juice was achieved by RAPD-PCR (Ong et al. 2012).

Aponte et al. (2012) presented a successful attempt to create an improved fermentation process of Spanish-style green olives by use of autochthonous starter cultures. Starter monitoring by RAPD-PCR using primer M13 provided valuable evidence of the adaptation of the *L. pentosus* strain used as a starter, even though its RAPD profile was recovered in 10 strains out of 17 isolated during natural fermentation. This occurrence came as no surprise since the strain used as a starter was isolated in the same ecological niche and may be considered autochthonous of that environment. *L. coryniformis* was never retrieved, suggesting the presence of a hurdle for strain prevalence, when drupes originated from irrigated fields.

12.4.3.4 Pulsed-Field Gel Electrophoresis (PFGE)

In PFGE, the genomic DNA is cut with a restriction enzyme, and then fragments are separated on an agarose gel (Figure 12.5). This method provides resolution at subspecies and strain level. The cells are embedded in agarose plugs and lysed. Then, genomic

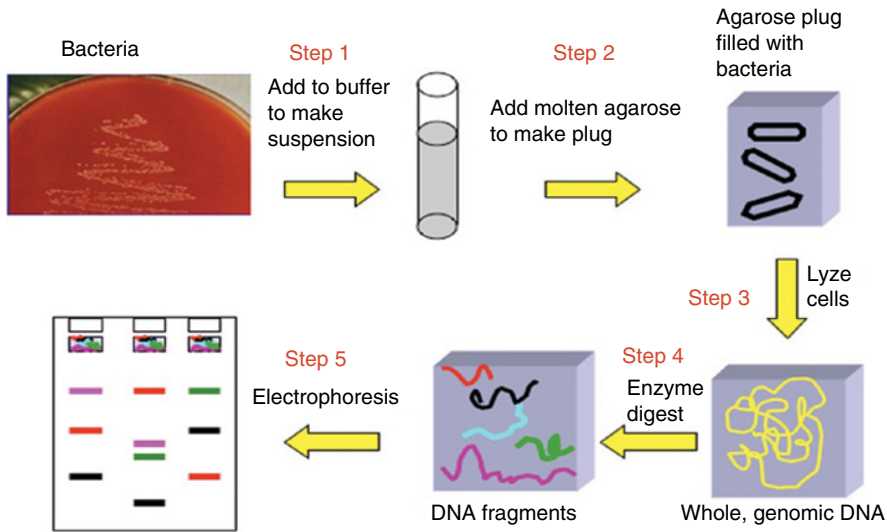


Figure 12.5 Pulsed-field gel electrophoresis (PFGE) methodology step by step. *Source:* www.foodsafetynews.com/2009/08/genetic-testing-1/#.VrK991KwQso.

DNA is digested with infrequent cutting restriction enzymes. PFGE is based on use of the alternating electrical field at predetermined intervals. The electrical field is changed at intervals called “pulse times.” PFGE can separate higher molecular weight DNA fragments (Bulut 2003).

PFGE alone is not adequate for differentiating LAB varieties. PFGE and DNA fingerprinting/typing allow comparison of large DNA fragments and these techniques have been successfully used for a variety of LAB. However, it is necessary to develop a particular approach to resolving intraspecies genetic relationships (Karahan & Çakmaçlı 2013).

Applications of PFGE for Fermented Fruits and Vegetables

To analyze the genetic diversity of LAB in Almagro (fermented eggplants), Sánchez et al. (2004) used RAPD combined with PFGE.

DNA fingerprinting approaches have been used to follow the progress of unmarked starter cultures in laboratory sauerkraut fermentations. RAPD-PCR was used for strain-specific identification of *Ln. mesenteroides* cultures (Breidt & Fleming 1996). A comparative analysis of RAPD banding patterns for fermentation isolates and starter cultures was performed using both genetically marked and unmarked cultures. Intergenic transcribed spacer (ITS)-PCR used for confirming the results from marked and unmarked starter cultures and strain identity was confirmed by PFGE patterns (Plengvidhya et al. 2004).

The first reported studies on olive fermentation using probiotic starter culture were by Saravanos et al. (2008). An important issue in inoculated olive fermentation is to control the survival of the specific inoculated strain during the process using molecular identification techniques. In this work, identification of LAB isolates using PFGE analysis revealed several changes in the survival of the inoculated LAB strains. Doulgeraki et al. (2012) exploited PFGE for the characterization of LAB communities present on

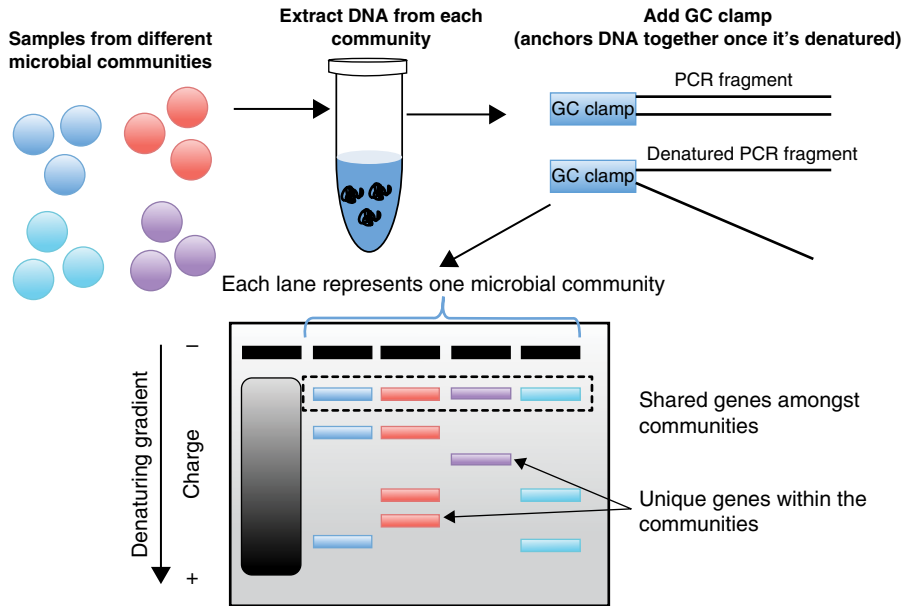


Figure 12.6 Assessment of different microbial communities by DGGE. https://en.wikipedia.org/wiki/File:Step-by-step_procedure_of_using_DGGE_analysis_in_microbiology.pdf. Licensed under CC BY-SA 3.0.

the surface of black olives packed with different compositions of gas and storage times. As far as the LAB were concerned, the two primary clusters of isolates belonged to the *L. plantarum* and *L. pentosus* species and molecular analysis at strain level revealed high genetic diversity among the isolates, in particular between the *L. pentosus* strains.

12.4.3.5 Denaturing Gradient Gel Electrophoresis

Based on sequence properties, DGGE separates amplicons of roughly the same size (Madigan et al. 2009) (Figure 12.6). These characteristics dictate the threshold at which DNA denatures. DGGE uses a gradient DNA denaturant (a mixture of urea and formamide) (Muyzer 1999). The fragment stops moving when it reaches its melting point (threshold of denaturant), due to the fact that a partially melted double-stranded DNA can no longer migrate through the gel (Fischer & Lerman 1983). A GC clamp (~40 bases with a high content of GC) is used to anchor the PCR fragments together once they have denatured (Rettedal et al. 2010).

Data Output and Interpretation

Each lane represents one microbial community and each “PCR-amplified fragment” band represents one microorganism. The shared bands among the microbial samples will be at the same vertical location on the gel because they are all roughly the same size (Muyzer et al. 1993). Another target may have greater variation in length, but the denaturant gradient uses the second factor (melting point) to distinguish further between the samples. DGGE will separate genes of the same size based on its sequences.

This technique addresses the important question: are the microbial communities the same or different in taxonomic composition? Each band in a different position on the gel represents a different phylotype (one unique sequence of an ancestral marker gene) (Madigan et al. 2009). DGGE profiles have many different 16S rRNA gene sequences for microbial communities (Ward et al. 1998). The number of bands at varying horizontal positions can be used to determine the biodiversity level in that sample and infer phylogenetic affiliation (Muyzer 1999). One can excise DGGE bands from the gel and then sequence them to know more about phylogenetic affiliation.

Applications of DGGE for Fermented Fruits and Vegetables

A PCR-DGGE technique was used to determine the composition of microflora during fermentation of kimchi (traditional Korean fermented vegetable food). DGGE profiles of partially amplified 16S rDNA were performed, and the most intense bands chosen for sequencing. LAB members, such as *Weissella confusa*, *Ln. citreum*, *L. sakei*, and *L. curvatus*, were the principal microorganisms responsible for kimchi fermentation (Lee et al. 2005).

Denaturing gradient gel electrophoresis as a culture-independent method has been employed to characterize LAB isolates from sunki (an unsalted, fermented vegetable traditional to Japan) without the need for previous culturing (Endo et al. 2008).

Aloreña table olives are naturally fermented indigenous green olives with a denomination of protection (DOP) from the Guadalhorce region in Spain. PCR-DGGE analysis carried out on these olives during fermentation revealed higher differences in microbial diversity (total mesophilic microbiota, yeasts and molds, and LAB) between vats, small and medium enterprises, and storage conditions (Abriouel et al. 2011).

A PCR-DGGE technique followed by sequencing of the 16S rRNA gene fragments eluted from the interested bands on denaturing gradient gels was used to monitor changes in the bacterial microflora of two commercial kimchi, salted cabbage, and mixed ingredient samples during 30 days of fermentation at 4°C and 10°C. *Leuconostoc* was the dominant LAB over *Lactobacillus* at 4°C. *W. confusa* was detected throughout fermentation in both samples at 4°C and 10°C. *Ln. gelidum* was the dominant LAB detected at 4°C in both samples (Hong et al. 2013).

In a study by Hong et al. (2014), the two identification methods, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and PCR-DGGE, produced distinct overall LAB profiles in salted Chinese cabbage as the main component of kimchi. The PCR-DGGE method detected a more differ microflora, including non-LAB strains.

12.4.3.6 Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

This is a recently developed technology providing a new generation of fingerprints used for identifying microorganisms. Like the other mass spectrometric methods, this is based on the ionization of a neutral molecule (e.g., nucleic acid) and the subsequent accurate determination of the resulting primary ions and their decay products in a high vacuum. The ion source, a mass analyzer, and the detector are typical components of the mass spectrometer (Pavlovic et al. 2013).

MALDI-TOF MS for Identifying LAB

Genotyping methods are rapid, highly sensitive and furnish concrete identification but require high levels of technical expertise and remain expensive. For these reasons, MALDI-TOF MS has become important as a chemotaxonomic tool for the identification of microbial isolates because it is rapid, robust, reliable, and cheap) (Pavlovic et al. 2013; Santos et al. 2013).

MALDI-TOF MS is a high-throughput technology based on comparison between the protein fingerprint obtained by microbial cells and the database of reference spectra by various algorithms integrated into systems (Figure 12.7). This tool has been increasingly studied and applied for the identification and typing of microorganisms (Fenselau & Demirev 2001; Lavigne et al. 2013; Welker & Moore 2011; Wieser et al. 2012).

The high sensitivity of MALDI-TOF MS makes it a fascinating method for the characterization and identification of a vast number of microbial isolates. Previous studies have demonstrated that it is a powerful, fast, reliable, and cost-effective method for the identification of LAB (de Bruyne et al. 2011; Doan et al. 2012; Tanigawa et al. 2010) which offers opportunities to identify the different members of fermented vegetable microbiota. Table 12.3 summarizes the strengths and weaknesses of MALDI-TOF MS compared to other systems.

Procedure and Interpretation

Mass analysis can be run indirectly, by enrichment steps to increase the recovery of injured cells and obtain pure cultures for subsequent analysis, or directly from single colonies. Generally, there are three possible approaches for cell disruption. Samples can be prepared using either the direct smear method, on target extraction, or an acetonitrile/formic acid extraction after an ethanol purification step. With the direct smear method, a single colony is picked and deposited on the target plate as a thin layer. Then

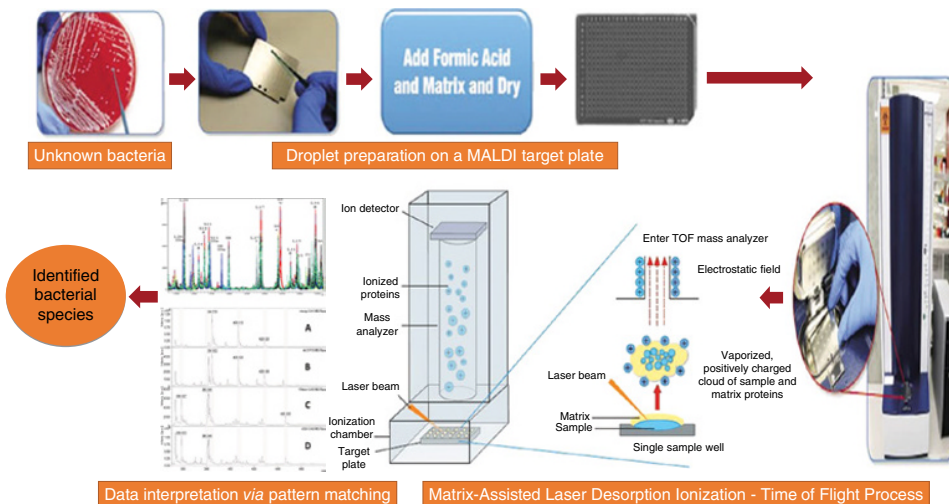


Figure 12.7 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) workflow for bacterial identification. Source: Theel (2013). Used with Permission of Mayo Foundation for Medical Education and Research. All Rights Reserved.

the sample is ready to dry and be covered with the matrix. After that, MALDI-TOF analysis can be done (Pavlovic et al. 2013; Theel 2013; Tonolla et al. 2010).

The target plate is inserted in the MALDI-TOF MS instrument. By comparing the spectra obtained with the reference database, the isolate can be identified. MALDI-TOF MS results based on the log(score) values calculated by the BioTyper software were compared to the 16S rRNA gene sequence similarity search results. Based on the calculated values, the identification level can be determined as follows.

- Score value >2 = the species can be identified
- Score value (1.7–2.0) = the genus can be identified
- Score value <1.7 = identification of the isolate is not possible.

The identification accuracy of strains is related strongly to the quality of sample (e.g., sample purity) and the number of reference spectra (Pavlovic et al. 2013; Seng et al. 2009; Theel 2013; Tonolla et al. 2010).

Table 12.3 Comparison between MALDI-TOF MS and other identification methods.

Method	Advantages	Disadvantages
MALDI-TOF MS	<p><i>1 Quick and simple</i> Starting from bacterial colonies, the result can be obtained within a few minutes Easy sample preparation High throughput</p> <p><i>2 Cost-effective</i> Cost of MALDI-TOF apparatus comparable to cost of medium-size sequencer, but running costs (consumables) are much less</p> <p><i>3 Reliable identification</i> Down to species or in individual cases even to subspecies and strain level Database-related problems can be overcome by including an increasing number of species or by providing, within a particular study, reference samples for comparison</p>	<p><i>1 Cultivation dependent</i> Colonies/growth must be obtained in order to perform analysis</p> <p><i>2 Fingerprinting-based method</i> Works only in comparison with a reference</p> <p><i>3 Identification limited by database</i> Most of the bacteria currently included in the database are clinically relevant species Environmental species are underrepresented</p>
Biochemical profiling	<p>Numerical taxonomy Automation possible Several commercial systems available Easy standardized operations</p>	<p><i>4 Cultivation-dependent identification limited by database</i> Database well developed for bacteria, but not for other kingdoms Most of the bacteria currently included in the database are clinically relevant species Environmental species are underrepresented</p>

Source: Tonolla et al. (2010).

Applications of MALDI-TOF MS for Fermented Fruits and Vegetables

Nguyen et al. (2013) provided an extensive and detailed description of the LAB diversity associated with assorted Vietnamese fermented vegetables using a polyphasic approach combining MALDI-TOF MS fingerprinting and sequence analysis of the phenylalanyl-tRNA synthase (*pheS*) gene. This was complemented with diversity data obtained through utilization of the DGGE culture-independent technique.

The growth of microbial contamination in fermented drinks can cause turbidity, haze, and off-flavors, often rendering the product undrinkable. MALDI-TOF MS, based on the generation of peptide mass fingerprint “distinctive protein peak patterns,” is a rapid, reliable, and powerful tool for identification of the bacterial spoilage encountered in drinks. *L. brevis*, *Ped. claussenii*, and *Ln. mesenteroides* were used to optimize sample preparation and MALDI-TOF MS settings. Different sample preparation methods were investigated, ranging from traditional cell smears to more elaborate extraction procedures including mechanical and enzymatic disruption of cells. Culturing time, availability of oxygen, and nutrient factors were studied on the acquired protein peak patterns (Kern et al. 2013).

Kern et al. (2013) underlined the capability, robustness, and convenience of MALDI-TOF MS as a tool for the rapid identification of unknown bacterial samples in food microbiology.

12.5 Future Applications

Fermented dairy products are considered the main reservoir of LAB and recently, fermented fruits and vegetables have demonstrated their suitability as novel non-dairy probiotic foods. Gene sequencing technology and relative genomics will play a role in the rapid identification of LAB strains and allow for increased study of the mechanisms and functionality of LAB as probiotics.

12.6 Conclusions

Many different fruit and vegetable fermentation processes are currently carried out on an industrial scale, most of which still rely on the autochthonous microbiota of the raw materials and fermentation plant. The implication of LAB in such processes has been deciphered by classic (e.g., microbiological) techniques in most cases. DNA-based culture-dependent (ribotyping, RAPD, PFGE) and culture-independent (DGGE, T-RFLP) applications may provide new insights into the microbial processes which take place during fermentation, as well as microbial diversity, including LAB. Furthermore, these data can be linked to other issues such as sensory properties (e.g., flavor development) or regional differences in fermented foods. DNA-based approaches can also help to evaluate the fitness of starter cultures used for fruit and vegetable fermentations.

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13

New Trends in Molecular Techniques to Identify Microorganisms in Dairy Products

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13.1 Introduction

Analyzing microorganisms in dairy products constitutes one of the main challenges for the food industry. Microorganisms in dairy products can play different roles (Quigley et al. 2013). From a safety point of view, the presence of pathogenic microorganisms in dairy products (e.g., *Listeria*, *Salmonella*, *Escherichia coli*, *Campylobacter*, and mycotoxin-producing fungi) is an immense risk to public health. Similarly, some contaminant microorganisms (e.g., *Pseudomonas*, *Clostridium*, *Bacillus*, and other spore-forming or thermophilic microorganisms) clearly influence the commercialization of these products. In contrast, other microorganisms facilitate dairy fermentations (e.g., *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Propionibacterium*, and fungal populations). Indeed, there is an increasing demand for “healthy” microorganisms (e.g., lactobacilli and bifidobacteria) in dairy products but it is necessary to control the amount of them after processing and subsequent storage.

Traditionally, the occurrence of microorganisms in a given environment or in an industrial process has been studied by culture-based methods (Justé et al. 2008) and the choice of a reference microorganism on which to carry out the studies has been necessary to establish the optimum thermal and non-conventional treatment conditions in a new product. In this context, *Pseudomonas fluorescens* has been used as it is a Gram-negative organism and the most important psychrotrophic bacterium, responsible for the deterioration of refrigerated milk (Villamiel & de Jong 2000). The most common treatment used to inactivate *Pseudomonas* in milk is commercial pasteurization (72–80°C for 10–21 seconds) (Wouters & Smelt 1997). Some authors

have investigated the inactivation of *P. fluorescens* in different substrates using non-thermal technologies (Gervilla et al. 1997; Villamiel & de Jong 2000).

However, traditional culture-based methods cannot characterize (minor) populations of microorganisms for which selective enrichment is needed before their enumeration. In addition, there is no good method to grow stressed and weakened cells which require specific culture conditions (Justé et al. 2008). Moreover, non-culturable cells are not detected with conventional methods and the percentage of active microbial community that can be cultured in vitro is low. This had led both food researchers and the food industry to investigate new techniques that can accelerate the identification and quantification of microbial species. This chapter critically describes some of the most common culture-independent techniques that are currently being used for these purposes.

13.2 Polymerase Chain Reaction (PCR)-based Methods

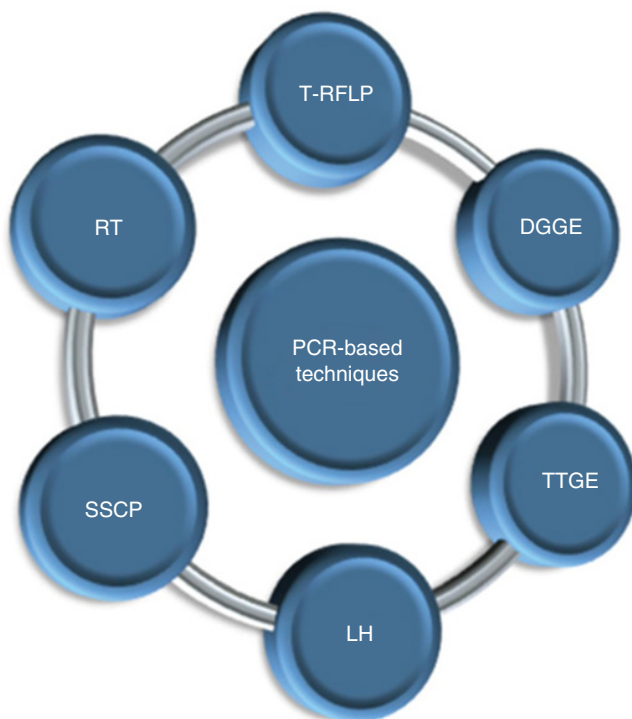
Over recent decades, PCR has become a fundamental aspect of molecular biology, and many molecular methods to study microbial communities have been based on this technique. In PCR, DNA serves as a template for PCR amplification of genetic targets with universal, genus- or species-specific primers to amplify target sequences of a given population. It should be noted that although fundamental PCR is based on three major steps (denaturation of DNA at 94–95 °C, annealing of the nucleotide primers at 37–70 °C, and polymerization (elongation) of the new DNA strand from nucleotides at 60–72 °C) (Kuchta et al. 2006), PCR conditions must be optimized as a function of microbial community and food matrix (Justé et al. 2008; Trmcic et al. 2008).

To effectively apply PCR-based methodologies in analyzing microbial community in dairy products, two important steps need to be considered: the extraction and purification of microbial DNA from the food matrix (Bonaiti et al. 2006; Rantsiou et al. 2008). In this context, insufficient homogenization of the dairy product, incomplete cell lysis that prevents the release of nucleic acid, and materials that inhibit PCR amplification can all cause difficulties in the correct identification of certain microbes in the samples (Bonaiti et al. 2006; Jany & Barbier 2008; Justé et al. 2008).

In addition, the selection of a gene or genetic marker that can be used for amplification in PCR constitutes one of the important steps for identification and differentiation of a wide variety of microbial communities in dairy products. The most common molecular marker used for these purposes is the bacterial ribosomal RNA operon, encompassing 16S rRNA and 23S rRNA genes (Justé et al. 2008). There are different PCR-based techniques for the identification and quantification of microbial communities in dairy products. Some of the most important assays are described below and listed in Figure 13.1.

For instance, PCR-denaturing gradient gel electrophoresis (DGGE) and PCR-temporal temperature gradient electrophoresis (TTGE) have been used to investigate microbial growth in dairy products. These methodologies are based on the direct extraction of genomic DNA and/or RNA from the sample, followed by amplification of the variable regions of the 16S gene. When the total DNA of the microbiota is used as a template in PCR amplification, the results reflect the complete genetic profile of the

Figure 13.1 Main PCR-based techniques used for identification and quantification of microbial growth in dairy products. DGGE, denaturing gradient gel electrophoresis; LH, length heterogeneity; RT, reverse transcription; SSCP, single-strand conformation polymorphism; T-RFLP, terminal restriction fragment length polymorphism; TTGE, temporal temperature gradient electrophoresis.



community diversity, including both live and dead cells. In contrast, when the total RNA is used, the results reflect the profile of metabolically active microbiota (Florez & Mayo 2006; Rantsiou et al. 2008).

13.2.1 DGGE/TTGE

Denaturing gradient gel electrophoresis/TTGE are based on the electrophoretic separation of PCR amplicons of equal length in a sequence-specific manner, using a polyacrylamide gel containing a denaturing gradient of urea and formamide (Justé et al. 2008; Trmcic et al. 2008). The basic steps of DGGE/TTGE are:

- extraction of total community DNA from the sample
- PCR amplification using specific oligonucleotide primers
- separation of the amplicons using DGGE/TTGE (Abriouel et al. 2008; Randazzo et al. 2009).

The principle that governs these techniques is that DNA fragments of the same length but with different sequences are separated, based on decreased electrophoretic mobility of partially melted double-stranded DNA molecules. The temperature used for PCR-DGGE electrophoresis is constant and generally between 55°C and 65°C, whereas for PCR-TTGE, the temperature varies over the time constituting the denaturation gradient (Ercolini 2004; Jany & Barbier 2008). These two PCR techniques are most often used for the comparison of complex microbial composition ecology and the investigation of their dynamics (Ogier et al. 2004; Parayre et al. 2007).

Different authors have evaluated the potential of PCR-DGGE/TTGE to identify and quantify microbial communities in dairy ecosystems (Abriouel et al. 2008; Arcuri et al. 2013; Bonetta et al. 2008; Dolci et al. 2008; El-Baradei et al. 2007; Ercolini et al. 2003; Licitra et al. 2007; Randazzo et al. 2006; Rantsiou et al. 2008; Temmerman et al. 2003). In this context, Ercolini et al. (2003) and Aponte et al. (2008) found that representatives of *Leuconostoc* community were identified in Stilton and Provolone del Monaco cheeses only by amplification of the V4–V5 and V6–V8 regions of the 16S rRNA gene, respectively, while targeting the V3 region failed. However, DGGE does not allow the individual detection of species in a mixed microbial community and/or species that constitute a low percentage (e.g., $<10^4$ CFU/g) of the population (Cocolin et al. 2007; Muyzer et al. 1993). This problem can be overcome when other primers are used in the PCR amplification step (Rantsiou et al. 2008).

Depending on the species and sometimes on a specific strain, the limit of detection in DGGE analysis may be variable. However, the use of specific primers, instead of universal bacterial ones, can minimize the sensitivity issue of the culture-independent PCR-DGGE method, and allows the detection of minor bacterial communities. The presence of either low DNA concentration or high competing DNA concentration may change the limit of detection by TTGE. Moreover, competition between the targeted DNA and the dominant species, for PCR primers, represents another factor limiting TTGE sensitivity. Nevertheless, minority species representing 1:100 or less of the total DNA concentration have been detected by TTGE, representing a very low threshold of detection (Ogier et al. 2004).

13.2.2 Reverse-transcription RNA

Reverse transcription RNA (RT-RNA) has been used by some authors to analyze the active microbiota of artisanal cheeses. Differentiation between the metabolically active (RNA-derived) and the total DNA-derived microbiota was successfully conducted by the combination of RT-PCR-DGGE and PCR-DGGE (Florez & Mayo 2006; Jany & Barbier 2008; Randazzo et al. 2009; Rantsiou et al. 2008). During ripening of artisanal Sicilian cheese, differences in metabolically active microbiota were identified by comparing the RNA-derived and DNA-derived DGGE profiles (Randazzo et al. 2006). Since different microbiota may be present during cheese ripening, RT-PCR-DGGE is therefore a promising and useful approach to study their microbial growth.

The detection principle is based on the low stability of RNA, being degraded in non-viable microorganisms, and the high stability of DNA in the cheese matrix even after cell death. Indeed, it is well known that RNA-based assays are more sensitive than DNA-based assays (Justé et al. 2008). In fact, a weakness has been observed when performing the PCR-TTGE DNA-based approach on Ragusano cheese from curd stretching at the 7th month of ripening, showing no changes in the microbiota profile (Licitra et al. 2007). The authors reported this observation as being due to the predominance of dead cell DNA, entering into competition for primers in PCR with minor growth of microbiota during ripening, and thus masking their presence. The consequence of this phenomenon is the non-amplification of the minor, new microorganisms in the population. The solution to overcome this problem would be the application of a RNA-based approach, due to the faster RNA degradation following cell death and the faster rate of accumulation for new cells than with DNA (Licitra et al. 2007).

13.2.3 Single-strand Conformation Polymorphism-PCR

Another PCR-based methodology with potential for identification and quantification of microbial growth in dairy products is single-strand conformation polymorphism-PCR (SSCP-PCR). This technique is based on the use of an acrylamide gel-based or a capillary-based automated sequencer for the separation of denatured (single-stranded) PCR products. Electrophoretic mobility in non-denaturing gels could show changes due to their folding into tertiary structures of single-stranded DNA according to their nucleotide sequences and their physicochemical environment (Jany & Barbier 2008). The SSCP approach uses universal primers and, like other culture-independent molecular methods, can be applied to dairy products without having information on the microbiota present in the sample (Duthoit et al. 2005). Sequence-dependent single-strand DNA of the PCR products could be obtained by heat denaturation. On the basis of their sequence differences, similar size fragments could be separated and detected using a genetic analyzer.

By using primers targeting different variable regions of the 16S rRNA, separation of numerous sequences from different species could be improved (Duthoit et al. 2005; Feurer et al. 2004). For instance, Callon et al. (2006) determined the diversity and succession of yeast populations in three different Salers cheeses using phenotypic diagnoses and SSCP. For SSCP analysis, they designed various yeast-specific primers for amplifying the V4 region of the 18S rRNA gene. *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Candida zeylanoides*, and *Debaryomyces hansenii* were reported as the most frequently encountered yeast species in the studied samples. The efficiency of detection of other less common species such as *Candida parapsilosis*, *C. silvae*, *C. intermedia*, *C. rugosa*, *Saccharomyces unisporus*, and *Pichia guilliermondii* was better using conventional methods. In another study, SSCP-PCR was performed to determine the microbiota of Salers cheese made from raw milk by amplifying the V2 and V3 regions of the 16S rRNA gene (Duthoit et al. 2005). Considerable diversity in microbial dynamics and sensory characteristics of chesses was reported in this study.

Saubusse et al. (2007) studied the feasibility of using SSCP to compare the bacterial communities of Saint-Nectaire cheeses with and without *L. monocytogenes* growth. They showed that SSCP is an effective method for differentiating between the bacterial communities of various cheeses prepared with similar technologies. After amplification of the V2 region of the 16S rRNA gene, all the studied cheeses with the lowest *L. monocytogenes* counts were determined on day 8 by the dominance in their SSCP profiles. It should be noted that although the SSCP-PCR approach is efficient at separating certain sequences, some species may be difficult to separate and overlap in the same SSCP peaks, and the microbial diversity may therefore be underestimated (Saubusse et al. 2007; Verdier-Metz et al. 2009).

13.2.4 Real-time PCR

Real-time PCR has been used to identify and quantify microbial communities in dairy products (Furet et al. 2004; Grattepanche et al. 2005; Justé et al. 2008; Mathys et al. 2008). This methodology is used to monitor the amplification of target microbial DNA in real time (Mohania et al. 2008; Zago et al. 2009). For this purpose, a fluorescent probe

is used to monitor the amplification of a target sequence. Two methods have been reported as the most commonly used for detection: DNA-binding fluorescent molecules (i.e., SYBR Green), and reporter-quencher system (i.e., TaqMan® probes). On the other hand, to quantify the gene copy numbers, a calibration curve using DNA as standard must be performed (Carey et al. 2007).

The main advantage of real-time PCR compared to other PCR-based techniques is that this assay avoids the need for post-PCR processing such as gel (agarose, polyacrylamide) or capillary electrophoresis, as in DGGE, TTGE, SSCP, and RFLP. For instance, real-time PCR has been used to identify and quantify microbial growth in different dairy products. In this context, *Streptococcus thermophilus* was quantified in plain yogurt and yogurt containing fruits using real-time PCR and the results were compared to those found after conventional plate count (Ongol et al. 2009). The authors found a significant positive relationship between the two methods. Moreover, other authors also demonstrated the feasibility of real-time PCR to quantify *Lactococcus lactis* subsp. *cremoris* ATCC 19257 strain from milk fermented by mixed cultures, and obtained a detection limit of 200 CFU/mL (Grattepanche et al. 2005). In addition, the absolute specificity of a RT-PCR assay to evaluate the presence and abundance of *Enterococcus gilvus* in Italian artisan and industrial cheeses has been demonstrated (Zago et al. 2009). Likewise, a method has been developed for extraction of RNA from cheese without culturing, and optimized real-time reverse transcription PCR for *Lactococcus lactis* (Monnet et al. 2008).

13.2.5 Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism (T-RFLP) is another PCR-based technique, which is based on the restriction endonuclease digestion of fluorescently end-labeled PCR products, most often the 16S rRNA gene, and allows fingerprinting of a microbial community. This assay has the potential to evaluate microbial growth in natural habitats or to determine how environmental and/or processing conditions can change the structure and dynamics of microbial communities. The main steps of T-RFLP analysis are listed in Figure 13.2.

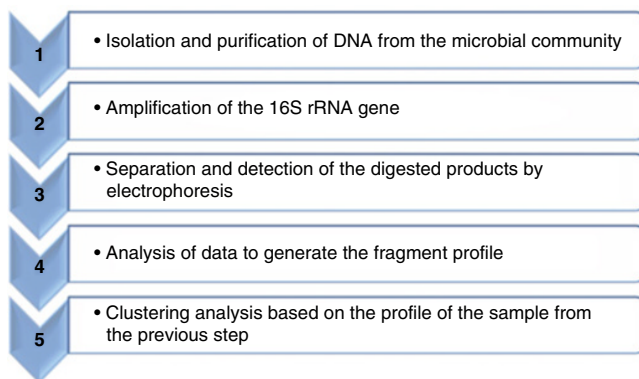


Figure 13.2 Steps of a T-RFLP analysis.

Several studies have evaluated the potential of this technique to study the bacterial flora of cheeses (Gouda-type and Maasdam) (Rademaker et al. 2005) and yogurt (Rademaker et al. 2006). It was found that this technique allowed the characterization of bacterial populations of Tilsit-type cheeses during the ripening period. Moreover, it was able to identify and quantify bacterial populations in yogurt production. The authors concluded that T-RFLP might be an important tool to consider when developing rapid semi-quantitative analyses of simple microbial ecosystems (i.e., simple dairy starter cultures).

However, T-RFLP can overestimate the number of species present in samples of unknown composition, such as complex ecosystems with high microbial diversity (Sanchez et al. 2006). In this context, the terminal restriction fragments (TRFs) technique has been used to identify individual strains of defined smear starters. During the 8-week ripening period of three Tilsit-type cheeses, the bacterial flora was studied by analyzing TRFs using *Hae* III and *Cfo* I as restriction enzymes. Results show that the maximum level of starter strains was reached after 2–4 weeks, with a total absence at the 8th week, except the *Corynebacterium* species, the most abundant genus on the surface of the fully ripened cheese (Rademaker et al. 2005). Other studies using T-RFLP have also been reported in the literature, such as those describing the analysis of hard cheeses (Gouda-type and Maasdam) and yogurt (Rademaker et al. 2006).

13.2.6 Length Heterogeneity PCR

Length heterogeneity PCR (LH-PCR) also has the potential to be used in the identification and quantification of microbial growth in dairy products. This method is similar to T-RFLP. However, LH-PCR analysis distinguishes different organisms based on natural variations in the length of the 16S rRNA sequences, while T-RFLP identifies PCR fragment length variations based on restriction site variability.

The quantification of the relative proportion of each fragment is performed by measuring the area of each peak corresponding to a fragment size, which is obtained by the conversion of fluorescence data into electropherograms. Using LH-PCR, results can be obtained in a relatively short time, around 30–40 minutes. However, care has to be taken when manipulating LH-PCR, which could present particular biases, like most PCR-based techniques. For example, increasing the number of PCR cycles can increase the incidence of chimeric PCR products.

Lactobacillus species are among the most important microorganisms in dairy products. Their identification has been relatively difficult and their biotechnological potential has been studied using LH-PCR (Martín-Platero et al. 2009). The authors reported that in Quesaila Arochena cheese, the most common *Lactobacillus* species identified using LH-PCR were *L. plantarum*, *L. paraplantarum*, and *L. curvatus*, whereas in Torta Arochena cheese, the most frequent species were *L. plantarum* and *L. curvatus*/*L. coryniformis*. Experiments performed using TTGE were not able to distinguish *L. curvatus* and *L. paracasei* in Torta Arochena, whereas when using LH-PCR, a low number of *L. paracasei* was detected (Martín-Platero et al. 2009). In another study, the dominant LAB species were identified using LH-PCR in whey starter cultures for Grana Padano cheese (Lazzi et al. 2004). This approach generated population fingerprints and allowed the evaluation of major microbial differences among several starters. Dominant

species found in Grana Padano cheese whey starter using LH-PCR were *L. helveticus*, *L. delbrueckii* ssp. *lactis/bulgaricus*, and *Streptococcus thermophilus* (Berrougui 2007).

Microbial dynamics of lyzed cells in fermented Parmigiano-Reggiano cheese during production and during 24 months of ripening were studied using LH-PCR (Gatti et al. 2008). The authors reported better understanding of microbial ecology and discussed the potential of recovered DNA as an indicator of complex microbial dynamics during subsequent stages of cheese making.

13.3 Fluorescent *In Situ* Hybridization

Another culture-independent molecular method, which can identify and inform about the distribution of microbial populations in dairy products, is fluorescent *in situ* hybridization (FISH). The potential of this assay to identify bacteria *in situ* without the need for isolation has been demonstrated for dairy products (Cocolin et al. 2007). FISH is a “non-PCR-based” molecular technique that allows the identification of microbial colonies present in a food product (e.g., cheese). FISH is based on the fluorescent labeling of a 16S rRNA bacterial domain probe that specifically hybridizes the target complementary sequence of intact cells. According to the target region of 16S rRNA, genus- and species-specific sequences and their taxonomy could be identified using designed oligonucleotide probes (Ercolini et al. 2003; Moter & Gobel 2000). The main steps of FISH analysis are listed in Figure 13.3 (Amann et al. 2001; Giraffa & Neviani 2001).

The potential of FISH to study bacterial community structure and location in Stilton cheese has been evaluated (Ercolini et al. 2003). For this purpose, fluorescently labeled oligonucleotide probes were developed to detect *Lactococcus lactis*, *L. plantarum*, and *Leuconostoc pseudomesenteroides*. The authors established the spatial distribution of several microbial species in the Stilton cheese matrix when they combined the fluorescently labeled oligonucleotide probes and the bacterial probe Eub338. Similarly, the microorganism population of Feta cheese using FISH with probes specific for eubacteria, *Streptococcus thermophilus*, *Lactococcus* spp., and *L. plantarum* was also investigated (Rantsiou et al. 2008).

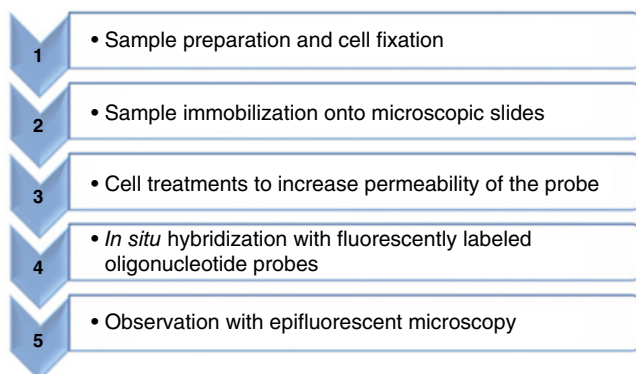


Figure 13.3 Main steps of FISH analysis for bacterial *in situ* identification.

13.4 Immuno-based Methodologies, Biochips, and Nanosensors

In the last 20 years, biochip-based systems have emerged as a potential tool for fast analysis of microbial contaminants in dairy products. For instance, a biochip assay has been developed based on DNA amplification of genes capable of detecting seven common species of mastitis-causing pathogens: *Corynebacterium bovis*, *Mycoplasma bovis*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *S. bovis*, *S. dysgalactiae*, and *S. uberis* (Lee et al. 2008). The authors demonstrated the ability of this biochip to detect these pathogens in bovine milk within 6 hours, with a limit of 10^3 CFU/mL.

Powdered infant formula (PIF) is a non-sterile product and may contain pathogenic bacteria (Agostoni et al. 2004). Due to its richness in nutritional compounds, it constitutes an excellent medium for bacterial proliferation. The detection of these pathogens should therefore be addressed. Ten pathogenic microbial strains (*E. sakazakii*, *Salmonella* spp., *Klebsiella pneumoniae*, *K. oxytoca*, *Serratia marcescens*, *Acinetobacter baumannii*, *Bacillus cereus*, *L. monocytogenes*, *Staph. aureus*, and *E. coli* O157) were identified in PIF based on the *wzy* (O antigen polymerase) gene and the 16S–23S rRNA gene internal transcribed spacer (ITS) sequences, using a DNA biochip (Wang et al. 2009). The authors reported high sensitivity and specificity against these highly restricted pathogens in PIF by regulatory standards, with 100% accuracy. The risk of potential infections by milkborne pathogens has thus been greatly reduced by the use of biochips as rapid and inexpensive tools for their detection with high accuracy.

Staphylococcus aureus is a serious milkborne pathogen that can be transmitted in different ways (e.g., cows suffering from mastitis, non-hygienic handling conditions) (Fagundes et al. 2010). For epidemiological investigations, rapid detection of *S. aureus* in milk is crucial due to its health-related issues caused by secreted toxins (de Oliveira et al. 2011). A system using *Staph. aureus* 16S rRNA-specific oligonucleotide probes was successfully applied to milk (He et al. 2010). The authors reported the sensitive and specific detection of *Staph. aureus* at 10^3 CFU/mL of the sample.

Other milkborne pathogens (e.g., *Yersinia pestis* and *B. anthracis*) have been specifically detected using DNA biochips, in experimentally inoculated milk samples with amounts as low as 1 ng (Goji et al. 2012). In a similar study, DNA biochips were used to detect the presence of *L. monocytogenes* after 24 hours in a modified *Listeria* enrichment broth at 37°C (Bang et al. 2013). A detection limit of ~ 8 log CFU/mL was obtained, and the authors reported the sensitivity and specificity of this method to identify *L. monocytogenes* among other *Listeria* spp. and pathogen species in laboratory media and milk.

In the same context, *L. monocytogenes* and enterococci have been used for a spore germination-based assay and specific detection in milk using a micro-well chip. To detect the presence of an analyte, the fluorescence is measured using an electron-multiplying charge-coupled device (EMCCD) following the specific action of active bio-sensing molecules on fluorogenic substrate. Based on this system, targeted enterococci with 5.66 log cells have been detected (Kumar et al. 2012).

Recently, a fast diagnostic assay has been developed to specifically detect *Brucella* IgG antibodies in milk samples, which involved the use of fluorescent, micellar silica nanosensors (Vyas et al. 2015). The authors demonstrated the ability of this technique to identify *B. abortus* antibodies through capture by fluorescent silica nanosensors

using spiked and raw milk samples validated by ELISA and PCR. The results obtained were promising as no sample processing procedures (e.g., isolation and separation) were required. The method was also highly specific and sensitive, with a short time (10 minutes) needed for recognition of the antigen.

These results pave the way for wide implementation of this method in the farming and food industries, as it could be applied as a rapid technique to detect pathogens in suspected bovine samples, as well as various disease-causing pathogenic strains based on antigenic components and surface biomarkers.

13.5 Benefits and Limitations of Molecular Techniques

Among the various benefits, molecular techniques can screen samples in a relatively short amount of time, are typically sensitive and specific, and can provide accurate and fast diagnosis. These techniques also have the potential to be automated. However, molecular techniques have important limitations. For example, not all pathogens can be easily detected. At this stage of development, only a few detection assays are commercially available for field testing of farm animals and milk samples. Moreover, these methodologies are expensive to develop and standardize. However, nanosensing techniques may allow precise detection of antibodies with low sample volumes.

13.6 Conclusions and Future Perspectives

The availability of molecular techniques opens the door to a new era of detection of microorganisms in dairy products. The increasing number of methods will help the food industry to optimize processing conditions, thus preserving both food safety and healthy aspects of dairy products. RNA-based RT-PCR could provide valuable data about the quantitative and metabolic status of the target. However, as RNA is less stable than DNA, an efficient and reproducible extraction of RNA still remains a challenge. Nevertheless, complete microbiota identification using culture-independent molecular approaches still presents bottlenecks, and more efforts are required to overcome the detection problems of these approaches and facilitate their use.

There are two innovative fields dealing with the study of gene expression and protein translation in natural environments that are particularly promising for the investigation of bacterial function. In addition to these fields, high-throughput parallel sequencing, metagenomics, and metatranscriptomics represent promising tools for deeper microbial diversity investigation. Gene profiles obtained by the combination of 16S rRNA and functional genes could create a relationship of structure/function between the microbiota and the ecosystem. Such structure–function studies will be interesting to investigate complex microbial communities.

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14

Molecular Techniques for the Detection and Identification of Yeasts in Wine

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14.1 Introduction

14.1.1 Natural Yeast Associated with Grapes, Must, and Wines

There is a wide range of microorganisms present during fermentation, involving sequential development. In general, when no starter cultures are used, non-*Saccharomyces* yeasts are the first group dominating the fermentation, followed by *Saccharomyces* yeasts that normally complete the process (Combina et al. 2005; Egli et al. 1998; Fugelsang 1997; Mora et al. 1990). Some of the most common wild yeasts reported on grapes are *Hanseniaspora uvarum* (and its anamorphic form *Kloeckera apiculata*), which represents 50–70% of the initial yeast flora on grapes (Amerine & Kunkee 1968; Fleet & Heard 1993; Jackson 2008), and those of the genera *Candida*, *Pichia*, *Rhodotorula*, *Kluyveromyces*, and *Hansenula* (Fleet & Heard 1993). However, the number of species and their presence during fermentation depend on the temperature, rainfall, altitude of the production area, pest control agents used in the vineyard (Amerine & Kunkee 1968), winemaking process (Cuinier 1978), and type of wine produced (Poulard 1984).

Saccharomyces cerevisiae is the principal yeast involved in alcoholic fermentation. It is usually absent or is rarely present on grapes but is instead associated with the winery environment and is incorporated into the must during fermentation through the natural handling of the vessels (Constantí et al. 1997; Frezier & Dubourdiou 1992; Longo et al. 1992; Vaughan-Martini & Martini 1995). Non-*Saccharomyces* yeasts grow well during the early stages of fermentation, when the ethanol concentration is still low, being later replaced by *Saccharomyces*, which is more tolerant to ethanol.

We found that differences in yeast diversity were often dependent on grape variety. This phenomenon can be attributed to several factors, including the different stages of berry ripening at harvest, physical damage to the grape surface, and pest management practices (Raspor et al. 2006). Although we studied different grape varieties grown in the same area and processed at the same winery, microclimatic conditions and viticultural practices may have influenced the yeast diversity we detected.

Most of the yeasts isolated from the vineyard air were also present in the grape juice at the beginning of fermentation. All the yeasts identified in the cellar were also found

later in the fermenting must. *Rhodotorula mucilaginosa* was found in air samples from both the vineyard and the cellar, and on the grape surface, but not on the tank surface. During 2008, *Zygosaccharomyces florentinus* was the only species found in all environmental samples (air and contact samples, from both the vineyard and the cellar).

The viable counts of the environmental samples showed the presence of only non-*Saccharomyces* species. Although *S. cerevisiae* and related species such as *S. bayanus* are predominantly responsible for fermentation, they represent only a small fraction of the diversity we identified, which is consistent with other reports showing that *S. cerevisiae* is rarely isolated from natural sources such as berry and leaf surfaces when using viable count methods (de La Torre et al. 1999; Martini 1993; Pretorius 2000; Vaughan-Martini & Martini 1995). The small number of species isolated from the cellar environment (air and tank surface) during 2009 compared to 2008 may have been caused by the sanitary conditions adopted by the winery after the sampling results in 2008.

The dynamic behavior of the yeast populations through the different stages of fermentation in steel tanks also differed among grape varieties. The detection of some yeast species only during the later stages of fermentation probably reflects their proliferation to cell numbers above the detection threshold of our assay, rather than their genuine absence at the beginning of fermentation. The relatively greater diversity of yeast species in red compared to white wines is consistent with the higher pH of red wines, providing favorable conditions for yeast growth (Deák & Beuchat 1993). In white wines, yeasts isolated from the grape skin were not found in the must, probably because they remained in the skin fraction during clarification, and this may also have contributed to the lower species diversity we observed.

The higher yeast diversity during the early stages of fermentation predominantly reflects the low ethanol tolerance of non-*Saccharomyces* species (Combina et al. 2005; di Maro et al. 2007; Egli et al. 1998; Fleet 2003; Satora & Tuszynski 2005; Torija et al. 2001). Nevertheless, we found that non-*Saccharomyces* yeasts such as *Pichia kluyveri*, *P. membrifaciens*, *R. mucilaginosa*, and *Metschnikowia pulcherrima* were active in the late fermentation stages in some must varieties. This is consistent with previous reports of ethanol tolerance in *M. pulcherrima* (di Maro et al. 2007; Querol et al. 1990; Torija et al. 2001), but *R. mucilaginosa* is usually found during the early stages of fermentation, and its presence along with the *Pichia* species later in the fermentation could add complexity but also reduce wine quality (Deák & Beuchat 1993; Loureiro & Malfeito-Ferreira 2003).

Considering the results from the 2008 and 2009 harvests together, we observed that the generally higher yeast diversity in the must at the beginning of fermentation was coincident with the rapid onset of the exponential growth phase. We evaluated the interrelation between the yeast species and the success of fermentation. We found that despite the diversity of yeasts in red and white varieties, white musts generally contained higher residual sugar levels than red musts and that sluggish fermentation was more likely. Such fermentations were characterized by the initial predominance of *Candida zemplinina* and *S. bayanus*, as well as lower levels of *M. pulcherrima* and *S. cerevisiae*, contrasting with the red wine musts. The impact of these properties on fermentation reflects the better performance of *S. cerevisiae* compared with the lower fructose uptake capacity of *S. bayanus* (Magyar & Tóth 2011), which is consistent with our results.

14.1.2 Importance of Monitoring Yeast During Fermentation

In wine making, yeasts play a pivotal role in the characteristics of the final product, being the predominant microorganism in the biochemical interaction with components of the must. Wine aroma is a unique and complex matrix of primary aromas given by the geography, geology (soil), and climatic conditions where the grapes are grown, which together are designated the “terroir” (Grifoni et al. 2006; Pagay & Cheng 2010; van Leeuwen et al. 2004), secondary aroma formed during the fermentation process, and tertiary aromas given by the aging and postfermentative process. The aroma complexity of a wine increases during alcoholic fermentation as a product of the synthesis of volatile compounds by wine yeast and the release of varietal aroma precursors (Swiegers et al. 2005).

Apart from the well-known *S. cerevisiae*, it is now recognized that non-*Saccharomyces* species contribute to enzymatic reactions occurring in the must during the early stages of vinification, enhancing the production of some volatiles (Heard & Fleet 1986). Non-*Saccharomyces* yeasts contribute to the aroma compound formation and are thought to result from the release of certain enzymes, such as pectinases (*Candida*, *Cryptococcus*, *Kluyveromyces*, and *Rhodotorula*), glucosidases, especially beta-glucosidase (*Candida*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, and *Zygosaccharomyces*), and esterases, among others (Esteve-Zarzoso et al. 1998).

Sometimes secondary aromas can be produced due to diverse causes such as failed or stuck fermentations, premature bottling, the presence of spoilage yeast, poor sanitary conditions, etc., producing undesirable aromas. Some non-*Saccharomyces* yeasts are considered spoilage yeasts due to the elevated amounts of these aromas, for example, *Hanseniaspora uvarum*, which is considered a spoilage yeast able to produce up to 2 g/L of acetic acid during fermentation, *Brettanomyces/Dekkera* spp. producing 4-ethylphenol, and other species as *Pichia* and *Candida* (Loureiro & Malfeito-Ferreira 2003). The most important spoilage metabolites produced by non-*Saccharomyces* wine yeasts are acetic acid, acetaldehyde, and ethyl acetate (Chatonnet et al. 1995).

In wine, it is difficult to determine the sensory influence of the individual compounds due to the existence of complex mixtures and interactions. Certain volatile compounds disappear, others remain unchanged, and others appear with the yeast metabolism. Indigenous non-*Saccharomyces* yeasts may have a significant and favorable effect on flavor development. The synergistic interaction among the different yeasts and their effects on sensory properties are yet to be fully investigated.

14.1.3 Traditional Enumeration and Isolation of Wine Yeast

Growth in nutrient media is the traditional method for isolation, enumeration, and detection of yeasts. Media should inhibit the growth of bacteria and molds and be nutritionally adequate to support all yeast species, including fastidious types. Although media with all these characteristics do not exist, several media have been formulated that satisfy general-purpose use, and several others have been devised to select specific groups or types of yeasts. Mycological media are also used in isolation and identification of yeasts (Deák 2008).

The procedure for the detection and enumeration of yeasts from food usually involves a number of steps after the preparation of media and equipment, such as obtaining and

Box 14.1 Media for the detection, enumeration, and isolation of yeasts from foods.**General, basal media**

Malt extract agar (MEA)
Sabouraud glucose agar (SGA)
Potato dextrose agar (PDA)
Tryptone glucose yeast extract agar (TGY)

Selective media for fungi

Acidified potato dextrose agar (APDA)
Acidified tryptone glucose yeast extract agar (ATGY)

Antibiotic supplemented

Oxytetracycline glucose yeast extract agar (OGY)
Chloramphenicol tryptone glucose yeast extract agar (CTGY)

Retarding molds

Rose Bengal chloramphenicol agar (RBC)
Dichloran Rose Bengal chloramphenicol agar (DRBC)

homogenization of the sample, serial dilution of the suspension, inoculation and incubation of media, and assessment of the original population size in the primary sample (enumeration, counting). These steps are followed by the isolation and purification of individual strains that can be subjected to a process of identification and typing by testing the morphological, physiological, biochemical, and molecular characteristics of the culture (Deák 2008). Numerous media have been developed for the detection, enumeration, and isolation of yeasts (King & Beelman 1986) (Box 14.1).

Traditionally, acidified media have been used for the cultivation of yeasts, such as malt extract with a pH of 3.5 to inhibit the growth of most bacteria. Acidified malt extract and tryptone glucose yeast extract broth or agar, as well as potato dextrose agar, are common general-purpose media. Comparing 10 media, Welthagen and Viljoen (1997) demonstrated that antibiotic-supplemented media resulted in higher counts than acidified media. Antibacterial antibiotics, such as oxytetracycline, gentamicin, and chloramphenicol, can be used alone or in combination (Koburger & Rodgers 1978; Mossel et al. 1975). Use of chloramphenicol is convenient because it is heat stable and can be added to other ingredients before autoclaving (Samson et al. 1992). These antibiotics in a concentration of 100 mg/L are usually not inhibitory to yeasts; however, Banks and Board (1987) observed that gentamicin prevented the growth of several yeasts at a concentration of 50 mg/L.

When agar media are used, viable counts are estimated by either the pour plate or spread plate technique. One advantage of the pour plate method is the somewhat greater sensitivity achieved by inoculating a 1 mL aliquot sample in contrast to the 0.1 mL that can be spread on the surface of agar in a standard Petri dish. However, plating is achieved by pouring molten agar media, heated to about 45 °C, which imposes a heat stress on yeast cells (Kennedy et al. 1980). Beuchat et al. (1992) indicated that spread plating results in better recovery of yeasts than pour plating. From comparative

studies, it can be concluded that spread plating is preferable to pour plate methods for giving significantly higher counts (Deák et al. 1986; Ferguson 1986; Seiler 1992).

The temperature of incubation can also influence the development of yeast colonies. In general, a temperature between 25°C and 28°C is appropriate for most yeasts, and even an ambient room temperature suffices in most cases. Five days at 25°C can be considered a standard incubation regime for yeasts (Beuchat et al. 1991; Hocking & Pitt 1992). Indirect estimates of cell populations can be made using various correlations of cell number with turbidity, metabolic activity, or dry mass (Deák 2008).

14.1.4 Yeast Genome and Importance of the ITS Region

These yeasts are particularly well adapted to harsh conditions prevailing in grape musts and wines (strong acidity, high sugar or alcohol concentration, presence of sulfites, etc.), which have shaped their genome (Pretorius 2000; Querol et al. 2003). Identification of the molecular basis of specific adaptation to the wine environment is therefore a key element in wine yeast genome research.

The genome sequence of a laboratory strain of *S. cerevisiae* was released in 1996 (Goffeau et al. 1996). The yeast genome is quite small, at only 12 Mb. It is highly packed, with about 6000 genes distributed over 16 chromosomes. *S. cerevisiae* also has two small cytoplasmic genomes: mitochondrial DNA and killer dsRNA. *S. cerevisiae* has a small (75 kb), circular mitochondrial DNA genome. The mitochondrial genome encodes a small set of proteins involved principally in respiration (Chen et al. 2000). Mitochondrial DNA is not essential for *S. cerevisiae* survival. Mutational loss of all mitochondria results in “petite colony” formation, whereby the constituent cells can only ferment, being unable to utilize molecular oxygen.

More recently, differences in the rRNA internal transcribed spacer (ITS) have been used to identify yeast species (Guillamón et al. 1998). The ITS region is the most widely sequenced DNA region for studying yeasts. It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other regions of rDNA (for small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and intergenic spacer (IGS) regions. In addition to the standard ITS1 + ITS4 primers used by most labs, several taxon-specific primers have been described that allow selective amplification of fungal sequences.

The ITS region, including the 5.8S rRNA gene (coding and conserved) and two flanking variable and non-coding regions ITS1 and ITS2, shows low intraspecific variability and high interspecific polymorphism. From the conserved sequences of 18S and 28S rRNA genes at the ends of the ITS region, two universal primers can be obtained (Bruns et al. 1991). The subsequent restriction analysis of the amplicons allows the identification of yeasts even though several species may be present simultaneously (Granchi et al. 1999).

14.2 Methods of Identification and Detection of Biodiversity

14.2.1 Culture-dependent Methods

Isolation and enumeration of yeasts from grapes, must, wine, and winery environment have traditionally involved plate counts. Numerous types of culturing media, either liquid broths or agar solids, have been used for the isolation, detection, or

enumeration of yeasts from grape juices and wines (King & Beelman 1986; Morris & Eddie 1957). In general, these media are complex, nutritionally very rich and generally contain antibiotics to prevent unwanted bacterial growth, and can be selective or non-selective. Other methods for the enumeration of yeasts include filtration procedures (e.g., membrane filtration), microscopy, dye reduction test, and most probable number method.

In vitro culture methods and conditions are often laborious but widely used to study yeast population dynamics. However, the phenotypic characteristics displayed by yeast are often influenced by the culture conditions and variability of the particular strain. This, alongside the existence of “viable but non-culturable” (VBNC) microorganisms present during vinification, leads to inaccurate classification and conclusions regarding population dynamics, especially when using plates (Díaz et al. 2013).

Molecular approaches have been used to identify yeast isolates after isolation and growth in pure culture. Numerous techniques have been used for this purpose, including application of the polymerase chain reaction (PCR) to 26S ribosomal DNA and sequencing, and PCR followed by restriction enzyme digestion of amplicons derived from ITS of 5.8s ribosomal DNA. These techniques still contain the bias inherent in the initial plating and isolation of the organism to be identified.

14.2.2 Culture-independent Methods

To circumvent the bias of culture-dependent methods, direct DNA sampling methods, coupled to molecular characterization of the total DNA and identification of different marker sequences, are being used to determine the numbers and types of yeast in an environmental sample. Techniques such as PCR combined with denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) have been used with great success to study the ecological succession of microorganisms during fermentation and to identify spoilage organisms in wine. These methods allow the identification of organisms that do not grow on a given medium under given conditions. However, these methods also have certain limitations, some of which have already been overcome. Initially, the analysis of DNA cannot distinguish between viable and non-viable cells, the methods are often limited to finding organisms only if they occur above a certain threshold frequency in the population, and, depending upon the technique used, are frequently limited to finding only those types of organisms that have been previously characterized molecularly. PCR-based methods typically rely upon specific primers that select only organisms of a certain genus and/or species. If an organism that is not expected to occur in the environment being examined is present, it may not be detected using specific primers.

14.2.3 PCR-based Methods

14.2.3.1 Sequencing the ITS Region

By definition, the ITS refers to the spacer DNA region (non-coding DNA) situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome or the corresponding transcribed region in the polycistronic rRNA precursor transcript.

14.2.3.2 Restriction Fragment Length Polymorphism of rDNA

A more precise method to identify isolated yeasts is restriction fragment length polymorphism (RFLP), a molecular technique based on the variations in homologous DNA sequences, from differing locations of restriction enzyme sites. In this analysis, the DNA sample is digested by restriction enzymes and the resulting fragments separated according to their size in an electrophoresis gel. With RFLP, it is possible to differentiate DNA between individuals in a population by the analysis of patterns derived from cleavage of their respective DNA. Thus, when DNA from two different individuals is cut with one or more restriction enzymes, fragments of different lengths are produced, and the pattern of those fragments is unique for different members of a population. In yeasts, the similarities and differences in the patterns generated can then be used to differentiate species and even strains (Cocolin & Ercolini 2007; Johansson et al. 1995).

Terminal restriction fragment length polymorphism (T-RFLP), combines restriction fragment analysis of a PCR-amplified product with automated sequencing gel technology. The amplification is performed with one or both the primers having their 5' end labeled with a fluorescent molecule. One primer used in PCR amplification of the marker gene is labeled at the 5' terminal with a fluorescent dye, in order that the terminal restriction fragments (TRFs) of the digested amplicon can be detected and quantified. In yeast analysis, genomic DNA has been amplified using ITS4 and ITS5 primers (first round) and ITS1 and ITS2 (nested). The size of both the digested and undigested PCR products is unique to particular yeast genera and also allows the differentiation of certain species, resulting in the unambiguous identification of up to 28 species.

Detailed evaluations of T-RFLP analysis have shown that, in most cases, both the sizes and relative signal intensities of the individual TRFs in a sample are highly reproducible (Díaz et al. 2013). Normally the methods are validated using pure cultures (for example, obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)). Even so, wild yeast species in wineries are often local subspecies that are subject to different environmental selection conditions and their sequences and PCR product sizes can differ slightly from purchased strains. In this case, sequencing of the obtained PCR products can validate the identification.

These methods have been widely used for monitoring yeast communities during wine fermentation. The T-RFLP technique is meant to be culture independent, and obtaining pure DNA that allows a proper read of the sequences in the genetic analyzer is still a challenge. In many studies, T-RFLP is only used as a fingerprinting tool or for identifying yeast species previously isolated from plates.

14.2.3.3 Random Amplified Polymorphic DNA (RAPD)

Several PCR-based techniques are used to identify strains of yeast in wine. The most commonly used is random amplified polymorphic DNA (RAPD)-PCR. Urso et al. (2008) used RAPD-PCR to follow *Saccharomyces* strains during the alcoholic fermentation of Picolit, an Italian sweet wine, and found that the inoculated *S. cerevisiae* starter culture actually performed the alcoholic fermentation in only one of the two fermentations studied.

Random amplified polymorphic DNA-PCR is fairly simple to perform, hence its extensive use in strain identification. It uses a single small primer to conduct PCR at a low annealing temperature. The small primer and low annealing temperature allow for

multiple hybridizations to the genome. When two primers land close to each other, a randomly amplified band will form. RAPD primers are usually selected so as to generate 3–12 such bands of varying length from a given genome. This allows for a survey of the polymorphisms found around a given genome, which are typically specific to a given strain of yeast or bacteria (Ivey & Phister 2011). This method has been used to group large numbers of strains isolated in ecological studies and provides a quick method for initial classification of isolates (Gadanhó et al. 2003).

While the technique is rapid, amenable to high-throughput analysis, and has the advantage of not needing any previous sequence information to compare strains, it does have a number of drawbacks. The most important of these is the difficulty in reproducing the banding patterns for a specific strain among laboratories. The procedures must be carefully standardized, as small variations in the DNA isolation or even the type of thermocycler can change the banding pattern (Ivey & Phister 2011).

14.2.3.4 Amplified Fragment Length Polymorphism

Amplified fragment length polymorphism (AFLP) is a technique most often used in relation to wine for the genotyping of grapes and molds (Ergül et al. 2006). It has seldom been applied for the strain typing of wine-related bacteria, although Cappello et al. (2008) found it to be a reliable method for the strain typing of *Oenococcus oeni*, formerly a *Leuconostoc*. With regard to strain typing of yeasts, few studies have been conducted. Azumi and Goto-Yamamoto (2001) used the method to study laboratory and industrial strains of *Saccharomyces sensu stricto* and found that *S. cerevisiae*, *S. bayanus*, *S. carlsbergensis*, and *S. paradoxus* all had species-specific banding patterns with some strain variation. Curtin et al. (2007) used the method to identify eight different genotypes of *Dekkera bruxellensis* from 31 wine-making regions in Australia. Other studies have examined *Saccharomyces* strains from different geographical origins (Lopandic et al. 2007).

While useful, this technique is laborious, and typically requires automated DNA sequencers, making it expensive. However, older versions of the technique, which relied on other less expensive detection methods, usually involving ultraviolet light, may also be used. DNA from the microbe of interest is digested with a restriction enzyme and adaptors are bound to the fragments. The fragments are then PCR amplified using primers, which target the adaptors and the restriction sites. The fragments are then separated using an automated DNA sequencer to detect a pattern. AFLP relies on the use of restriction enzymes to digest the DNA, as done for RFLP, but the difference is that unique oligonucleotides are ligated to the ends of the cuts and serve as priming sites for PCR amplification of the fragments (de Barros Lopes et al. 1998).

14.3 Enumeration of Wine Yeasts

14.3.1 Quantitative PCR

Quantitative real-time PCR (qPCR) represents a fast and reliable alternative to identify and quantify yeast during fermentation. The method is based on the amplification of a DNA target which is linked to a fluorescence reporter molecule. There are several reporters that can be used, but SYBR Green is the one most commonly employed for detection of wine-related microorganisms (Díaz et al. 2013; Fleet 1993). The main advantage of using qPCR is its low detection limits, as low as 10 CFU/mL.

The first application of qPCR to wine was by Phister and Mills (2003) for the detection of *Dekkera bruxellensis*. Other assays were developed by Delaherche et al. (2004) not only to *D. bruxellensis* but also to “ropy” *Pediococcus damnosus* in samples of spoiled wine. The *P. damnosus* assay targeted the *dps* gene, which is specific for exopolysaccharide production. Neeley et al. (2005) used this technique to quantify wine-related LAB as a group. A primer set, WLAB1-2, was created to detect *L. plantarum* and *O. oeni* at cell densities as low as approximately 100 CFU/mL, even in the presence of *S. cerevisiae*, *G. oxydans*, or *A. aceti*. The method has since been used to follow just about every microbe related to wine. Care must be taken, however, as a number of compounds present in wine may interfere with the assay (Wilson 1997). Tessonnière et al. (2009) examined six different DNA isolation methods in developing a qPCR assay for *D. bruxellensis* and found that polyvinyl pyrrolidone was able to eliminate most of the PCR inhibitors.

Quantitative PCR is rapid, taking a few hours, and is also sensitive. In some cases, it can detect as few as 10 organisms per milliliter, where other methods such as DGGE or microscopy generally require at least 1000 organisms per milliliter (Madigan & Martinko 2006). qPCR can even be multiplexed to detect a number of organisms in one assay (Selma et al. 2009). The major disadvantage outside cost and personnel training is centered on the method’s inability to differentiate viable and non-viable microbes. One of the few studies to address this issue was conducted by Hierro et al. (2006). They designed a real-time PCR assay to detect and quantify the total yeast population of a wine sample. While the assay itself was useful, the limit of detection for yeast grown in YEPD media was 10^2 and 10^3 CFU/mL in wine. The most informative part of the study used reverse transcriptase PCR (RT-PCR) to measure the viability of the cells. These studies suggest that mRNA may make a better target for differentiating viable and non-viable cells than rRNA or DNA. While traditional qPCR is not able to differentiate between living and non-living cells, it is still important to quantify non-culturable cells. Regardless of whether such cells are truly VBNC or simply sublethally injured, they continue to influence wine flavor and palatability (Cocolin & Ercolini 2007; Fleet 1993).

14.3.2 Ethidium Bromide Monoazide PCR

One possible solution to this issue may be found in the use of ethidium bromide monoazide (EMA), which is a fluorescent photoaffinity label that covalently couples to nucleic acids upon exposure to light. EMA is a dye that can bind to DNA of dead cells and prevent its amplification by PCR. An EMA staining step prior to PCR allows for the effective inhibition of false-positive results from DNA contamination by dead cells (Figure 14.1) (also described by Rudi et al. 2005).

Ethidium bromide monoazide can only enter cells with compromised walls and membranes (Nocker & Camper 2006). Therefore, it is believed to be a good indicator of cell viability, as only viable cells will have intact membranes, thus keeping the dye out. Once a sample is treated with EMA, real-time PCR can then be performed and only the viable cells will be quantified. Rawsthorne and Phister (2009) used an EMA assay to distinguish viable cells of *Zygosaccharomyces bailii* in different fruit juices. It was determined that the assay could detect as few as 12.5 viable cells in the presence of 10^5 CFU/mL of heat-killed *Z. bailii* cells.

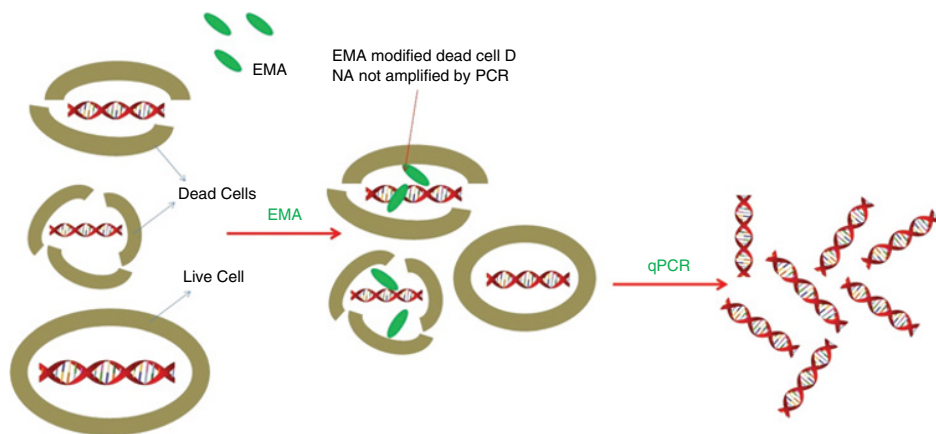


Figure 14.1 Schematic representation of EMA-PCR. Exclusion of EMA from live but not dead cells leads to cross-linking of DNA in dead cells by EMA and then amplification of DNA sequences of live but not dead cells by PCR.

14.4 Diversity of Wine Yeasts

14.4.1 PCR-DGGE

In the 1980s, DGGE was developed by Fischer and Lerman, and this technology was used as a method of detecting mutations in microbial DNA (Fischer & Lerman 1983). DGGE has been used for species identification of yeast populations in foods and beverages. The technique is based on separation of DNA fragments of differing nucleotide sequences (e.g., species specific) through decreased electrophoretic mobility of partially melted double-stranded DNA amplicons in a polyacrylamide gel containing a linear gradient of DNA denaturants (i.e., a mixture of urea and formamide).

Applications of DGGE have included identification and population dynamics of yeasts in sourdough bread (Meroth et al. 2003), in coffee fermentations (Durand et al. 2013; Masoud et al. 2004) and on wine grapes (Prakitchaiwattana et al. 2004). This method relies on the amplification of yeast 26S rDNA by using universal primers U1 (linked with a GC clamp) and U2. Amplification fragments are separated according to their length and nucleotide composition in a denaturing polyacrylamide gel (gradient from 20% to 60% of urea and formamide). Amplification fragments of interest are excised directly from the gel and sequenced for microbial species identification, taking the sequence band of yeast 26S rDNA as reference. One of the advantages is the possibility of identifying viable but not culturable yeasts whose DNA is also amplified (Cocolin et al. 2001). Levels of detection are often around 10^3 CFU/mL but 10^2 CFU/mL have been reported (El Sheikha 2010), which compares favorably with standard plate count methods (Prakitchaiwattana et al. 2004). Masoud et al. (2004) and Prakitchaiwattana et al. (2004) reported detection by DGGE of species that were not recovered by plating, suggesting that some yeasts may establish significant populations in a product and then die.

This technique has been used for the analysis of microbial populations in both grape and wine samples and has led to the identification of different yeast species

(*Candida diversa*, *C. sorboxylosa*, *Dekkera bruxellensis*, *Hanseniaspora occidentalis*, *Issatchenkia hanoiensis*, *Issatchenkia occidentalis*, *Issatchenkia orientalis*, *Kluyveromyces thermotolerans*, *Metschnikowia pulcherrima*, *Pichia kluyveri*, *S. cerevisiae*, *Saccharomyces ludwigii*, *Torulaspota delbrueckii*, *Z. bailii*). PCR-DGGE is not a quantitative tool (Cocolin et al. 2001). According to Cocolin et al. (2000), the method is relatively sensitive, being able to detect yeast populations in wine which represent at least 0.01% of the dominant *Saccharomyces* population.

In the most comprehensive use of DGGE to date, Renouf et al. (2007) monitored the microbial population through the entire wine-making process from berry to wine. Fifty-two different yeast species were found on the surface of grapes sampled from eight different vineyards. DGGE analysis demonstrated that the yeast population declined significantly through the process in three distinct phases.

- Phase 1: the total population increased during initial fermentation to about 10^8 CFU/mL with *S. cerevisiae* being dominant.
- Phase 2: after the first racking, the population declined.
- Phase 3: during aging, the population rose again to around 10^3 – 10^4 CFU/mL and stabilized, with *D. bruxellensis* dominating the population (Renouf et al. 2007).

14.4.2 Temperature Gradient Gel Electrophoresis

A related technique is temperature gradient gel electrophoresis (TGGE), in which the gel gradient of DGGE is replaced by a temperature gradient (Muyzer & Smalla 1998). DNA fragments of the same length but with different nucleotide sequences are separated on polyacrylamide gels using TGGE as a result of differing electrophoretic mobilities caused by partial denaturing along a linear temperature gradient (Riesner et al. 1992). When a molecule reaches its melting point (T_m), the double helix undergoes a conversion to a partially denatured molecule and stops migrating. For two fragments with the same size, the DNA melting point will depend upon the proportion and position of the G + C bases. To discriminate between bands with the same mobility, heteroduplex formation generated during PCR amplification of the DNA of the two strains considered is used (Tsuchiya et al. 1994). The lack of homology at a given point in the sequence results in distortion of the usual structure, which can be detected by TGGE as a result of slower migration than for the corresponding homoduplexes. This method has been used to analyze 16S and 18S rDNAs in studies of microbial ecology and sometimes in taxonomic studies (Felske et al. 1999).

Temperature gradient gel electrophoresis based on the principle of “one sequence, one band” holds out new prospects for yeast taxonomy. Some reports have been published on the use of TGGE-PCR for studies of population dynamics in artificial habitats able to support high cell densities, such as food and wine (Fernández-González et al. 2001; Hernán-Gómez et al. 2000; Mills et al. 2002). Fernández-González et al. (2001) used TGGE to characterize *Saccharomyces* and non-*Saccharomyces* species from wine must during fermentation. Among the isolates used, 58% were shown to be members of the genus *Saccharomyces* using restriction enzyme analysis. TGGE was used to distinguish the genus of the non-*Saccharomyces* isolates: *Candida*, *Kluyveromyces*, and *Hanseniaspora* (Fernández-González et al. 2001). Manzano et al. (2004) were able to study the ecology of the genus *Saccharomyces* directly in wine at

the strain level. Again using the ITS1 and ITS2 regions as PCR targets, TGGE analysis showed seven different strains of *S. paradoxus*, all with similar migration patterns (Manzano et al. 2005). This technique was also able to distinguish those strains from samples of *S. cerevisiae*. TGGE patterns were identical for samples drawn directly from musts and those obtained from plated colonies, demonstrating the effectiveness of the technique for direct identification. This method has also been shown to be effective at verification of dry yeast strains before they are used for fermentation (Manzano et al. 2006).

14.5 Conclusions and Future Perspectives

This chapter has described cultural, direct, and indirect molecular methods, provided examples of their impact on the study of the microbial ecology of wine, and also discussed their strengths and limitations. We can conclude that the cultural methods for identifying yeasts rely on morphological, physiological, and biochemical characteristics and require in-depth expertise in conducting and evaluating high number diagnostic tests. These methods are complex and time-consuming.

A number of advances in molecular detection techniques hold promise for applications in the identification of wine-related microbial communities. Real-time PCR is a rapid, direct, sensitive, and reliable technique for enumeration of the total yeast population in wine. RT-PCR can be used to quantify yeasts during industrial wine fermentation and to rapidly control the risks of wine spoilage. Today, DGGE/TGGE is a well-established molecular tool in environmental microbiology that allows study of the complexity and behavior of microbial communities. The technique is reliable, reproducible, rapid, and inexpensive. DGGE/TGGE technique is used for synchronous analysis of numerous samples, allowing to control community changes over time, as well as for identification of community members by sequencing of excised bands. Another emerging method which could find application in studying the ecology of wine yeasts is known as deep sequencing or pyrosequencing.

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Section V

Foodborne Pathogens and Food Safety

15

Rapid Detection of Food Pathogens Using Molecular Methods

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15.1 Introduction

Food- and waterborne diseases are recognized as one of the most serious public health concerns in the world. The risk of foodborne pathogenesis has risen continuously over the years due to increased international travel and trade, economic development, and globalization. This risk is higher in developing countries because of lower levels of sanitation, poor socioeconomic conditions, and lack of awareness of health issues and practices. In addition to diseases caused by direct infection by pathogens, some foodborne diseases are caused by the presence of toxins produced by microorganisms in food (Centers for Disease Control and Prevention [CDCP] 2015). There are four main types of microbial pathogens which contaminate food and water: viruses, bacteria, fungi, pathogenic protozoa and helminths (CDCP 2015).

Food can be contaminated by pathogenic organisms during preharvest, harvest or postharvest processing. Some pathogenic microorganisms are only transferred by food while others are transmitted through other methods as well. In order to minimize the risk of these microbial infections, microbiological quality control programs should be applied throughout the food chain. So, the availability of reliable, rapid and cost-effective test systems to detect the presence or absence and the degree of pathogen contamination has become increasingly important in the food industry (Nugen & Baeumner 2008).

However, all over the world, routine tests are still mostly done by conventional microbial methods. The problems associated with these conventional detection techniques include low reliability in identification and quantification, inaccurate microbial numbers as some bacteria which are viable in the environment can enter a dormancy period in media, and, most importantly, the long analysis time. In order to overcome these problems, a number of rapid detection methods for foodborne pathogens are being developed. Again, these methods have their own associated problems which include low detection sensitivity, inability to differentiate between viable and non-viable cells, etc.

The reasons for low sensitivity in molecular detection techniques include difficulties in isolating microorganisms from food matrices, inhibition of detection by chemical inhibitors in food samples, and the need for minimum number of cells to give detectable results. The inability of molecular detection techniques like the PCR to differentiate between viable and non-viable cells is due to the persistence of nucleic acid (DNA and rRNA) in food samples, even after cell death.

Considerable research has been carried out to solve these problems, such as chemical and magnetic immobilization of microbial cells, improvement of DNA and RNA extraction methods, breakdown of DNA from lysed cells using DNAase enzymes, improvements in molecular detection methods, etc. This chapter will highlight some of the recent efforts made in this field.

15.2 Methods Used to Detect Foodborne Pathogens

Foodborne pathogenic microorganisms can be found all over the world at different levels. Detection and quantification of pathogens in contaminated foods are the major concerns in the food industry. Many methods have been developed for this, including conventional methods, immunology-based methods, and molecular detection techniques such as the PCR to amplify the DNA of pathogens, hybridization of complementary strands of DNA, and nanotechnological methods, etc. These methods will be discussed in the following sections.

15.2.1 Conventional Methods

Culturing and microscopic observation of specific colony characteristics is the traditional method used in pathogenic microorganism detection. Enrichment cultures are often used to increase specific types of microorganisms before they are identified. These cultures provide specific conditions for the growth of a particular type of microorganism and help to isolate microorganisms that can metabolize a particular substrate or can live under certain conditions even though they may be present in very small numbers in the original sample (McMeekin 2003).

In order to detect selected pathogenic microorganisms, many specific culture media have been developed and optimum culture conditions identified. These culturing techniques are used not only for identification but also for quantification of pathogenic microorganisms in food samples (McMeekin 2003). In quantification of laboratory microbial cultures, standard curves are developed from optical density (OD) of microbial cultures measured using a spectrophotometer or from dry weight of microorganism or by colony count (CFU/mL). The standard curve method makes the quantification method easy but inapplicable for microorganisms present in food samples because of the effect on OD values of many other particles and solids present in the food matrix.

Conventional detection methods have been modified to be more user-friendly, sensitive, and reliable, and to reduce the cost of materials as well as the need of skilled personnel. All steps of conventional detection methods, including sample preparation, plating, and colony counting, have been modified by introducing equipment such as gravimetric diluters, stomacher machines, spiral platters, automated colony counting systems, image analysis systems, etc. Specific culture media have been developed for

the identification of foodborne microbial pathogens, including Rambach agar, Rainbow agar *Salmonella*, and XLT4 agar which are used for *Salmonella*, and EMB agar, Endo agar, and HiCrome *E. coli* agar B which are used for *E. coli* (Fratamico 2003). Information on specific culture media for most of the foodborne pathogens is freely available in the literature (HIMEDIA 2012; Sigma 2014).

There are several drawbacks in the identification and quantification of pathogenic microorganisms by culturing techniques. The main problems are low reliability in identification and quantification and long analysis time. Moreover, some viable bacteria in the environment can enter a dormancy period in media which can lead to underestimation of microbial numbers. Different chemical components and mixed microflora in the food matrix can affect the isolation of pathogenic microorganisms from food samples. This could lead to false-negative results or underestimation of microbes in food samples (Vunrczant & Pllustoesser 1987). The time required for the detection of pathogenic organisms is too great. For example, the detection of *Salmonella* by culturing techniques requires a 24-hour long enrichment step which is a major problem especially when rapid food pathogenic detection is required. Some of the methods used for the rapid detection of food pathogens are given in the following sections.

15.2.2 Immunological Methods

Immunological methods are based on specific binding of an antibody to an antigen. Many antibodies have been identified for the detection of specific microorganisms or microbial toxins. These methods can be classified as homogeneous or heterogeneous. In the homogeneous methods, also called marker-free assays, there is no need to separate the bound from the unbound antibody. The antigen–antibody complex formed is directly visible or measurable and the incubation time is usually very short. This method can be used to obtain both qualitative and quantitative results. In the heterogeneous assay, the unbound antibody must be separated from the bound antibody using labeled reagents (Ng et al. 2010).

Immunological methods can be performed with minimum requirements in laboratory experiments but detection is possible only when the sample contains $10^3 - 10^5$ microbes/mL. This often requires enrichment for 16–24 hours. In order to avoid the enrichment step, immune capture-based separation of microorganisms using immunomagnetic separation (IMS) can be applied. In this technique, samples are mixed with beads coated with antibodies for the target organisms which then bind to the immunomagnetic beads and are separated. To obtain results easily and cost-effectively with immunological methods, automated systems or kits that are easy to handle can be used (Uyttendaele et al. 2000). There are many different immunological methods and other techniques combined with immunological routes commercially available for the detection of pathogenic microorganisms in food samples.

The enzyme linked immunosorbent assay (ELISA) is one of the mostly commonly used immunological methods for the detection of food- and waterborne pathogens. This method has been applied by many scientists to detect different types of foodborne pathogens (López-Campos et al. 2012). Hybridoma technology, which allows the limitless production of highly specific antibodies (monoclonal antibodies) which can be used to identify pathogens to the species or even subspecies level (Thornton 2009), has helped in the development of this technique for a number of applications.

15.2.3 Nucleic Acid-based Assay Methods

Recently, significant progress has been made in nucleic acid-based assays and molecular detection techniques used for microbial detection. These techniques are mainly based on the identification of polymorphism in DNA and RNA sequences and the use of a unique polymorphism in target organisms to detect such microorganisms. These methods have become more popular because of their high sensitivity, reliability, and low time requirement compared to other methods used in microorganism detection.

Nucleic acid-based methods used in microorganism detection can be categorized into three main categories: molecular subtyping, nucleic acid hybridization, and amplification.

15.2.3.1 Molecular Subtyping

Molecular subtyping methods are mainly used in molecular taxonomic studies to identify the taxonomical relationship of strains between and within species. This is particularly used in the identification of different individuals having the same or very similar phenotypic characteristics which other characterization methods cannot identify. These methods are also used in epidemiological studies and DNA fingerprinting. Another application is the development of genetic markers to identify plants or animals for breeding purposes. There are many molecular subtyping methods including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP), etc. Each of these methods has its own advantages and disadvantages, so the selection of a method depends on the specific application (European Food Safety Authority 2013).

15.2.3.2 Hybridization Methods

Tagged hybridization techniques use preprepared probes which are 15–30 base pairs in length to hybridize with complementary sequences of DNA or RNA of target microorganisms. Figure 15.1 shows the steps followed during hybridization of DNA fragments obtained by reverse transcription of RNA present in cells. After hybridization, the hybridizing nucleic acids are detected by different techniques such as antibody–antigen detection, detection by labeled DNA probes, direct detection by radioactive and fluorescent probes, and indirect detection by enzyme reporters.

Depending on the hybrid recognition system, many hybridization methods have been developed, including dot-blot assay, *in situ* hybridization, Southern blotting (DNA sequences), Northern blotting (RNA sequences), and fluorescent *in situ* hybridization (FISH). FISH is a promising method in food microbial detection and has received a great deal of attention in recent years (Fusco & Quero 2012).

Fluorescent *in Situ* Hybridization

Fluorescent *in situ* hybridization is a molecular method commonly used for microbial identification and quantification in a wide range of samples (both solid and liquid based) (Amann & Fuchs 2008; Amann et al. 1997). This technique is a combination of (a) specificity of DNA/RNA hybridization, which allows detection of specific microbial species, and (b) microscopic observation, which allows visualization of morphological characteristics of microbial pathogens (Justé et al. 2008; Nath & Johnson 2000). Generally, the probes used in FISH are short (15–20 nucleotides) and fluorescently labeled at their 5'

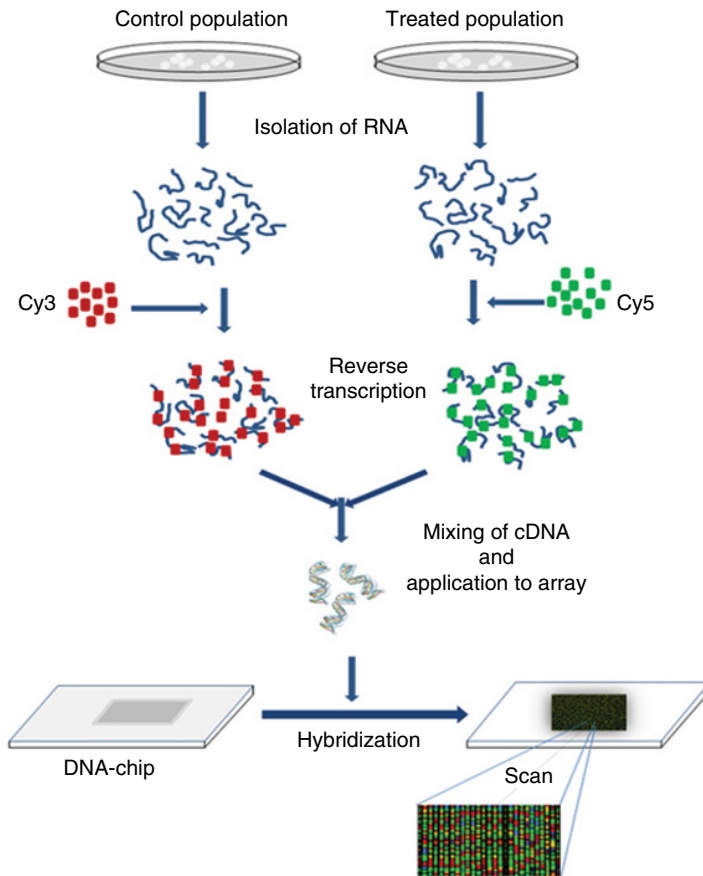


Figure 15.1 Steps of nucleic acid hybridization process using reverse transcription for production of DNA fragments from RNA.

end. The fluorescent probe binds specifically to the complementary DNA/RNA sequence of a pathogen. After hybridization, specifically stained cells are detected and identified using fluorescent microscopy. In addition, specific RNA targets (miRNA, mRNA, lncRNA) in cells and tissue samples can be localized and detected by this method (Wagner et al. 2003). FISH has high sensitivity (detection limit 10^4 CFU/g) and can be performed in a short time (1–3 h) (Bottari et al. 2006).

In recent years, peptide nucleic acid (PNA) probes have been used instead of nucleic acid-based probes to improve the efficiency of FISH technology as they enable more rapid and specific hybridization (López-Campos et al. 2012).

In the context of food safety, FISH technology can detect a variety of pathogens and beneficial microbes. For example, it has been used in the quantification of probiotic bifidobacteria in fermented food, detection of specific lactic acid bacteria in natural whey cultures for producing cheeses and also in wine (Machado et al. 2013). In combination with flow cytometry, FISH has been used to detect *Salmonella* sp. on the surfaces of tomatoes and other fresh foods (Bisha & Brehm-Stecher 2010).

Fluorescent *in situ* hybridization differs from other non-PCR molecular detection techniques (Ercolini et al 2003). In this method, cells containing a threshold amount of rRNA can be detected microscopically using rRNA probes (Regnault et al. 2000). Most bacterial cells contain ribosomes and a high number of rRNA copies (Amann et al. 1995) which can be detected by this technique with very high assay sensitivity. Since dead bacterial cells lose their membrane integrity, their ribosomes degrade quickly inside the dead cells (Hannig et al. 2007). This degradation of rRNA targets can be used for the detection of viable microorganisms separately from dead microorganisms by FISH (Vieira-Pinto et al. 2007). Sometimes dead bacteria can maintain some metabolic activity until they are lysed (Regnault et al. 2000), which could lead to overestimation of viable bacterial count. On the other hand, there are reports which state that some live bacteria could be in an inactive state and not produce enough rRNA for detection by FISH, which may lead to negative results or underestimation of viable cell numbers (Vieira-Pinto et al. 2007). Vivification of microorganisms by antibiotic treatment prior to detection by FISH has been used in some studies to increase the detectable signal by increasing the amount of rRNA in the cells (Regnault et al. 2000). This treatment could activate inactive cells and reduce the underestimation of viable count due to inactive but viable cells. Moreover, it could increase the fluorescent signal of live cells compared to dead cells, which could lead to more reliable detection of viable cells.

The ability of FISH to differentiate viable from non-viable cells depends on the rapid degradation of rRNA in non-viable cells. In our work (Rathnayaka & Rakshit 2010b), *Salmonella enterica* that were heat-killed at 80 °C, 100 °C, and 121 °C were examined for the presence of rRNA using FISH at various times ranging from 5 minutes to 48 hours after heat treatment. rRNA was detected by FISH in heat-killed bacteria after treatment at 80 °C, 100 °C, and 121 °C for 12 hours, 3 hours, and 1 hour respectively. Results showed strong correlation between temperature and stability of rRNA in heat-killed bacteria. We concluded that FISH was a suitable method for differentiation of viable from non-viable cells, especially for samples subjected to extreme heat.

15.2.3.3 Amplification Methods

Compared to other methods, detection methods based on amplification of DNA are important because of their high sensitivity and specificity. The most popular DNA amplification method is the PCR. PCR-based detection methods are a powerful diagnostic tool in the detection of food- and waterborne pathogens. In the PCR, first the double-stranded DNA are denatured into single strands by high temperature, then specific short DNA fragments called primers are annealed to these denatured single DNA strands, followed by extension of the primers to produce complementary new single-stranded DNA. This process is catalyzed by thermo-stable DNA polymerase. This technique can be used to produce more than a billion copies of a target fragment of a DNA strand in a couple of hours, starting from a single target DNA molecule. Since this technique produces such a huge number of DNA copies, PCR products can be visualized as a band on an ethidium bromide-stained electrophoresis gel. The polymorphism in the band size visualized in gel electrophoresis can be used in pathogen detection and identification (Iwobi et al. 2012).

Polymerase chain reaction-based assays have been identified as powerful diagnostic tools for the detection of microorganisms present in food samples (Malorny et al. 2003). The capacity of PCR to detect microorganisms depends on the purity of the template

used as a target and the presence of a sufficient number of target molecules (Lucero Estrada et al. 2007). The presence of PCR inhibitors in food samples is the main limitation in PCR-based assays due to the production of false-negative results. The removal of inhibitory substances and rapid and efficient DNA extraction in the preparation of samples for PCR-based detection of food pathogens is important (Jeníková et al. 2000). Hence, the application of PCR-based methods is closely linked to the selection of suitable methods for DNA extraction (Amagliani et al. 2007).

One drawback in PCR-based detection is its low detection sensitivity for foodborne pathogenic bacteria. Difficulties in acquiring bacterial cells from food samples and insufficient product amplification in PCR reaction are the reasons for this low sensitivity. Different methods such as immunomagnetic separation and metal hydroxide immobilization have been used to increase the efficiency of acquiring cells from food samples prior to DNA extraction for PCR (Jeníková et al. 2000; Lucero et al. 2000). Nested PCR, a modified PCR method, has been used to increase the amount of amplified products in the PCR reaction and the sensitivity of PCR-based detection. In this method, two sets of primers are used in two successive runs of PCR; the first set of primers amplifies the selected gene sequence in the sample DNA and the second set amplifies a secondary target within the first run product. This secondary amplification increases the sensitivity and specificity of detection.

In a study carried out in our laboratory (Rathnayaka & Rakshit 2010a), we evaluated the effectiveness of metal hydroxide immobilization using different DNA extraction methods for the detection of *Salmonella enterica* in pork sausage samples by nested PCR. Immobilization of bacterial cells by $Zr(OH)_4$ and $Ti(OH)_4$ was carried out prior to DNA extraction by five DNA extraction methods. The *fliC* gene and enterotoxin (*stn*) gene of *S. enterica* were amplified by nested PCR. Both metal hydroxide immobilization and nested PCR amplification were able to increase detection sensitivity. DNA extraction by a modified Kapperud method (Kapperud et al. 1993; Lucero Estrada et al. 2007) was found to be the most effective. Using nested PCR, the enterotoxin (*stn*) gene was found to be more sensitive for detection than the *fliC* gene. This study shows that the use of metal hydroxide immobilization and nested PCR is able to increase the sensitivity of *S. enterica* detection in meat food samples.

Another important concern in *Salmonella* detection from food samples is differentiation of dead and live microorganisms. It is important to obtain positive results when only live microbes present. The plate count method traditionally used for determination of the viable count of *Salmonella* has disadvantages such as the long incubation period, and clumping and inhibition of cells by neighboring cells which leads to underestimation of cell numbers (Lahtinen et al. 2006). Although many rapid, reliable, and specific methods have been developed for the detection of *Salmonella* in food samples, they are not applicable for the determination of viable bacterial count. The DNAase-treated DNA PCR method (DTD-PCR), in which bacterial cells are treated with DNAase enzyme prior to DNA extraction in order to degrade DNA in dead cells, is one of the methods used to attempt to overcome this problem (Mukhopadhyay & Kumar 2002).

In our laboratory, we found that bacterial immobilization by metal hydroxides can be used for enrichment of various bacterial strains, thus reducing the time for their detection (Do et al. 2009). The application of DNase I to eliminate DNA from dead cells and subsequently detect the presence of viable pathogens by conventional PCR was studied. The results indicated that the treatment of immobilized cells with

DNase I (1 h at 37 °C) prior to DNA extraction could efficiently eliminate false positives caused by the presence of non-viable cells. The technique was used to detect various pathogens with whole milk as a food model. The detection limits for *E. coli* O157:H7 (384 bp), *Listeria monocytogenes* (482 bp), and *E. coli* wild type (580 bp) were 5×10^1 cells and that for *Salmonella Typhimurium* (685 bp) was 5×10^2 cells in 10 mL of whole milk.

Different types of PCR-based assays have been applied for the detection of microbial pathogens, such as the combination of PCR and immunomagnetic separation (IMS-PCR), the combination of PCR and ELISA, DTD-PCR, multiplex PCR, nested PCR, fluorescent PCR, reverse transcriptase PCR and real-time PCR (Fach et al. 2002; Jeníková et al. 2000; Zhou et al. 2013).

15.2.4 Comparison of Rapid Methods and Conventional Methods

Analysis of food pathogens is challenging due to the complexity and heterogeneity of food matrices, non-uniformity in pathogen distribution, and interference by non-pathogenic microbes (Doyle 2001). As food safety management continues to have strict objectives, the efficacy of detection/quantification methods will be subjected to scrutiny, especially for accuracy, specificity, sensitivity, rapidity, and on-site applicability (Vunrczant & Pllustoesser 1987). The safety agency will utilize data generated from rapid detection methods to disseminate information quickly, identify dormant and/or viable pathogens, and regularly monitor the food processing chain to reduce labor costs and human errors. Traditional microbiological methods are unable to provide timely data to meet the safety objectives of current food production and distribution networks. In addition, they are expensive, require highly specialized skills and longer analysis time, and cannot perform on-site analysis. In view of these limitations, alternative rapid but accurate methods for pathogen detection are constantly being developed. Rapid detection is critical, especially when finished products are in transit, thereby preventing the outbreak of foodborne illness.

15.2.4.1 Challenges and Limitations of the Rapid Methods

Advances in biotechnology have led to the emergence of rapid diagnostic methods (Lübeck & Hoorfar 2003). In addition to their rapidity and sensitivity, they have several advantages including the need for less hands-on manipulation, automation, multi-sample analysis, and miniaturization (Abubakar et al. 2007). Although convenient, rapid detection methods have their own merits and drawbacks (Abubakar et al. 2007).

Results Interpretation

Generally, rapid methods are based on damaging cells and are therefore unable to provide definitive information on the relative counts of viable and non-viable cells. Most of the rapid techniques are designed specifically to detect a particular pathogen, therefore making them expensive for multipathogen analysis. The results obtained by rapid methods are classified by AOAC International as follows: if results are “negative” then they are considered definitive and if results are “positive” then they are considered presumptive –requiring further verification by traditional culture-based methods (Mandal et al. 2011).

Sensitivity and Rapidity

Sensitive detection is often achieved through traditional culture methods (qualitative), wherein the presence or absence of a pathogen is determined in a known quantity of food. As small numbers of pathogens are enough to cause diseases, the general requirement will be to detect <1 cell per 10–20 g of food material. Most probable number (MPN) techniques are suitable to detect pathogens with <10–100 counts/g. In plate counting, pathogens can be detected when their number is >10 CFU/g and accuracy increases when pathogens are >100 CFU/g, whereas in rapid detection methods, the lower detection limit will be 1000 CFU/g. Thus, rapid methods lack sensitivity compared to traditional methods. Another disadvantage is that rapid methods require an appropriate official standard as a reference for comparison, which involves culturing in many cases. In addition, rapid methods involve cell damage during sample preparation, thereby limiting information on the viability of pathogens.

Nevertheless, rapid detection methods can save time by obtaining interpreted results within a few minutes to a day compared with traditional methods. Therefore, commercially available rapid detection methods (ELISA, PCR, LFD, and biosensor) are of great use in food industries (Feng 2007; Leon-Velarde et al. 2009).

Specificity

Conventional methods generally use liquid or solid culture media to grow target microorganisms, thereby restricting the growth of other pathogens in food matrices. In nucleic acid-based assays, specificity depends on the selection of target primer or probe DNA sequences and hybridization conditions. In immunological antibody techniques, the drawback lies with cross-reactivity and species specificity in the assays.

Processing Time and Cost

One of the challenging problems for direct detection of pathogens in food is sample processing cost. For example, in quantitative PCR (qPCR), the major drawback is sample preparation. If the test sample has a low pathogen count and the amount of sample taken for analysis is small, then there is a high chance that the pathogen may not be included in the test sample. Similarly, biosensor-based techniques work well in fluid systems with low organic content and their efficacy decreases as the amount of fat and protein content increases. In microarray techniques, a similar problem can be found with sample preparation and validation of results.

Although rapid methods are gaining popularity, their practicality as a pathogen monitoring tool, especially for on-site analysis, is still a long way from realization.

15.2.5 Present Situation and Future Developments of Foodborne Pathogen Detection by Rapid Methods

Some of the most recent novel methods and their developments are discussed below.

15.2.5.1 Quantum Dots

Quantum dots (QDs) are semiconducting nanoparticles (2–10 nm in diameter) with narrow, highly specific, and stable emission spectra that are useful in the detection of food pathogens. Due to their photo-stability, stained fluorescence, larger effective Stokes shifts, narrow emission and wider excitation bandwidths, they are considered

better than other organic fluorophors (Resch-Genger et al. 2008). In addition, QDs offer several advantages in food pathogen detection due to their high specificity, rapid detection, ease of mass fabrication, and adaptability to on-site analysis. Analysis using QDs requires less time (<2 h) compared to traditional methods (at least 24 h). However, this method requires initial enrichment to achieve sensitivity in differentiating live and dead cells.

15.2.5.2 Nanomechanical Cantilevers

Nanomechanical cantilevers are made from silicon and are attached with phage-derived peptides, which specifically bind to pathogenic bacteria. These peptides bind surface molecules of pathogens, resulting in deflection of the cantilever, which gives rise to a detectable signal (Wang et al. 2014). For example, commercial gold-coated atomic force microscopy microcantilevers immobilized with a monoclonal antibody have been used to detect *V. cholerae* O1 in concentrations ranging from 1×10^3 to 1×10^7 CFU/mL (Sungkanak et al. 2010).

15.2.5.3 Phage-based Assays

Bacteriophages have been explored recently as a probe for food pathogen detection, due to their high specificity and selectivity towards their host and their ease of amplification. As phages recognize surface molecules of specific bacteria, they can be used as a probe for detecting food pathogens. Genetic engineering of phages can be performed to increase the surface property of phages for immobilization. In addition, reporter genes can be inserted into phages that are expressed after infection. This could be an indicator to differentiate live and dead cells. However, using phages has some limitations such as the initiation of host cell lysis, the drying effect, which results in poor host pathogen capturing ability, and our limited knowledge of surface functionalization (Ripp 2010).

15.2.5.4 Pyrosequencing

Many well-known bacterial strains are characterized by their signature sequences and can be identified by pyrosequencing to specifically detect single nucleotide polymorphisms (SNPs). The DNA-sequencing reaction is initiated by adding an oligonucleotide primer to a single-stranded PCR product. As the polymerase enzyme adds a complementary base to the newly double-stranded product, a light signal is emitted which can be further detected. This technology can be miniaturized (e.g., 96-well plate), thus providing the option to analyze multiple food pathogens (Tenover 2007). Some of the disadvantages of pyrosequencing technology are that:

- it can only sequence a short length of nucleotide sequence
- the light signal will become non-linear after the incorporation of more than 5–6 identical nucleotides
- primer designs can be challenging.

15.2.5.5 Biochip/Microarray Techniques

Biochips can be used to detect a variety of pathogens by imprinting antibodies against or DNA molecules representing specific pathogens on a chip for simultaneous detection of pathogens. These chips can detect minute quantities of target molecules. However, they have certain limitations such as requiring cell enrichment and being

easily contaminated, therefore restricting their real-time analysis. In addition, this method is unable to provide information on cell viability. Although biochip/microarray technology has great potential, it requires further research (Nollet & Toldra 2010). Similarly, lectin-based arrays are designed specifically to detect the glycan component of lipopolysaccharide present in the cell walls of Gram-negative bacteria. Using this technique, multiple bacterial cells can be detected. The lectin arrays can be considered as an emerging tool for the identification of bacterial strain typing. However, commercial availability of the number of glycan-specific lectins is limited.

15.2.5.6 Laser Sensor

A scattered image can be obtained by passing light from the laser through a bacterial colony grown on an agar and capturing the scattered light using a sensor. The obtained image is a unique fingerprint of a particular organism, and the test sample can be compared with the database to determine the type of organism immediately. The advantage of this technology is that the analysis is fast and it avoids excessive reagents used in the detection process. However, total identification time can be long as it depends on the amount of time needed to culture a colony (Singh et al. 2014).

15.2.5.7 Lightcycler and Smartcycler

These are advanced PCR product detection systems alternative to regular fluorescent-based molecular beacons. For example, these systems show high specificity for methicillin-resistant *Staphylococcus aureus*, and also interference from *mecA* gene in coagulase-negative staphylococci is obviated (Shakya 2013).

15.2.5.8 Hyphenated Methods

Other recent advances in high-throughput technologies for laboratory use include denaturing high-performance liquid chromatography and nucleic acid analysis by mass spectrometry. These technologies can detect SNPs, including those related to antibiotic resistance phenotypes (Cifuentes 2012).

15.2.5.9 Biosensors

Biosensor-based techniques are gaining interest due to their fast detection and adaptability to on-site diagnostics. Biosensors consist of an element which can recognize a biological response or a bioreceptor (e.g., enzyme, antibody, tissue, among others) and a transducer (e.g., electrochemical, optical, thermometric, among others) (Su et al. 2011). For microbial toxins, biosensors offer sensitivity in the range of ng/mL. Most commonly studied biosensors are based on bioluminescence, piezoelectric principle and electric impedance. Biosensors based on carbon nanotube material are in focus due to sensitivity of detection (in ppb) and rapidity (Nollet & Toldra 2010). When DNA samples come in contact with the nanotube biosensor, there are changes in conductivity which can be measured to detect responsible pathogens. In the food industry, biosensors can find several applications such as identifying GMO food products and detecting transgenic genes and pesticides/toxins in food. Practically, electrochemical transducers are the closest to meet the requirements of onsite analysis such as sensitivity, accuracy, ruggedness, portability, and ease of use (Nugen & Baeumner 2008).

Although real-time food pathogen analysis is still in the future, recent advances in pathogen analysis based on combined biotechnology, sequencing, bioinformatics

(next-generation pulsenet and array track), omics technologies (proteomics, transcriptomics, genomics, metabolomics/metabonomics), and medical diagnostic tools have allowed us to measure food pathogens in a short time. The development and application of modern pathogen detection tools will contribute to better food safety systems in the marketplace. In the future, the focus will shift from classic detection parameters (characterization of genus/species or serotype of pathogen) to more specific disease-causing or virulence factors. The development of rapid pathogen separation techniques (e.g., membrane filters) and their genetic material analysis by automated machines (nucleic acid sequencing) will enable higher sensitivity and rapidity of analysis. The development of user-friendly analytical tools (e.g., colloidal gold flag for ELISA) will allow rapid expansion of information about food pathogens (Unnevehr et al. 2004).

Another main challenge is to improve sample preparation time and enrich analyte concentration prior to analysis. For example, biosensors are generally developed in buffer solutions, but food matrices contain a mixture of matrix molecules which can hinder the detection process. Due to complex food matrices, the sample preparation time itself may take longer than the biosensor detection time. Similarly, gene amplification based tools such as PCR and nucleic acid sequence-based amplification are very sensitive to inhibitory molecules. Therefore, the need for rapid/clean sample preparation and separation/enrichment steps is emphasized (Batt 2007).

For example, immunomagnetic separation is promising for isolating pathogens from food systems such as meat products, mashed potatoes, and green salads (Warren et al. 2006), with a reduced detection time. In this technique, antibodies are immobilized on a magnetic bead (micron scale) that captures the analytes present in the sample. In another study, *L. monocytogenes* in ham was detected with a detection limit of 1.1×100 CFU/g using a 25 g sample (Hudson et al. 2001; Jackson et al. 1993; Warren et al. 2006). Similarly, buoyant density gradient centrifugation has been used to separate pathogens and toxins from complex food matrices, thereby aiding in rapid detection methods (e.g., PCR) (Fukushima et al. 2007). In addition, several environmentally friendly green extraction methods are being developed, such as subcritical water extraction and supercritical fluid extraction. These methods provide higher selectivity and reduced extraction time. Separations based on chromatographic techniques combined with spectrometry (hyphenated techniques) are also being investigated. In future, techniques based on immunomagnetic separation and alternative bioaffinity ligands such as bacteriophages, carbohydrates, and aptamers will be explored for improved target capture and sample preparation.

15.2.5.10 Automated Detection Methods

Many automated direct detection methods based on biosensors have been developed for the detection of foodborne microbial pathogens. These biosensors have different detection mechanisms such as detection of optical changes, chemical changes, mass changes, etc. Among these, the cell counting methods including flow cytometry and direct epifluorescent microscopy can be considered to be in the same category of detection techniques.

Flow cytometry is an optical method used to analyze individual cells in complex mixtures. In this method, a laser light beam passes through microorganisms suspended in a liquid medium, so that the microorganisms scatter the light. Then, the scattered light is collected by a system of lenses and photocells. The nature of scatter is an

intrinsic character of microorganisms and is used in the identification of number, size, and shape of microorganisms present in the sample. The determination of bacterial cell number by flow cytometry could be used for the rapid determination of viable bacterial numbers (Vunrcrzant & Pllustoesser 1987).

The direct epifluorescent filter technique (DEFT) is also a direct method used for the enumeration of microorganisms. It is based on the binding properties of fluorochrome acridine orange with microorganisms. In this technique, samples are first pretreated with detergents and proteolytic enzymes. Then the samples are filtered onto a polycarbonate membrane, stained with acridine orange, and examined under a fluorescent microscope. The number of viable bacteria can be determined by counting the orange cells visualized (Pettipher 1985).

Automated methods used for microbial pathogen detection have been developed continuously with the objective of increasing sensitivity of detection and reducing detection time. The US FDA has archived a list of many of the commercially available rapid methods (Feng 1998), classified by the principles underlying the procedure used. The AOAC status of rapid tests is indicated for those methods that have been validated or evaluated by the AOAC.

15.3 Conclusions

Rapid methods are generally used as screening techniques, with negative results accepted as they are but positive results requiring confirmation by appropriate official methods that vary from country to country. In many instances, attempts to validate the rapid methods follow simultaneous statistical confirmation using conventional methods for periods as long as 5 years in a real industrial or food testing facility.

Overall, due to stringent food safety regulations, there will be continuous developments in pathogen detection technologies. The predominance of one method for detection of all types of pathogens in different food matrices is not likely. Further changes in the food and medical industries will drive the need for specific rapid tests. The development of antibiotic-resistant strains will add another dimension to the need for rapid food pathogen detection methods using molecular technology.

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16

Biosensor-Based Techniques: A Reliable and Primary Tool for Detection of Foodborne Pathogens

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16.1 Introduction

Food quality is a subject of increasing concern and is related to nutritional value, acceptability, and safety of food. Problems that affect food freshness and quality include exposure time in an inadequate environment, incorrect design of food packaging, inadequate management of temperatures, and the level of oxygen during the handling of fruit and vegetables in modified atmospheres.

Food safety is one of the most important areas of public health worldwide. Foodborne diseases, foodborne infection, and/or foodborne poisoning result from infection with viruses, bacteria or parasites. Foodborne pathogens include microorganisms (i.e., bacteria, viruses, and fungi) as well as a number of parasites capable of infecting humans via contaminated food or water (Dwivedi & Jaykus 2011). Many of these organisms have an essential function in nature but certain potentially harmful microorganisms can have a profound negative effect on both animals and humans, costing the food industry (and indirectly, the consumer) many millions of dollars each year. Velusamy et al. (2010) identified Norovirus, *Salmonella*, *Campylobacter*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Clostridium perfringens*, *Toxoplasma gondii*, and *Escherichia coli* as pathogens causing foodborne illnesses and reported that these pathogens are generally found to be responsible for the vast majority of illnesses, hospitalizations, and deaths. In particular, foodborne bacteria such as *E. coli* O157:H7, *S. enterica*, *Staph. aureus*, *L. monocytogenes*, *C. jejuni*, *Bacillus cereus* and other shiga-toxin producing *E. coli* strains (non-O157 STEC) and *Vibrio* spp. are leading causes of foodborne diseases.

The World Health Organization (WHO) defines foodborne illnesses as diseases, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food. Although the global incidence of foodborne disease is difficult to estimate, it has been reported that in 2005, 1.8 million people died from diarrheal diseases and a great proportion of these cases can be attributed to contamination of

food and drinking water (WHO 2007). In industrialized countries, the percentage of the population suffering from foodborne diseases each year has been reported to be up to 30%.

Diseases caused by foodborne pathogens have become an important public health problem in the world, producing a significant rate of morbidity and mortality (Oliver et al. 2005). Although the safety of food has dramatically improved over time, progress is uneven and foodborne outbreaks from microbial contamination, chemicals, and toxins are common in many countries (WHO 2007). Table 16.1 provides information on foodborne outbreaks, including food types, places, and sources. The World Trade Organization (WTO) reported in 2007 that Europe accounted for 46% of world exports of agricultural products, where food represents 80% of agricultural exports (WTO 2007). Trading of contaminated food across countries increases the potential for outbreaks and consequent health risks posed by microbial pathogens in food are a major global concern.

Although food security has been significantly enhanced in recent years, improvements are uneven, and microbial contamination, chemicals, and toxins leading to foodborne outbreaks are widespread in several countries.

Food products and their raw materials are composed of complex compounds, so in order to guarantee high quality and security, quality control is the foremost task allied with the food industries.

Potentially threatening bacteria in food, soil, and water have historically outrun any detection effort, resulting in unwarranted deaths and illness. The food industry is the main party concerned with the presence of pathogenic microorganisms, where failure to detect a pathogen could lead to a catastrophic effect. Detection and quantification of microbial pathogens are usually the first procedures performed before the application of any strategy for combating them. Traditional microbiological methods are powerful, error proof and dependable, but these lengthy, cumbersome methods are often ineffective because of their short shelf-life and non-compatibility with the speed at which products are being manufactured.

Recently, various rapid detection, identification, and monitoring methods have been developed for foodborne pathogens, including nucleic acid-based methods, immunological methods, biosensor-based methods, etc. (Xihong et al. 2014). Automation is highly desirable in detection methods, but is not achievable with traditional methods. Therefore, biosensor-based tools offer the most promising solutions and address some modern-day needs for fast and sensitive detection of pathogens in real time or near real time (Leonard et al. 2003). These rapid and precise methods are some of the most effective ways to control and prevent human foodborne infections. In order to address these problems, various food quality management systems enable food companies and authorities to carry out fast and reliable quality testing of all kinds of food through the use of biosensors. Biosensors can also detect indicators of processes such as lactulose, a disaccharide, which is formed in the thermal treatment of milk. It allows for distinguishing between milk that has been submitted to ultra high temperature (UHT) treatment and milk sterilized in the container (Cock et al. 2009).

Biosensors are devices that detect biological or chemical complexes in the form of antigen-antibody, enzyme-substrate or receptor-ligand. Biosensors in their simplest form are analytical devices that convert a biological response to a measurable electrical

Table 16.1 Foodborne outbreaks caused by pathogenic microorganisms.

Pathogen	Food consumed	Place and year of outbreak	Source of outbreak	References
<i>Campylobacter jejuni</i>	Cooked chicken	Japan, 2005	Secondary contamination in cooking practice	Yoda & Uchimura (2006)
<i>Salmonella</i> spp.	Egg, squash and seafood	South Korea, 2007	Infected food handler	Kim et al. (2007)
<i>Salmonella</i> spp.	Cake	Germany, 2006	High ambient summer temperatures and failure to keep the cake refrigerated	Frank et al. (2007)
<i>Salmonella</i> spp.	Confectionery (éclairs)	Berlin, 2006	Insufficient cooling of the éclairs during transport and before sale could have enhanced bacterial growth	Wichmann-Schauer et al. (2006)
<i>Salmonella</i> spp. and <i>Shigella</i>	Mixed rice and chicken	Bangkok, 2005	Mixed contamination	Chanachai et al. (2008)
<i>Salmonella</i> spp.	Preprocessed raw beef	Netherlands, 2005	Imported contaminated beef	Kivi et al. (2007)
<i>Salmonella</i> spp.	Rucola lettuce	Norway, 2004	Imported Rucola lettuce	Nygård et al. (2008)
<i>Salmonella</i> spp.	Cheese, lettuce, and roma tomatoes	North-eastern United States, 2004	Contaminated roma tomatoes	Gupta et al. (2007)
<i>Salmonella</i> spp.	Hard pastry with vanilla cream	Spain, 2002	Inadequate handling of foods containing eggs	Camps et al. (2005)
<i>E. coli</i> O157:H7	Hamburger patties	USA, 2007	Contaminated beef patties distributed by meat processing plant	Belson & Fahim (2007)
<i>E. coli</i> O103:H25	Cured mutton sausage	Norway, 2006	Contaminated mutton	Schimmer et al. (2008)
<i>L. monocytogenes</i>	Turkey meat	USA, 2002	Contaminated turkey distributed by turkey processing plant	Gottlieb et al. (2006)
<i>L. monocytogenes</i>	Cheese	Japan, 2001	Contaminated cheese products distributed by cheese producing plant	Makino et al. (2005)
Norovirus	Raw oysters	Canada, 2004	Not specified	David et al. (2007)
Hepatitis A	Green onions	USA, 2003	Contaminated green onions	Wheeler et al. (2005)
Norwalk-like viruses	Salad sandwiches	India, 2002	Not specified	Girish et al. (2002)

signal proportional to the concentration of the analytes. A biosensor consists of a bioreceptor or biorecognition element and a transducer. A bioreceptor can be a tissue, microorganism, organelle, enzyme, cell, antibody, etc., while the transducer may be optical, electrochemical, thermometric, etc. Ironically, geometric growth in biosensor technology is fueled by the imminent threat of bioterrorism through food, water, and air and by funding through various governmental agencies. The trend in pathogen testing emphasizes the need to commercialize biosensors for the food safety industry as legislation creates new standards for microbial monitoring. With quicker detection times and reusable features, biosensors will be important to those interested in real-time diagnostics of disease-causing pathogens. As the world becomes more concerned with safe food and water supply, the demand for rapid detecting biosensors will rise.

16.1.1 Conventional Versus Molecular Techniques

Conventional methods for the detection and identification of bacteria mainly rely on specific microbiological and biochemical identification. While these methods can be sensitive and inexpensive and give both qualitative and quantitative information on the number and nature of the microorganisms tested, they are greatly restricted by assay time with initial enrichment needed in order to detect pathogens which typically occur in low numbers in food and water. Some standard methods such as the NF EN ISO and 11290-1 method for the detection of *L. monocytogenes* can require up to 7 days to yield results, as they rely on the multiplication of microorganisms to visible colonies (Artault et al. 2001; de Boer & Beumer 1999). Biosensors offer an exciting alternative to more traditional methods, allowing rapid “real-time” and multiple analyses that are essential for the detection of bacteria in food, especially perishable or semi-perishable foods.

The need for a more rapid, reliable, specific and sensitive method of detecting a target analyte at low cost is the focus of a great deal of research, especially for applications outside the laboratory environment. Since its inception in the 1970s, hazard analysis critical control point (HACCP) methodology has evolved as the leading food safety strategy used by the food industry. HACCP identifies where potential contamination, time, and temperature problems can occur (the critical control points). However, key technologies needed to successfully implement any HACCP program are real-time microbial detection, traceability, and source identification.

16.1.2 Biosensors: An Introduction

Quantification of biological or biochemical processes is important for medical, biological, and biotechnological applications. However, it is not easy to convert biological data directly to an electrical signal. However, biosensors can overcome the difficulty of converting biological data or process to an electrical signal.

A sensor can be defined as a device or system including control and processing electronics, software, and interconnection networks that responds to a physical or chemical quantity by producing an output which is a measure of that quantity (Patel 2002). Sensors can be divided into three categories: physical sensors, chemical sensors, and biosensors. Physical sensors are used for measuring distance, mass, temperature, pH, etc. (Eggins 2002) while chemical sensors are compact devices that transform chemical

information into an analytically useful and measurable signal. Chemical sensors usually contain two basic components connected in series: a chemical recognition system and a physicochemical transducer. The selective and reversible detection of the chemical sensor is accompanied by the electrical signal that is obtained from the physicochemical transducer.

Biosensors are special chemical sensors in which the recognition system utilizes a biochemical mechanism (Thévenot et al. 2001; Vastarella 2001). Biosensors can also be defined as analytical devices incorporating biological materials such as enzymes, tissues, microorganisms, antibodies, cell receptors or biologically derived materials or a biomimic component in intimate contact with a physicochemical transducer or transducing microsystem (Malhotra et al. 2005; Mello & Kubota 2002). A biosensor should be clearly distinguished from a bioanalytical system, which requires additional processing steps such as addition of reagent. Furthermore, it should be distinguished from a bioprobe which is either disposable after one measurement, i.e., single use, or unable to continuously monitor the analyte concentration. It converts a biological response into an electrical signal. A biosensor is a device which is composed of two elements:

- a bioreceptor that is an immobilized sensitive biological element (e.g., antibody, enzyme, DNA probe) that has the ability to recognize the analyte (e.g., antigen, enzyme substrate, complementary DNA)
- a transducer which is used to convert the biological or biochemical signal resulting from the interaction of the analyte with the bioreceptor into an electrical signal.

Transduction may be optical, electrochemical, thermometric, piezoelectric, magnetic, and micromechanical or combinations of one or more of these techniques. The generated signal intensity is directly or inversely proportional to the analyte concentration present in the sample. Electrochemical transducing elements are often used to develop biosensors. They offer certain advantages such as low cost, simple design, and small dimensions. Biosensors can also be based on calorimetric, gravimetric or optical detection (Sassolas et al. 2012). Advances in antibody production and the emergence of phage-displayed peptide biosensors (Chaplin & Bucke 1990; Goldman et al. 2000) offer increased possibilities for the rapid detection of pathogens. According to the International Union of Pure and Applied Chemistry (IUPAC), a biosensor is precisely defined as a self-contained integrated device, capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element which is retained in direct spatial contact with a transduction element (Vastarella 2001).

Figure 16.1 shows a schematic diagram of a biosensor. The bioreceptor recognizes the target analyte and the corresponding biological responses are then converted into equivalent electrical signals by the transducer. The amplifier in the biosensor responds to the small input signal from the transducer and delivers a large output signal that contains the essential waveform features of an input signal. The amplified signal is then processed by the signal processor where it can later be stored, displayed, and analyzed.

Biosensors have been widely applied to a variety of analytical problems in medicine, environment, food processing industries, security, and defense. Biosensors can be directly applied for the detection of pathogens in processed food matrices. Such processing methods usually include mincing and homogenization of food samples in the presence of detergents and/or proteolytic enzymes and the choice of processing method depends on the type and complexity of the sample. Biosensors do not require the

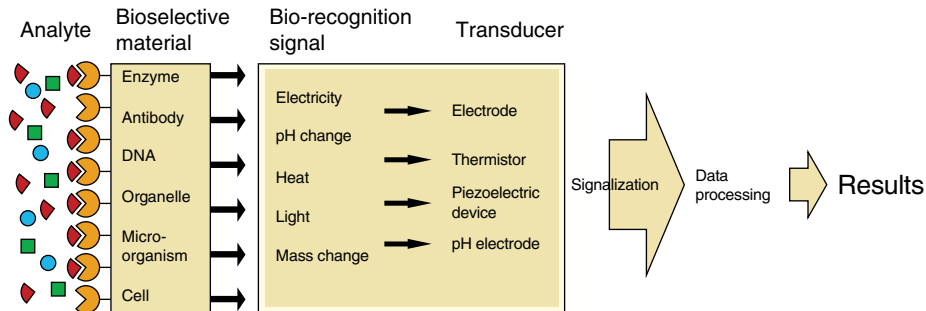


Figure 16.1 Components of biosensor. *Source:* Kivirand et al. (2013). www.intechopen.com/books/state-of-the-art-in-biosensors-general-aspects/calibrating-biosensors-in-flow-through-set-ups-studies-with-glucose-optrodes. Licensed under CC BY 3.0.

time-consuming sample pre-enrichment and secondary enrichment steps and therefore can accurately predict the level and type of food contamination much faster than conventional microbiological, immunological, and molecular biological methods. Biosensors for monitoring food and water samples have not yet been commercialized, unlike those available for medical diagnostics, but recent developments show tremendous possibility.

16.2 Ideal Requirements for Biosensor-Based Microbial Detection Assay

- *Accuracy:* false-positive and false-negative results must be low or preferably zero, especially when detecting pathogenic organisms.
- *Linearity:* linearity of the biosensor should be high for detection of high substrate concentration.
- *Assay time:* the biosensor should produce a “real-time” response, especially when perishable foods are being tested.
- *Sensitivity:* failure to detect false-negative results lowers the sensitivity of the assay, which cannot be tolerated in food microbiology.
- *Specificity:* the biosensor should easily discriminate between the target organism or toxin and other organisms.
- *Reproducible:* each assay should be highly reproducible and easy to calibrate.
- *Robust:* the biosensor must be able to resist changes in temperature, pH, and ionic strength and could be sterilized.
- *User friendly:* the assay should be fully automated and require minimal operator skills for routine detection.
- *Compatible interface:* the biointerface should be compatible with the transduction principle, resist non-specific binding, and should be freely accessible in three-dimensional space.
- *Validation:* the biosensor assay should be evaluated against current standard techniques and LOD obtained.

16.3 Need for Rapid Method

Conventional pathogen detection methods such as microbiological and biochemical identification are time-consuming and laborious, while immunological or nucleic acid-based techniques require extensive sample preparation and are not amenable to miniaturization for on-site detection. Biosensors have shown tremendous promise to overcome these limitations and are being aggressively studied to provide rapid, reliable, and sensitive detection platforms for such applications. According to Vunrcrzant and Pllustoesser (1987), the food industry is in need of more rapid methods which are sensitive for the following reasons.

- To provide immediate information on the possible presence of pathogens in raw material and finished products.
- Low numbers of pathogenic bacteria are often present in complex biological environments along with many non-pathogenic organisms.
- The presence of even a single pathogenic organism in food may be an infectious dose.
- For monitoring process control, cleaning, and hygienic practices during manufacture.
- To reduce human error and to save time and labor cost.

16.4 Classification of Biosensors

All biosensors rely on highly specific recognition events to detect target analytes and suitable transducers to obtain measurable signal for the analyte of interest. Figure 16.2 shows some analytes that can be analyzed in a biosensor system (Mello & Kubota 2002).

Figure 16.2 demonstrates that there are various combinations of biological material and transducer, depending on the sample of interest and the type of physical magnitude to be measured. Biosensors are classified based on their biological recognition elements or transducers or alternatively the combination of these two aspects (Vastarella 2001).

Biosensors are indicators of biological compounds that can be as simple as temperature-sensitive paints or as complex as DNA-RNA probes. The science of biosensors is a multidisciplinary area. The potential application of biosensor technology to food testing offers several attractive features. Many of the systems are portable and hence can be used for field testing or on-the-spot analysis and are rapid tests capable of testing multiple samples simultaneously.

Biosensing methods for pathogen detection are centered on four basic physiological or genetic properties of microorganisms: metabolic patterns of substrate utilization, phenotypic expression analysis of signature molecules by antibodies, nucleic acid analysis, and analysis of the interaction of pathogens with eukaryotic cells. The first biosensor was fabricated by Clark and Lyons in 1962. A Clark oxygen electrode was combined with the enzyme glucose oxidase to monitor glucose levels. Uptake of the co-reactant oxygen could be monitored amperometrically while glucose underwent enzymatic oxidation (Yoo & Lee 2010). Alternatively, the production of hydrogen peroxide during the enzyme reaction could be measured.

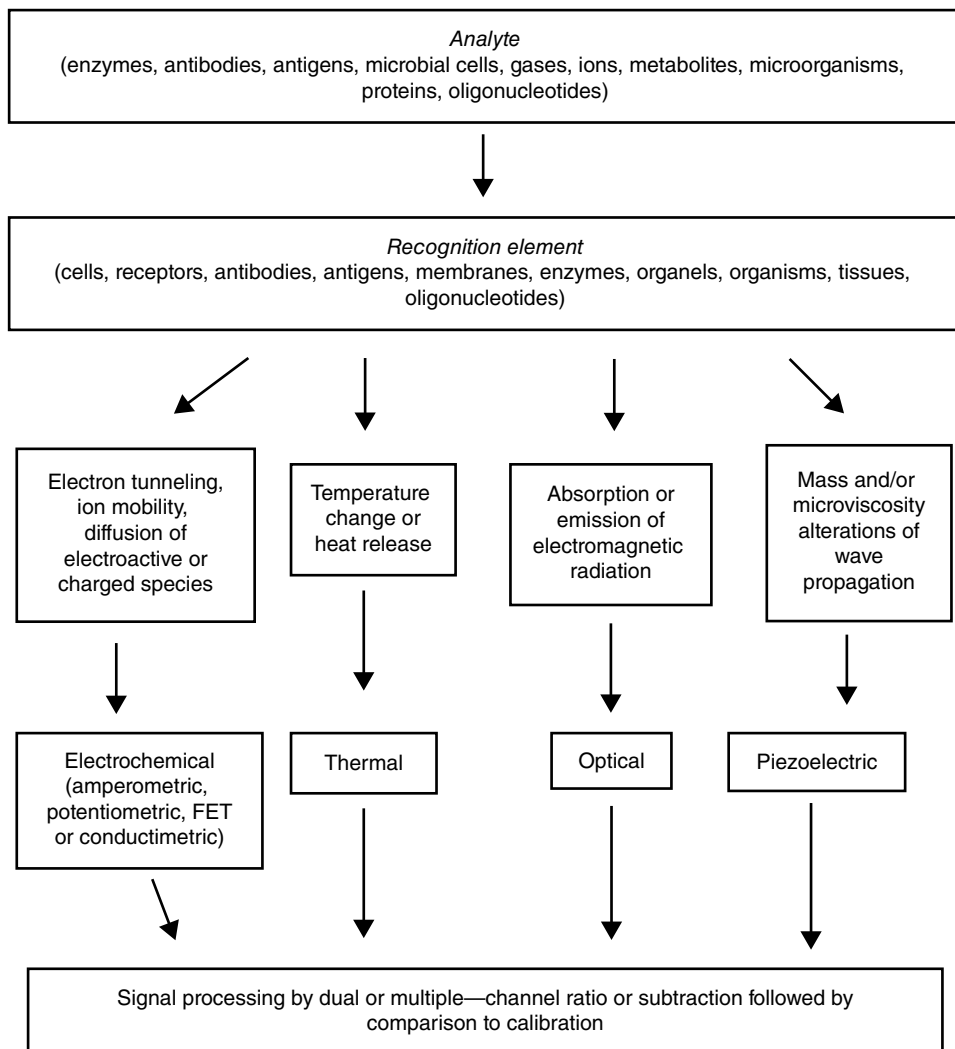


Figure 16.2 Biocomponents and transducers in a biosensor system. FET, field-effect transistor. Source: Mello & Kubota (2002). Reproduced with permission of Elsevier.

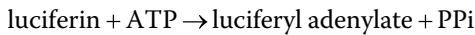
16.4.1 Bioluminescence Biosensor

A bioluminescence biosensor measures the change in luminescence emitted by living microorganisms. There are two general types of bioluminescence used in the food industry: adenosine triphosphate (ATP) bioluminescence and bacterial bioluminescence

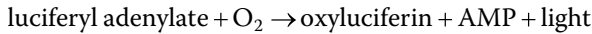
16.4.1.1 ATP Bioluminescence

Adenosine triphosphate bioluminescence techniques are commonly used to measure the effectiveness of cleaning surfaces and utensils. The general procedure takes a swab sample and combines it with a mixture of luciferase/luciferin (enzyme/substrate).

As summarized in the following two reactions, the reaction of luciferin with luciferase requires the presence of ATP.



Light is produced once luciferyl adenylate reacts with oxygen. Other products of this reaction include oxyluciferin and AMP.



The main advantages of using a bioluminescence biosensor to detect foodborne pathogens include extreme sensitivity and speed in detection, ease of implementation, and their ability to be used in portable field devices (Guisán 2006). One of the disadvantages of this method is the non-specificity of ATP assays since ATP is present in all living cells. For this reason, bioluminescence sensors alone do not provide any information regarding the origin of the ATP source. This problem has been addressed by coupling the sensors with other detection and identification techniques (Fratamico et al. 2005).

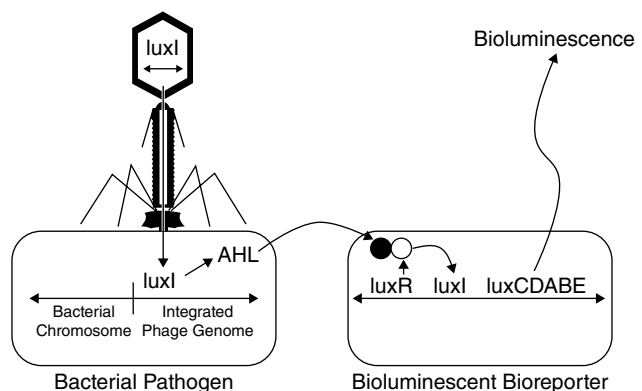
16.4.1.2 Bacterial Bioluminescence

The gene responsible for bacterial bioluminescence is known as the *lux* gene. DNA carrying this specific gene can be introduced into host-specific phages, as shown in Figure 16.3. Once the *lux* gene is transferred to a host bacterium during infection, bioluminescence occurs. Bioluminescence will only occur once the *lux* gene is transferred, since the host-specific phage does not have the necessary intracellular components to express the gene (Mandal et al. 2011). Luminometers are used to accurately detect the light emission.

16.4.2 Fiberoptic Biosensor

The basic principle of fiberoptic biosensors is that when light propagates through the core of an optical fiber, i.e., waveguide, it generates an evanescent field outside the surface of the waveguide. The waveguides are generally made up of polystyrene fibers or glass slides. When fluorescent-labeled analytes such as pathogens or toxins bound to the surface of waveguide are excited by the evanescent wave generated by a laser

Figure 16.3 Bacterial bioluminescence. *Source:* Mungroo & Neethirajan (2014). www.mdpi.com/2079-6374/4/4/472. Licensed under CC BY 4.0.



(635 nm) and emit fluorescent signal (Bhunja 2007; Taitt et al. 2007), the signal travels back through the waveguide in high order mode to be detected by a fluorescence detector in real time. The research and high-tech development of optical biosensors have grown exponentially during the last decade because of the linear, real-time, and label-free detection of many chemical and biological substances by this technique (d’Orazio 2011).

16.4.3 Bioreceptors

A bioreceptor is a molecular species that exploits a biochemical mechanism for recognition. They are accountable for binding the concerned analyte to the sensor for measurement (Velusamy et al. 2010). Bioreceptors can broadly be classified into distinct classes: antibody-antigen bioreceptor, enzymatic bioreceptor, nucleic acid (DNA) bioreceptor, cellular structures or cellular bioreceptor, biomimetic bioreceptor, and bacteriophage bioreceptor.

16.4.4 Antibody-antigen Bioreceptor

Antibodies are universal bioreceptors used in biosensors. The antibodies may be polyclonal, monoclonal or recombinant based on their selective properties and synthesis. Nonetheless, they are usually immobilized on a substrate which can be the detector surface, its vicinity or a carrier (Hoa et al. 2009). An antigen-specific antibody binds its exclusive antigen in an extremely specific way similar to a lock and key (Willis et al. 2013), so that the three-dimensional structures of antigen and antibody molecules are corresponding. This unique high level of antibody specificity is responsible for the advantages of immunosensors as an analytical tool, allowing antibodies to bind exclusively to their corresponding antigen, be it a chemical, biomolecule or specific microorganism.

16.4.5 Enzymatic Bioreceptor

Enzymes as bioreceptors offer numerous advantages over fluorescently labeled and radiolabeled substances; enzyme immunoassay reagents are highly stable and sensitive and there are no health hazards. Enzyme immobilization has emerged as a fundamental way to evolve competent biosensors with relevant properties such as good operational and storage stability, immense sensitivity, high selectivity, short response time, and high reproducibility (Sassolas et al. 2012). The most frequently used enzymes are horseradish peroxidase (HRP) and beta-galactoxidase. The detection of pathogenic bacteria such as *L. monocytogenes*, *E. coli*, and *C. jejuni* can be done by labeling the antibody with these enzymes.

16.4.6 Bacteriophage Bioreceptors

Bacteriophages (phages) are viruses 20–200 nm in size (Singh et al. 2013) that bind to specific receptors on the bacterial surface in order to infuse their genetic material inside the bacteria. Phages identify the bacterial receptors via their tail spike proteins. Phages offer various advantages including the specificity of the synergy of this sort of virus with

its target host cell, the ability to lyse and kill the host plus ability to reproduce throughout the infection process (Zourob 2010). In addition, they are omnipresent, innocuous to humans, economical, are conveniently produced, and have a long shelf-life as they endure harsh environments diminishing environmental limitations and enabling regeneration of the biosensor surface. Researchers have proclaimed the function of phages as a biorecognition component for the exposure of various pathogens such as *Staph. aureus* (Singh et al. 2013) and *Bacillus anthracis* spores by adopting diverse sensing platforms based on their ability to increase in numbers, resulting in 200–300 mature phage particles after infection of one host cell and thereby greatly increasing the sensitivity of detection of the targeted bacterial species.

16.4.7 Nucleic Acid Bioreceptors

The precise biorecognition in DNA biosensors depends on the complementarities of adenine:thymine (A:T) and cytosine:guanine (C:G) pairing in DNA which are generally referred to as genosensors. Nucleic acid-based biosensors have been utilized by several researchers for the detection of food pathogens such as *E. coli* O157:H7 (Liu et al. 2011), *Salmonella* spp. (Zhang et al. 2012), etc. An additional type of biosensor employs a peptide nucleic acid as the biorecognition element. Peptide nucleic acid (PNA) is a synthetic oligo amide that is capable of binding incredibly firmly to complementary oligonucleotide sequences. One major drawback of PNA is that its synthesis is very costly but its key disadvantage is that purine-rich PNA oligomers tend to accumulate and are weakly soluble in aqueous media.

16.4.8 Cell-based Biosensors

Cellular structures and cells have been utilized in the evolution of biosensors and biochips. Isolation of cell organelles can be done to utilize them as bioreceptors. In cell-based biosensors (CBBs), a whole cell serves as the molecular recognition element and requires two transduction phases. The cells serve as the primary transducer, converting the detected analyte into a cellular response. A second transducer is required to convert the cellular signal into an electronic signal that can be processed and analyzed. The second transduction is dependent on the type of cellular signal to be monitored.

There are many reasons why living cells are well suited for recognition. First, cells provide sensitivity to a wide range of biochemical stimuli since they contain many highly evolved biochemical pathways. Second, cells provide a functional assay for biochemical agents. Because CBBs make use of direct measurements of physiological function (and changes induced by toxins), they provide detection capability for previously unknown agents. The third major advantage associated with cells as bioreceptors for incorporation into biosensors is that detection limits can be very low, because of signal amplification. The above properties distinguish CBBs from molecular biosensors that rely on the detection of molecular events such as antibody binding, DNA hybridization or enzymatic reactions.

Cell-based assays (CBAs) continue to serve as a reliable method for detection of pathogens in food samples. CBA systems can report perturbations in the normal physiological activities of mammalian cells as a result of exposure to an external or environmental challenge. For this, mammalian cells are used as electrical capacitors.

Electrical impedance (EI) uses the inherent electrical properties of cells to measure parameters related to the tissue environment. The mechanical contact between cell-cell and cell-substrate is measured via conductivity or EI. The cell can be equated to a simple circuit since it is nothing more than conductive fluid encapsulated by a membrane surrounded by another conductive fluid. The conductive fluids make up the resistance elements of the circuit, while the membrane acts as a capacitor. Changes in impedance indicate changes in cell density, growth or cellular behavior. These biosensors are able to provide detailed information about the growth characteristics of the tissue culture, including information on spreading, attachment, and cellular morphology.

16.4.9 Mass Sensitive Biosensors

Assessment of minute transformation in mass is a distinct characteristic of transduction that has been exploited for biosensors. The fundamental mode of mass analysis relies on the response of piezoelectric crystals (Pramanik et al. 2013). This results in the vibration of crystals at a distinct frequency with the operation of an electrical signal of explicit frequency. Therefore, the frequency of oscillation depends on the electrical frequency which is applied to the crystal and its mass (Velusamy et al. 2010). Thus, in simple words, binding of chemicals results in increased mass which in turn changes the oscillation frequency of the crystal which can be measured electrically and utilized in the determination of the additional crystal mass. The detection of *L. monocytogenes* has been conceivable with the development of a quartz crystal microbalance biosensor (Singh et al. 2013).

16.4.10 Electrochemical Biosensors

These are adaptations of conventional antibody-based enzyme immunoassays (ELISA), which comprise the catalysis of substrates by an enzyme conjugated to an antibody and the production of products which in turn can be detected in the pattern of pH change, ion or oxygen consumption due to generation of electrical signals on a transducer (Velusamy et al. 2010).

16.4.11 Amperometric Biosensors

Amperometric transduction is a universal electrochemical detection method which has been well exploited for pathogen detection. This technique is integral to optical detection methods such as fluorescence, which is considered as the most precise of the optical techniques (Konradi et al. 2012). These sensitive biosensors can also be used to identify various foodborne pathogens including *E. coli* O157:H7 (Singh et al. 2013), *Salmonella* (Iles & Kallichurn 2012), *L. monocytogenes* (Davis et al. 2013), and *C. jejuni* (Yang et al. 2013).

Electrochemical biosensor technology has developed rapidly in the last few years. There have been immense breakthroughs in the development of electrochemical sensors for detecting virus infections and bacterial contamination (Velusamy et al. 2010). Reymond et al. (2007) devised an amperometric detection method for the determination of the presence, amount, and concentration of an analyte in a microfluidic

sensor. There have also been studies related to the evolution of a biosensor for the estimation of protein and amino acids (Chalova et al. 2009). Electrochemical biosensors developed on the basis of amperometric detection have been linked to other biosensing techniques; for example, a bioenzyme electrochemical biosensor was helpful in the detection of pathogens including *E. coli* O157:H7 (Settingington & Alocilja 2012) and *Salmonella Typhimurium* (Liu et al. 2011). Electrochemical biosensors have advantages in that they have the ability to sense materials without damaging the system (Arora et al. 2011).

The use of biosensors in various industries and for environmental analysis has become very important (Kuila et al. 2011). Some of the applications of biosensors include the control of food manufacturing processes, control of fermentation processes, evaluation of food quality and monitoring of organic pollutants, such as an electrochemical biosensor that can be used to detect *Salmonella* and *E. coli* O157:H7 in less than 90 minutes (Arora et al. 2011). Electrochemical biosensor studies are performed using electrochemical cells.

16.4.12 Electrochemical Cells

With electrochemical cells used in electrochemical sensor studies, the electrodes play an important role. The electrode material, its surface modification or its dimensions affect the detection ability of the electrochemical biosensor. Three kinds of electrodes are used in the electrochemical cell (Figure 16.4):

- reference electrode
- auxiliary (counter) electrode
- working electrode.

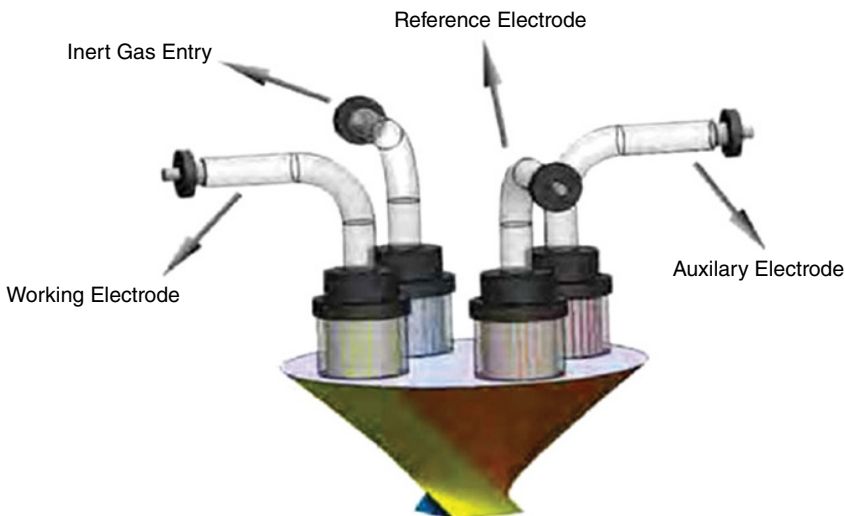


Figure 16.4 Electrochemical cell. Source: Koyun et al. (2012). www.intechopen.com/books/a-roadmap-of-biomedical-engineers-and-milestones/biosensors-and-their-principles. Licensed under CC BY 3.0.

16.4.12.1 Reference Electrode

Types of reference electrode:

- type 1: hydrogen electrode
- type 2: calomel electrode
- type 3: glass electrode.

The reference electrode is a standard hydrogen electrode that is potentially explosive and is not very suitable for use as an electrode with hydrogen gas for routine measurements. So there are two commonly used and commercially available reference electrode types.

- Ag/AgCl electrode: this is a silver wire coated with AgCl and dipped into NaCl solution.
- Saturated-calomel electrode: calomel is the other name of mercurous chloride (Hg_2Cl_2).

The calomel electrode consisting of a paste mixture of (I) mercuric chloride powder, potassium chloride, and saturated potassium chloride solution.

16.4.12.2 Auxiliary (Counter) Electrode

In a two-electrode system, when a known potential or current is applied between the working and auxiliary electrodes, the other variables may be measured. The auxiliary electrode functions as an anode whenever the working electrode is operating as a cathode and vice versa. The auxiliary electrode usually has a surface area which is much larger than that of the working electrode. The half-reaction occurring at the auxiliary electrode should be fast enough so as not to limit the process at the working electrode. The potential of the auxiliary electrode is not measured against the reference electrode but is used to balance the reaction occurring at the working electrode. This configuration allows the potential of the working electrode to be measured against a known reference electrode. The auxiliary electrode is often coated with an electrochemically inert material such as platinum, gold or carbon.

16.4.12.3 Working Electrode

The working electrode is where the reaction occurs in an electrochemical system (Allen & Faulkner 2000; Kissinger & Heineman 1996; Zoski 2007). In an electrochemical system with three electrodes, the working electrode can be referred to as either anodic or cathodic depending on the reaction occurring. There are different kinds of working electrodes: glassy carbon electrode, Pt electrode, screen-printed electrode, gold electrode, silver electrode, indium tin oxide-coated glass electrode, carbon nanotube paste electrode, carbon paste electrode, etc. Screen-printed electrodes are prepared with ink deposits on the electrode substrate (glass, plastic or ceramic) in the form of thin films. Different inks can be used to obtain different dimensions and shapes of biosensors. Screen-printed (electrochemical) cells are widely used for the development of amperometric biosensors because these biosensors are cheap and can be produced at large scale (Figure 16.5) (Koyun et al. 2012).

16.4.13 Potentiometric Biosensors

Potentiometric biosensors use ion-selective electrodes in order to transduce the biological reaction into an electrical signal. Thus, they are simply composed of an

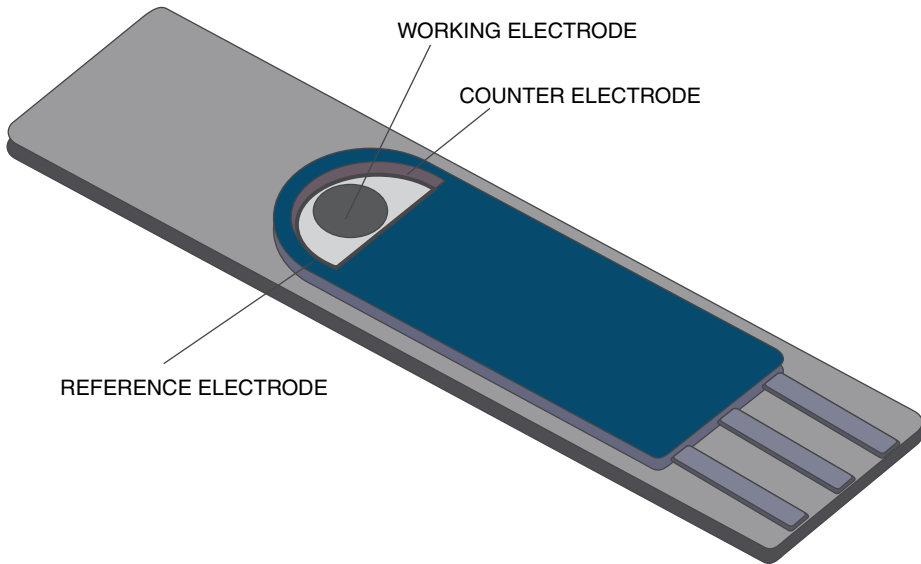


Figure 16.5 Electrochemical biosensor as screen-printed electrode. *Source:* Koyun et al. (2012). www.intechopen.com/books/a-roadmap-of-biomedical-engineers-and-milestones/biosensors-and-their-principles. Licensed under CC BY 3.0.

immobilized enzyme membrane which surrounds the probe from a pH meter and the hydrogen ions are generated or absorbed here via a catalyzed reaction. The reaction happening adjunct to the thin sensing glass membrane directs the change in pH which can be read directly from the pH meter's display. A light-addressable potentiometric sensor (LAPS) for the detection of pathogens has been reported (Byrne et al. 2009). Gehring et al. (1998) developed an immune-ligand assay (ILA) in conjunction with a LAPS for the rapid detection of *E. coli* O157: H7 cells in buffered saline. Zhang et al. (2013) developed a potentiometric flow biosensor based on ammonia oxidizing bacteria for the detection of toxicity caused by the presence of pollutants in water.

16.4.14 Calorimeters

Calorimetric transducers measure the heat of a biochemical reaction at the sensing element. These devices can be classified according to the way heat is transferred. Isothermal calorimeters maintain the reaction cell at constant temperature using Joule heating or Peltier cooling and the amount of energy required is measured. Heat conduction calorimeters measure the temperature difference between the reaction vessel and an isothermal heat sink surrounding it. Using highly conducting materials ensures quick heat transfer between the reaction cell and the heat sink. Finally, the most commonly used is the isoperibol calorimeter that also measures the temperature difference between the reaction cell and an isothermal jacket surrounding it. However, in this case, the reaction cell is thermally insulated (adiabatic). This calorimeter has the advantage of being easily coupled to flow injection analysis systems (Kröger & Danielsson 1997).

16.4.15 Surface Plasmon Resonance-based Sensors

Surface plasmon resonance (SPR) is a phenomenon that occurs during optical illumination of a metal surface and can be harnessed for biomolecular interaction analysis (BIA) (Liedberg et al. 1995). It is best described as a charge density oscillation at the interface between two media with oppositely charged dielectric constants. Plasmons represent the “excited” free electron portion of the surface metal layer. This resonant excitation is provided by compatible light energy photons. The amplitude of the resulting plasmon electromagnetic or evanescent wave is maximal at the interface between the plasmon generating (metal) and the emergent (ambient) medium (Salamon et al. 1999). The ambient medium is generally aqueous phase and thus less dense, with correspondingly lower refractive indices, and is penetrated by the evanescent wave to a depth of approximately one wavelength. Typically, guided waves propagate in a confining structure such as an optical fiber, whereas the surface plasmon wave (SPW) is guided by the metal–dielectric interface (Tubb et al. 1997). Figure 16.6 provides a simplified overview of the detection principle.

16.4.16 Piezoelectric Biosensors

The piezoelectric principle usually describes the properties of crystals and their ability to generate electrical potential in response to a mechanical force. This makes piezoelectric biosensors suitable for direct label-free detection of specific nucleic acid targets, making them useful for foodborne pathogen detection. A popular type of piezoelectric biosensor is the quartz crystal microbalance shown in Figure 16.7. Specific oligonucleotide patterns are immobilized on the surface of the quartz crystal and placed in a solution containing potential target nucleic acids. Once the target nucleic acids start to bind to their complementary oligonucleotides, the mass of the piezoelectric biosensor increases with

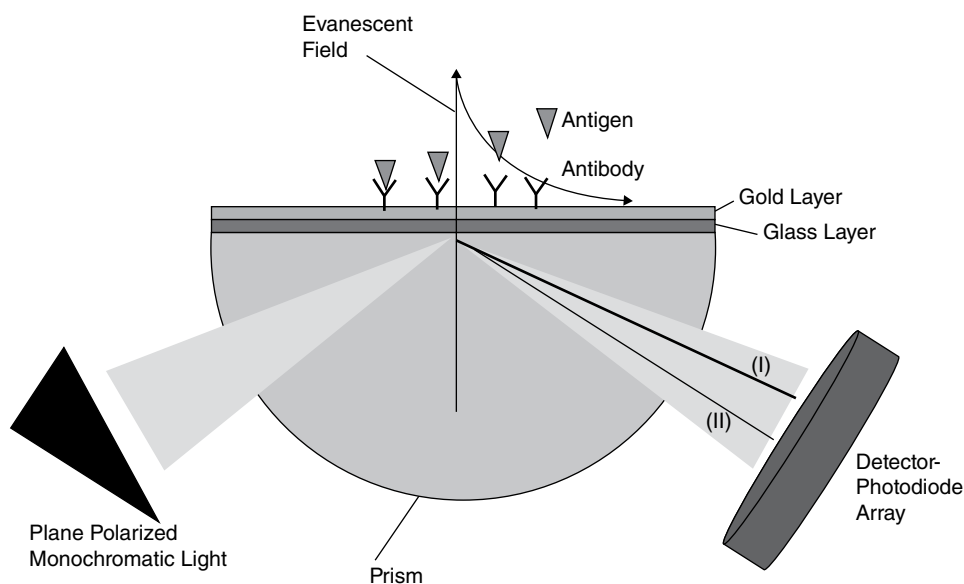


Figure 16.6 Diagrammatic illustration of the SPR principle (Quinn & Kennedy 1999) showing the Kretschmann (Kretschmann 1971) prism arrangement of the type used in BIA core instrumentation. Source: Leonard et al. (2003). Reproduced with permission of Elsevier.

a proportional decrease in the resonance frequency of the quartz oscillation (Zourob 2010).

Piezoelectric biosensors have a number of advantages including real-time output, cost-effectiveness, and ease of use. However, they are generally difficult to regenerate after hybridization. It is postulated that due to a decrease in manufacturing costs, these detectors may become a cost-effective one-time tool. Other disadvantages include lack of specificity and sensitivity, and interference at the sensor surface (Zourob 2010).

16.4.17 Acoustic Wave-based Biosensors

Electroacoustic devices used in biosensors are based on the detection of a change of mass density, elastic, viscoelastic electric, or dielectric properties of a membrane made of chemically interactive materials in contact with a piezoelectric material. Bulk acoustic wave (BAW) and surface acoustic wave (SAW) propagation transducers are commonly used. In the first, a crystal resonator, usually quartz, is connected to an amplifier to form an oscillator whose resonant frequency is a function of the properties of two membranes attached to it. The latter is based on the propagation of SAWs along a layer of a substrate covered by the membrane whose properties affect the propagation loss and phase velocity of the wave. SAWs are produced and measured by metal interdigital transducers deposited on the piezoelectric substrate as shown in Figure 16.8.

Acoustic wave biosensors are based on the detection of mechanical acoustic waves and incorporate a biological component. These are mass sensitive detectors which

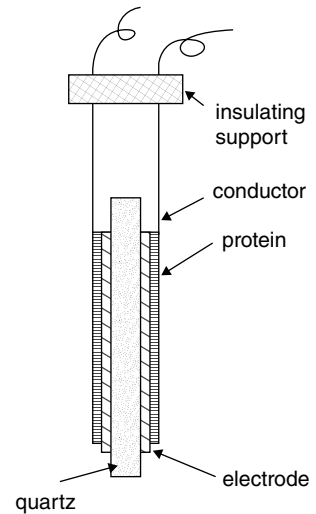


Figure 16.7 Piezoelectric biosensor. *Source:* Mungroo & Neethirajan (2014). <http://www.mdpi.com/2079-6374/4/4/472>. Licensed under CC BY 4.0.

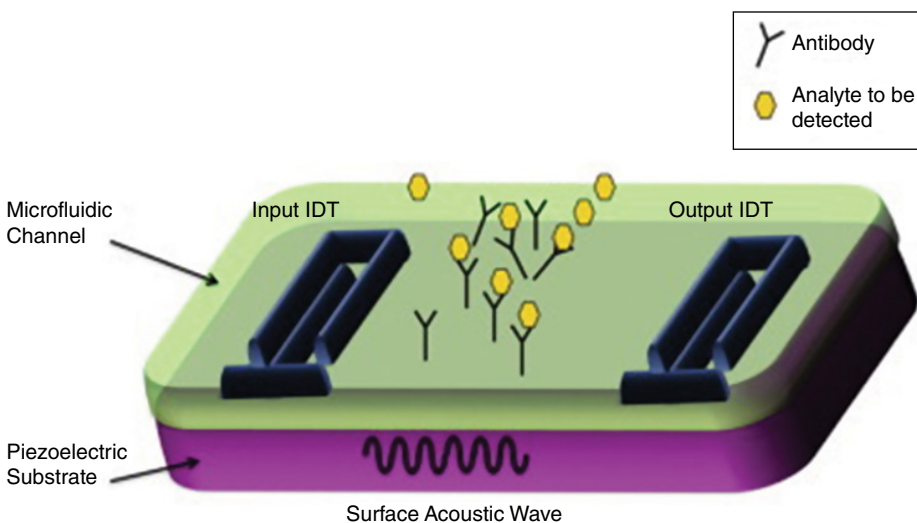


Figure 16.8 Surface acoustic wave propagation sensor. *Source:* Voiculescu & Nordin (2012). Reproduced with permission of Elsevier.

operate on the basis of an oscillating crystal that resonates at a fundamental frequency (Babacan et al. 2000). After the crystal has been coated with a biological reagent (such as an antibody) and exposed to the particular antigen, a quantifiable change occurs in the resonant frequency of the crystal which correlates to mass changes at the crystal surface (Griffiths & Hall 1993). The vast majority of acoustic wave biosensors utilize piezoelectric materials as the signal transducers. Piezoelectric materials are ideal for use in this application due to their ability to generate and transmit acoustic waves in a frequency-dependent manner (Babacan et al. 2000). The physical dimensions and properties of the piezoelectric material influence the optimal resonant frequency for transmission of the acoustic wave. The most commonly used piezoelectric materials include quartz (SiO_2) and lithium niobate (LiTaO_3) (Griffiths & Hall 1993). In order to acquire an active surface for use in a piezoelectric biosensor, the surface must be stable chemically, contain a high number of actively immobilized biological elements, and the coating surface should also be as thin as possible (Babacan et al. 2000).

Acoustic wave biosensors offer label-free, on-line analysis for antigen–antibody interactions and also provide the option of several immunoassay formats, which allow increased detection sensitivity and specificity. Other advantages include cost-effectiveness combined with ease of use. Disadvantages include relatively long incubation times for the bacteria and biosensor surface, problems with crystal surface regeneration, and the number of washing and drying steps required (Invitski et al. 1999).

16.4.18 Bioactive Paper Sensor

Organophosphates are the pesticides most often found on fresh fruits and vegetables. Bioactive paper sensors have been developed to determine the presence of neurotoxins and organophosphates. These are paper-based solid-phase biosensors containing acetylcholinesterase (AChE) that utilizes piezoelectric inkjet printing of biocompatible, enzyme-doped, sol-gel based inks to create colorimetric sensor strips (Kapoor et al. 2015). Their construction includes entrapping of AChE and a chromogenic substrate, indophenyl acetate (IPA), using biocompatible sol-gel derived silica inks in two different zones (sensing and substrate zones). Polyvinyl amine (which captures anionic agents) is first printed and then AChE is overprinted by sandwiching the enzyme within two layers of biocompatible sol-gel derived silica on paper. The sample is first introduced to the sensing zones via lateral flow of a pesticide-containing solution. Following an incubation period, the opposite end of the paper support is placed into distilled deionized water to allow lateral flow in the opposite direction to move paper-bound IPA to the sensing area to initiate enzyme-catalyzed hydrolysis of the substrate, causing a yellow to blue color change. AChE inhibitors, including paraoxon and aflatoxin B1, were detected successfully using this sensor by measuring the residual activity of AChE on paper, using a colorimetric assay, which provided good detection limits and rapid response times of less than 5 minutes (Hossain et al. 2009).

16.5 Conclusions and Future Perspectives

The most important task for the food industry is to produce safe food and in response to this, food companies frequently have a quality control section that deals with pathogen detection using culture techniques and bioassays such as ELISA for determining

and enumerating pathogens in the food product. Although conventional methods for the detection and identification of microbial contaminants can be very sensitive, inexpensive and present both qualitative and quantitative information, they may require several days to yield results. Biosensors offer an exciting alternative to the more traditional methods, allowing rapid “real-time” and multiple analyses that are essential for the detection of bacteria in food, especially perishable or semi-perishable foods.

One of the major applications of biosensors is in the poultry industry as poultry products are a major cause of human foodborne illness due to contamination with pathogens such as *Salmonella* spp. and *Campylobacter* spp.

Biosensors that are used for the measurement of carbohydrates, acids, and alcohols are commercially available. These biosensors are mostly used in quality assurance laboratories or on-line coupled to the processing line through a flow injection analysis system. Their implementation in-line is limited by the need for sterility, frequent calibration, analyte dilution, etc.

Potential applications of enzyme-based biosensors to food quality control include measurement of amino acids, amines, amides, heterocyclic compounds, carbohydrates, carboxylic acids, gases, co-factors, inorganic ions, alcohols, and phenols. Biosensors can be used in processes making yogurt, soft drinks, wine, and beer. Immunosensors have an important potential in ensuring food safety by rapidly detecting pathogenic microorganisms in fresh meat, poultry or fish.

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17

Molecular Identification and Detection of Foodborne and Feedborne Mycotoxigenic Fungi

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17.1 Mycotoxigenic Fungi

Foods such as grains, cereals, and derivatives are highly susceptible to fungal contamination throughout the world. A number of these contaminating fungal species also produce mycotoxins, which are defined as small molecules of low molecular weight, which are natural products of secondary metabolism. These metabolites rarely affect fungal growth and reproduction under laboratory conditions, but play important roles in fungal ecology and adaptation, providing protection to UV radiation and serving as repellants against predators (Reverberi et al. 2010). As a toxigenic set of different chemical classes, mycotoxins can have deleterious effects on vertebrates and other groups of animals, even at low concentrations (Bennett 1987). Mycotoxins vary in toxicity, with some lethal and others causing little impact on human and animal health.

Over 300 mycotoxins have been reported to date (Jestoi et al. 2004). On the basis of extensive analytical studies and detailed distribution studies in nature for these fungi, the International Agency for Research on Cancer (IARC) has highlighted aflatoxins, fumonisins, ochratoxins, zearalenone, and deoxynivalenol (trichothecenes) as the most important mycotoxins in agriculture. In addition to being carcinogenic, these toxins are also immunosuppressive, neurotoxic, and nephrotoxic. Aflatoxins are produced mainly by the fungi *Aspergillus flavus* (teleomorph *Petromyces flavus*), *Aspergillus parasiticus* (teleomorph *Petromyces parasiticus*), and *Aspergillus nomius* (teleomorph *Petromyces nomius*) (Horn et al. 2009, 2011). These fungi are distributed worldwide and are frequently found on substrates such as Brazil nuts and peanuts. Fumonisins are mainly produced by *Fusarium moniliforme* (teleomorph *Gibberella fujikuroi*), *Fusarium proliferatum* (teleomorph *Gibberella intermedia*), and *Fusarium verticillioides* (teleomorph *Gibberella moniliformis*) (Munkvold & Desjardins 1997; Rheeder et al. 2002) which are typically found on corn, wheat, and cereals. Ochratoxins (OTA) are

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produced by *Aspergillus carbonarius* (teleomorph *Sterigmatocystis carbonaria*) and *A. ochraceus* (teleomorph *Sterigmatocystis ochracea*) (www.mycobank.org). OTA contamination is commonly associated with cereals, fresh grapes, dried vine fruit, wine, beer, cocoa, and coffee (Durand et al. 2013). *Penicillium nordicum* and *P. verrucosum*, contaminate substrates such as grape, coffee, and corn. The zearalenones are produced by *Fusarium graminearum* (teleomorph *Gibberella zeae*), *F. culmorum*, and *F. sporotrichioides*, and are frequently found on corn, sorghum, and wheat. The deoxynivalenols are known to be produced by *F. graminearum*, *F. culmorum*, and *F. sporotrichioides*, with contamination common on wheat, corn, and barley (Council for Agricultural Science and Technology [CAST] 2003).

17.2 Polymerase Chain Reaction-based Characterization of Mycotoxigenic Fungi

Fungal taxonomy is dynamic and sometimes controversial. Morphological approaches can group different species due to convergence of characteristics, as well as being limited in taxonomic resolution, unable to detect species divergence observed in genotypes when species are morphologically indistinguishable (Peterson 2012). Morphotaxonomic methods can also be laborious and time-consuming, making molecular tools universally more applicable to fungal taxonomy.

The polymerase chain reaction (PCR) is a molecular tool that enables DNA amplification from just a few cells or fungal spores. Through comparison of polymorphisms in amplified DNA regions, differences that may not be apparent based on phenotypic characteristics may be revealed. Given the potential sensitivity and accuracy in this method, PCR can enable the accurate identification and sensitive detection of fungal DNA from picogram (pg) or femtogram (fg) quantities of target material (Abdin et al. 2010).

Various PCR-based methods have been applied in the characterization and identification of mycotoxin-producing fungi and have revolutionized agricultural and clinical diagnosis technology. These methods utilize small oligonucleotides for the characterization of target genomic regions for the specific microorganism, which can reveal differences at the interspecific and intraspecific levels.

Random amplified polymorphic DNA (RAPD) is a technique that allows analysis of genotypic variability in samples based on comparison of amplified random segments of genomic DNA. No prior knowledge of the target sequences is required with this approach, with the short oligonucleotide primers binding to unknown regions in the genome. RAPD has been employed for characterization of genetic variability in mycotoxin-producing fungi such as *F. graminearum* and *A. flavus*, revealing fungal population structures from different geographic regions and correlation with mycotoxin-producing strains, as well as pathogenicity (Carter et al. 2002; Jamali et al. 2012).

Restriction fragment length polymorphism (RFLP) is a technique that can reveal differences in homologous genomic regions based upon differences in restriction enzyme recognition sites. PCR-RFLP, which is often based on PCR amplification of the ribosomal DNA (rDNA) internal transcribed region (ITS) and subsequent digestion with endonucleases, has been widely employed for the identification of fungal contaminants.

In the case of mycotoxin-producing fungi, restriction endonuclease digestion analysis of the ITS region for *Aspergillus* spp. collected from vineyards enabled identification of the ochratoxin A-producing fungi *Aspergillus niger*, *A. carbonarius*, *A. tubingensis*, *A. japonicus*, and *A. aculeatus* (Spadaro et al. 2012).

Microarray technology has also been employed for molecular identification and detection of mycotoxin-producing fungi. In addition to the speed and sensitivity, where detection limits of femtograms (fg) can be reached, microarrays allow for detection of more than one parameter in a single analysis. A typical microarray consists of numerous specific oligonucleotides, homologous to target genomic regions of interest, which are fluorescently labeled and immobilized on a solid support. Since the development of the first Affymetrix multi-pathogen identification (MPID) microarray chip (Wilson et al. 2002), for identification of 18 prokaryotic, eukaryotic and virus pathogens, microarray chips have been created for molecular identification and detection of mycotoxin-producing fungi. Genomic regions encoding the mycotoxin biosynthetic pathway enzymes and products have been the target for microarray identification of potential mycotoxin-producing fungi. For example, Schmidt-Heydt and Geisen (2007) developed a microarray with oligonucleotides for trichothecene type A and B coding genes in *F. sporotrichioides* (type A) and *F. graminearum* (type B), aflatoxin coding genes in *A. flavus*, ochratoxin A coding genes in *P. nordicum*, fumonisin coding genes in *F. verticillioides*, and patulin coding genes in *P. expansum*. Species-level variation in rDNA ITS sequences has also been adapted in microarray format for differentiation of trichothecene-producing species *F. graminearum*, *F. pseudograminearum*, *F. culmorum*, *F. avenaceum*, *F. poae*, *F. sporotrichioides*, *F. equiseti*, *F. langsethiae*, and *F. tricinctum* (Nicolaisen et al. 2005).

Offering even greater sensitivity, quantitative PCR (qPCR) relies upon the simultaneous amplification and quantification of target DNA molecules. Two possible methods of amplicon detection can be employed, the first using a non-specific fluorescent dye, which may bind to any double-stranded DNA, and the second a fluorescent-labeled specific DNA probe, which reveals amplicon quantification only after hybridization. A number of qPCR systems based on multiple diagnosis of mycotoxin-producing fungi have been described. For example, Vegi and Wolf-Hall (2013) developed a method using TaqMan probes for simultaneous detection and quantification of *Fusarium*, *Penicillium*, and *Aspergillus* mycotoxin-producing species on cereal grains, targeting trichothecene synthase (*Tri5*) genes, rRNA genes and a polyketide synthase (*PKs*) gene, for the respective fungi. As well as offering specificity, a limit of detectability of 3 pg of genomic DNA was observed.

Another technique that has been employed for molecular diagnosis of mycotoxin-producing fungi is PCR-ELISA. In this technique, specific PCR amplicons are hybridized with fluorescent probes, with signal detection based on ELISA immunological techniques. Similar to qPCR, PCR-ELISA quantifies PCR products specific to the probes. Applications have focused on molecular identification for differentiation of mycotoxin-producing and non-producing fungi. For example, Grimm and Geisen (1998) developed a PCR-ELISA assay targeting the ITS rDNA region for fumonisin-producing species, differentiating between *Fusarium* species *F. moniliforme*, *F. proliferatum*, *F. nygamai*, *F. napiforme*, *F. poae*, and *F. solani*. Although PCR-ELISA offers potential for widespread uptake in terms of cost and simplicity, problems with reproducibility and false-negative results have been reported (Zheng et al. 2006).

Amongst the different PCR-based molecular diagnostic tools developed for detection and identification of mycotoxigenic fungi, two classes of genes have been widely exploited as appropriate targets, namely those involved in cellular function and genes involved in mycotoxin biosynthesis (Morcia et al. 2013).

17.2.1 Genes Involved in Cellular Function

17.2.1.1 Nuclear Ribosomal DNA (rDNA)

The nuclear ribosomal DNA (rDNA) cluster region in eukaryotic organisms has been widely employed over the last 20 years in phylogeny, molecular identification, and detection of fungi (White et al. 1990). This target region consists of three conserved transcribed rRNA-encoding genes, namely 18S, 5.8S, and 28S, together with two variable non-coding internal transcribed spacers (ITS1 and ITS2), which are eliminated following transcription. In addition to the tandem repeat nature of this region, which can facilitate PCR amplification, the presence of both conserved and variable regions has enabled widespread application for evolutionary divergence analysis. The ITS rDNA region is today a recognized molecular marker by the Barcode of Life Consortium (www.barcodeoflife.org/) for resolving fungal species. In the case of mycotoxigenic fungi, application has included PCR-RFLP analysis of the ITS rDNA region for identification of *Aspergillus niger*, *A. tubingensis*, *A. japonicus*, *A. aculeatus*, and *A. carbonarius*, the major ochratoxin A producer collected from vineyards (Spadaro et al. 2012). Similarly, Midorikawa et al. (2008) developed a specific PCR system with an internal amplification control that targeted regions of the rDNA ITS region for *A. flavus* on peanut and Brazil nut.

In a large study focusing on diversity of *Aspergillus* section *Flavi* member species on Brazil nut samples from the Amazon forest, macro- and micromorphology, mycotoxin quantification, and partial sequencing of ITS rDNA, beta-tubulin, and calmodulin genes enabled accurate identification of *A. flavus*, *A. parasiticus*, and *A. nomius* as the principal contaminants on this host (Baquião et al. 2013). Specific primers designed for qPCR for the ITS2 rDNA region have been developed for molecular identification, detection, and quantification of aflatoxigenic species *A. flavus* and *A. parasiticus* from wheat flour (Sardiñas et al. 2011).

In the context of multiplex PCR-based methods for identifying mycotoxin-producing fungi, target genes such as the ITS rDNA region have been used for the design of species-specific primers or probes for the identification of various mycotoxigenic species. Suanthie et al (2009) reported a system based on TaqMan probes for mycotoxin-producing species of *Fusarium*, *Penicillium*, and *Aspergillus* on distillers' grain material. Over 40 *Aspergillus* species, 23 *Fusarium* species, and 32 *Penicillium* species were identified, as well as 64 other fungal species. The sensitivity of the method for detection ranged from 1 pg to 10 ng of genomic DNA.

17.2.1.2 Mitochondrial DNA

Mitochondrial DNA (mtDNA) has also been employed for molecular identification and detection of mycotoxin-producing fungi. MtDNA codes for messenger RNA, transfer RNA, and ribosomal RNA, as well as essential proteins and enzymes for formation of functional mitochondria involved in electron transfer and ATP synthesis processes, such as the enzymes cytochrome b, cytochrome oxidase, and ATPase subunits. The mtDNA

rRNA genes and non-coding regions have been employed in fungal molecular diagnosis, frequently at an intermediary taxonomic level such as fungal genus (Bruns et al. 1992). MtDNA is also an attractive target for molecular identification of fungal species. Varga et al. (1994), for example, developed a simple mtDNA RFLP approach for differentiation of *Aspergillus* species *A. niger*, *A. tubingensis*, and *A. brasiliensis*. Specific primers for the fungal mtDNA region are also molecular tools that contribute to the identification of mycotoxin-producing fungi. In an attempt to develop a PCR-based method for identification of aflatoxigenic *Aspergillus* section *Flavi* aflatoxin-producing species *A. flavus*, *A. tamarii*, and *A. nomius* on Brazil nuts, primers targeting the mtDNA SSU rRNA region and specific for the genus *Aspergillus* were developed, with differentiation at the species level based on RFLP polymorphisms (Midorikawa et al. 2014).

In addition to applications in molecular identification, mitochondrial DNA can also be appropriate for analysis of fungal population diversity. For example, Cunnington (2006) employed the intergenic mitochondrial region *nad5-arg2* in multi-locus sequence typing (MLST) of *F. oxysporum* populations, with this intergenic region as variable as the mtDNA SSU rRNA region.

17.2.1.3 Beta-tubulin Gene

As with the nuclear rDNA ITS and mtDNA rRNA regions, the beta-tubulin gene region is widely employed in molecular taxonomy and phylogeny. The best-known tubulins in eukaryotes are alpha-tubulin and beta-tubulin, which perform important cellular functions related to cell division, as components of microtubules. Given that the beta-tubulin gene is a multicopy gene, which facilitates amplification in PCR reactions and is also highly variable between fungal groups, this gene has become a frequent target region for phylogenetic studies and molecular identification (Hubka & Kolarik 2012). For the genus *Fusarium*, for example, which contains many mycotoxigenic species, the beta-tubulin gene has 3.5 times more variable information than the mtDNA SSU rRNA gene, considered a favorable region in taxonomic and phylogenetic studies as well as taxonomic analysis for large groups of eukaryote comparison (Baldauf & Palmer 1993; O'Donnell et al. 1998). In the case of the genus *Penicillium*, which also contains a number of mycotoxigenic species, the beta-tubulin gene has also been employed in resolving phylogenetic relationships (Frisvad & Samson 2004; Samson et al. 2004; Seifert & Louis-Seize 2000).

Amplification of specific target regions of the beta-tubulin gene has also enabled molecular identification and detection of mycotoxigenic fungi in contaminated food and grain. For example, a specific qPCR detection method has been developed for *F. graminearum* on wheat, with sufficient specificity and sensitivity to detect early stages of infection (Reischer et al. 2004).

Molecular identification of species present in different populations of *Aspergillus* section *Nigri* from maize has also been reported. Recently, the putative fumonisin biosynthesis gene cluster involved in the production of fumonisin B2 and B4 was discovered in some *Aspergillus* section *Nigri* (Frisvad et al. 2011). The data for the beta-tubulin gene, together with the *fum8* and calmodulin gene, enabled the identification of *A. niger*, *A. tubingensis*, and *A. welwitschiae* (Susca et al. 2014).

17.2.1.4 Calmodulin Gene

Molecular markers related to gene regions with cellular functions are variable in intronic portions and conserved in exonic portions, with the resolution level varying according

to fungal taxa. Calmodulin is a protein expressed in all eukaryotic cells that binds calcium ions and functions as an intermediary messenger, modifying its interactions with various target proteins (Braun & Schulman 1995). Sequence variability in the calmodulin gene intron regions can be appropriate for species identification, supporting multitarget sequence-based identification based on sequence data for regions such as the previously described rDNA ITS, mtDNA rRNA, and beta-tubulin gene.

In the case of aflatoxigenic *Aspergillus* section *Flavi* species *A. nomius*, *A. flavus*, and *A. parasiticus* from Brazil nut, numerous molecular approaches have been developed for species identification, including RAPD, AFLP, RFLP, sequence data for the rDNA ITS region, beta-tubulin, and calmodulin genes (Baquião et al. 2013; Gonçalves et al. 2012; Massi et al. 2014; Midorikawa et al. 2014). Geiser et al. (2007) suggested that beta-tubulin and calmodulin gene regions are the most promising for molecular identification of the species *Aspergillus*.

For mycotoxigenic *Fusarium* species, primers for the corn contaminants *F. proliferatum*, *F. subglutinans*, and *F. verticillioides* have been developed based on specific ITS rDNA and calmodulin gene regions, with a detectability limit of 12.5 pg of genomic DNA for each species (Mulè et al. 2004).

17.2.2 Barcode of Life Data (BOLD) System

Mycotoxin-producing fungi diagnosis from gene regions with cellular functions is mostly targeted for species identification and these gene regions can be developed for group-specific barcode markers. Ratnasingham and Hebert (2007) developed software called the Barcode of Life Data System (BOLD), a computer platform to help in species identification from DNA barcoding, which offers ways to store, manage, analyze, and display barcode data, integrating molecular, morphological, and geographical data.

The universal DNA barcode marker for fungi is the nuclear ribosomal ITS region and there are more than 134 000 specimen records for fungi in BOLD. The BOLD database for *Fusarium* comprises 937 species and a total of 2424 specimens with barcodes. The *Penicillium* genus comprises 683 species in BOLD with a total of 2539 specimens with barcodes, and the *Aspergillus* genus has 371 species with a total of 1860 specimens with barcodes. This database is easily used for DNA sequence-based species level fungi identification and it has improved the diagnosis of mycotoxigenic fungi.

17.2.3 Metabolic Pathway Genes

Effective molecular identification and detection of mycotoxigenic fungi requires resolution sufficient for closely related taxa. Although sequence data for genes involved in cellular function is widely applied for identification and differentiation of fungal species, variability in such genes can occasionally be insufficient for species differentiation. For example, rDNA ITS regions, whilst differentiating *A. sojae*/*A. parasiticus* from *A. oryzae*/*A. flavus*, cannot differentiate between the individual species. Similarly, whilst the TEF-1-alpha marker is frequently employed as a species level marker, resolution is insufficient for *F. cerealis* and *F. culmorum* (Kristensen et al. 2005).

Genes involved in the synthesis of secondary metabolites are usually clustered along the genome, comprising enzyme-encoding genes and often transcription factors which regulate secondary metabolite synthesis (Osbourn 2010). For mycotoxin-producing

fungal species, molecular identification and detection can target mycotoxin biosynthetic pathway genes as an approach for distinguishing not only at the species level but also at the strain level, differentiating mycotoxigenic and non-mycotoxigenic strains based on insertions or deletions (indels) which can affect gene transcription and protein synthesis (Kim et al. 2008).

17.2.3.1 Aflatoxins/Sterigmatocystin

According to the IARC, aflatoxins (AF), fumonisins (FUM), ochratoxins (OTA), zearalenone (ZEA), and deoxynivalenol (trichothecenes) (TRI) are the mycotoxins most harmful to human and animal health.

In the case of aflatoxins, the gene clusters and regulatory mechanisms responsible for their biosynthesis are well elucidated for *A. flavus*, *A. nomius*, and *A. parasiticus*, which are the main aflatoxin-producing species (Georgianna & Payne 2009). Aflatoxin B1 (AFB1) biosynthesis involves 23 enzymatic reactions, starting from acetyl-CoA synthesis. A total of 15 intermediate precursors are described in the pathway, involving 25 genes clustered in a region of 75 kb DNA (Bhatnagar et al. 2006). This gene cluster also has two transcription factors, *aflR* and *aflS* (*aflJ*). Although known to be activated independently by different promoters, Georgianna and Payne (2009) suggest that the protein synthesized by the *aflS* gene increases the pathway gene transcription when attached to activator *aflR*.

The initial conversion step in the aflatoxin pathway is the conversion of acetate to norsolorinic acid (NOR), involving *aflA* (*fas-2*), *aflB* (*fas-1*), and *aflC* (*pksA*) genes (Trail et al. 1995). This is then followed by a series of conversions, beginning with the conversion of versicolorin B (VERB) to versicolorin A (VERA) by the action of *aflL* gene products (*verB*). The formation of VERA from VERB is a branch point separating biosynthesis of AFB1 and AFG1 (aflatoxin G1) from that of AFB2 and AFG2 (Bhatnagar et al. 1991; Yabe & Hamasaki 1993). The final steps in AFB1 synthesis are driven by two enzymes; the first is an O-methyltransferase A, encoded by the *aflP* gene (*omtA*), which converts sterigmatocystin (ST) in O-methylsterigmatocystin, and the second an oxidoreductase, encoded by the gene *aflQ* (*orda*), which converts O-methylsterigmatocystin to AFB1 (Yu et al. 2004).

In addition to the genes in the aflatoxin biosynthetic pathway cluster, global regulatory genes such as *laeA* can act on the aflatoxin metabolic pathway. The *laeA* gene, known to encode a putative methyltransferase, contains an S-adenosylmethionine (SAM) binding motif. The disruption of *laeA* in *A. nidulans* resulted in loss of the *aflR* gene for sterigmatocystin expression (Bok & Keller 2004). Another global regulator gene involved in aflatoxin production is the *veA* gene, which in addition to its involvement in light-dependent conidiation (Mooney & Yager 1990) and sclerotia formation (Duran et al. 2007), also modulates *aflR* gene expression in *A. flavus*, *A. parasiticus*, and *A. nidulans* (Calvo et al. 2004; Duran et al. 2007; Kato et al. 2003). Multiplex or individual PCR systems using primers developed for the aflatoxin biosynthetic pathway genes *aflD*, *aflR*, *aflS*, *aflM*, *aflO*, and *aflP* have been created for identification and detection of aflatoxigenic species (Rahimi et al. 2008; Shapira et al. 1996). Based on the analysis of *aflR*, *aflS*, *aflE*, and *aflK* genes, a system that differentiates non-aflatoxigenic species such as *A. oryzae* and *A. sojae* from aflatoxigenic species *A. flavus*, *A. toxicarius*, and *A. parasiticus* was also developed (Nakamura et al. 2011).

Genes that code for enzymes that participate in critical steps of the aflatoxin biosynthetic pathway, such as *aflP* and *aflQ*, are useful candidates for developing identification and detection methods that distinguish between aflatoxigenic and non-aflatoxigenic species. In *A. flavus* and *A. parasiticus*, these genes are necessary for the conversion of ST into AFB1. In *A. nidulans*, the lack of aflatoxin-producing ability is related to the loss of functionality or absence of these genes. *A. ochraceoroseus* is an aflatoxin producer which presents an aflatoxin/sterigmatocystin gene cluster which is very similar to that in the non-aflatoxigenic *A. nidulans*, although its aflatoxin-producing ability may be attributed to the presence of the *aflP* and *aflQ* orthologous genes in the genome (Cary et al. 2012).

In many cases, the loss of ability to produce aflatoxins is due to large deletions in the distal biosynthetic pathway, suggesting that these genes may be under low selection pressure (Cary & Ehrlich 2006). Chang et al. (2005) identified eight different patterns of deletion positioned in the distal biosynthetic pathway of non-aflatoxigenic strains of *A. flavus*, with strains of the same vegetative group possessing identical deletion patterns.

Geisen (1996) developed a molecular diagnostic system for aflatoxin-producing fungi based on multiplex PCR, combining specific primers for *aflD*, *aflM*, and *aflP* genes from the *A. parasiticus* biosynthetic pathway. The method distinguished aflatoxin/sterigmatocystin-producing species *A. flavus*, *A. parasiticus*, and *A. versicolor* from non-producing species based on amplicon pattern. Transcription factors encoding genes such as *aflR* and *aflJ*, together with final precursor pathway genes *aflP* and *aflQ*, have also been target regions for identification and detection of aflatoxigenic *A. flavus* and *A. parasiticus* (Manonmani et al. 2005; Paterson 2006; Rahimi et al. 2008; Rodrigues et al. 2007).

17.2.3.2 Fumonisin

Fumonisin, commonly found in corn, are produced by a number of *Fusarium* spp., of which *F. verticillioides* and *F. proliferatum* are considered to be the principal fumonisin-producing species. The fumonisin gene cluster of 17 genes is located over a 45 kb genome region. Based on amino acid sequences, the *fum21* gene is the only Zn(II)2Cys6 transcription factor that regulates gene expression in the *fum* cluster (Brown et al. 2007).

Fumonisin biosynthesis requires three initial crucial steps in order to form the toxin. In the first step, the *fum1* gene encodes a polyketide synthase (*pks*), leading to synthesis of a 10,14-dimethyl-octadecanoic acid-like polyketide. The second and third steps comprise hydroxylation from an alpha-oxoamine synthase (*fum8*) followed by a reductase and cytochrome P450 monooxygenase (*fum6*). Other genes such as *fum13*, encoding a C-3 carbonyl reductase, and *fum3* and *fum2*, which encode enzymes utilizing molecular oxygen to catalyze the C-5 and C-10, as well as *fum7*, *fum10*, *fum11*, and *fum14* genes, which are required for tricarballic acid esterification, also form part of the fumonisin biosynthetic pathway. Additional genes that encode transporter proteins such as *fum19*, *fum17*, and *fum18* are also indirectly involved in fumonisin biosynthesis or are not essential for the production of fumonisin B1 (FB1), fumonisin B2 (FB2), fumonisin B3 (FB3), and fumonisin B4 (FB4) (Alexander et al. 2009).

In an attempt to identify diverse mycotoxigenic fungal species, a multiplex PCR method was developed to enable detection of aflatoxin-producing fungi (*aflD*), trichothecene producers (*tri6*), fumonisin producers (*fum13*), and ochratoxin A

producers (*otanps*) based on the presence of target biosynthetic pathway genes. The method incorporated a competitive internal amplification control and was optimized with total DNA isolated from pure cultures from maize contaminated by mycotoxigenic fungal samples. Multiplex PCR results confirming the presence of mycotoxigenic species were supported by high-performance liquid chromatography analysis (HPLC) for mycotoxin presence (Rashmi et al. 2013).

A similar approach was conducted using multiple markers based on essential genes for the fumonisin biosynthetic pathway (*fum6* and *fum8*), trichothecene pathway (*tri5* and *tri6*) and zearalenone pathway (*zea2*). A total of 96 isolates representing several filamentous fungal species were analyzed. Results showed detection reliability and sensitivity for trichothecene-, zearalenone-, and fumonisin-producing strains (Dawidziuk et al. 2014).

17.2.3.3 Trichothecenes

The main trichothecene-producing fungi are *F. culmorum*, *F. graminearum*, *F. poae*, *F. sambucinum*, and *F. sporotrichioides* (Rheeder et al. 2002; Starkey et al. 2007). The metabolic pathway and genes involved in trichothecene biosynthesis are well elucidated for *F. graminearum*. Located over a 25 kb cluster and composed of 12 genes, the trichothecene pathway contains two regulatory genes, *tri6* and *tri10*. *Tri6* is a classic Cys2His2 zinc finger protein that regulates most trichothecene genes, with expression under regulation by *tri10* (Proctor et al. 1995; Tag et al. 2001).

The first step in trichothecene biosynthesis is the cyclization of farnesyl pyrophosphate (FPP) into trichodiene, resulting from a catabolic processes conducted by a trichodiene synthase encoded by the *tri5* gene. The *tri4*, *tri101*, *tri11*, and *tri3* genes encode enzymes involved in nine catabolic reactions, which result in formation of calonectrin. Catabolysis can then occur, resulting in trichothecene type A (T-2 toxin) and trichothecene type B (NIV and DON) (Alexander et al. 2009; Kimura et al. 2007).

In addition to molecular techniques based on specific PCR, a loop-mediated isothermal amplification (LAMP) method for specific DNA synthesis has been described, with amplicon detection based on color reaction. This methodology has been used in detection assays for numerous bacterial pathogens and fungal contaminants. Denschlag et al. (2014) developed a LAMP method for the diagnosis of trichothecene-producing *Fusarium* species *F. graminearum*, *F. culmorum*, *F. cerealis*, *F. sporotrichioides*, *F. poae*, and *F. langsethiae*. The combination of two sets of LAMP specific primers for the genes *Tri6* and *Tri5* in a multiplex PCR was capable of detecting *Fusarium* species mentioned.

17.2.3.4 Zearalenones

As with fumonisins and trichothecenes, zearalenones are also produced by a number of *Fusarium* spp. such as *F. culmorum*, *F. sporotrichioides*, *F. esquiseti*, and *F. graminearum*. Zearalenone is a polyketide produced from an acetate-malonate unit by the polymalonate acetate biosynthetic pathway. The *F. graminearum* zearalenone biosynthetic pathway possesses the reducing and non-reducing polyketide synthases, *pks4* and *pks13*, transcription factor *zeb2*, and an isoamyl alcohol oxidase *zeb1*, all of which are essential genes for the production of zearalenone (Gaffoor & Trail 2006; Kim et al. 2005; Lysøe et al. 2006, 2009).

Baturo-Ciesniewska and Suchorzynska (2011) developed a PCR-based system for trichothecenes, nivalenol, and zearalenone-producing mycotoxigenic fungi on cereals, with species presence correlating with mycotoxin presence based on HPLC MS/MS analysis.

17.2.3.5 Ochratoxins

Ochratoxins are produced by fungi from the genera *Aspergillus* and *Penicillium*, with *A. ochraceus*, *A. carbonarius*, and *P. verrucosum* considered the main producing species. In contrast to the advances made in characterization of the aflatoxin and fumonisin biosynthetic pathways, the OTA biosynthetic pathway is not yet completely elucidated (Gallo et al. 2012). Studies so far have characterized the *pks* gene region in *P. nordicum*, which includes genes that encode *pks* (*otapksPN*), the non-ribosomal peptide synthetase (*otanpsPN*), a homolog to a non-ribosomal peptide synthase, a gene (*otachlPN*) homologous to chlorinating enzyme, and a gene (*otatraPN*) homologous to the carrier protein involved in OTA export (Karolewicz & Geisen 2005). Of the additional *Penicillium* producing species, the orthologous gene (*otapksPV*) has been identified in the OTA biosynthetic pathway in *P. verrucosum* (Geisen et al. 2006; O'Callaghan et al. 2013).

Gene deletion studies with these genes in OTA-producing strains of *A. carbonarius* strains confirmed their involvement in production of this mycotoxin. *Aspergillus* species such as *A. carbonarius* also harbor genes encoding *pks* (*AcOTApks*) (Gallo et al. 2014), as well as NRPS (*AcOTAnrps*), both of which are involved in the initial OTA pathway steps (Gallo et al. 2012). Further candidate genes involved in OTA production have been identified through comparative gene expression analysis in OTA-producing and non-producing strains of *A. westerdijkiae*, with cytochrome P450, 3-hydroxyphenylacetate-6-hydroxylase and endoplasmic reticulum oxidoreductin encoding genes positively modulated in OTA-producing strains (Sartori et al. 2014).

The *pks* gene is currently the principal gene marker for OTA producer identification. Spadaro and colleagues (2012) developed a specific method based on *pks* gene markers for *Aspergillus* and *Penicillium* species associated with *Vitis vinifera* contamination. Storari and colleagues (2013) also developed an identification and detection system for OTA-producing *A. niger*, *A. awamori*, and *A. carbonarius* on the same host. The methodology, which was based on loop-mediated isothermal amplification (LAMP) combined with specific primers for *pks* genes, enabled greater detectability limits compared to conventional PCR. Markers for the genes *otapksPN* and *otanpsPN* have also been described, enabling specific identification and detection of *P. nordicum* and *P. verrucosum*, differentiating from other *Penicillium* species commonly encountered on meat products (Bogs et al. 2006).

17.3 Genomics of Mycotoxigenic Fungi

Whole genome sequences and resequenced genomes have been made available for a number of mycotoxigenic fungi, with a particular focus to date on member species of the genus *Aspergillus*. The large variability between species and importance of these fungi as phytopathogens and mycotoxin producers, as well as in the enzyme industry, have made the genus *Aspergillus* a model clade for comparative studies in functional genomics between eukaryotes (Gibbons & Rokas 2013).

Aspergillus nidulans, historically considered the model species for the genus, with complete elucidation of its sexual cycle, has been the focus of whole genomic sequencing (WGS) (Galagan et al. 2005). The species *A. oryzae* and *A. niger*, classified as GRAS status (Generally Regarded as Safe), with wide industrial applications (Machida et al. 2005; Pel et al. 2007), have also been the focus of WGS, with data available in the genome databases at the Broad Institute (www.broadinstitute.org), the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the *Aspergillus* Genome Database (www.aspgd.org/). The *A. nidulans* genome, with a total size of 30.06 Mb and 9396 predicted genes, contrasts with the larger genomes of *A. oryzae* and *A. niger*, with respective genome sizes at 37.12 Mb and 37.2 Mb, and gene models totaling 12 336 and 11 200 (Machida et al. 2005; Pel et al. 2007). The *A. flavus* genome is estimated to be similar in size to *A. oryzae*, with 36.8 MB coding 12 424 predicted genes (Gibbons & Rokas 2013; Payne et al. 2006). Comparative analysis between *A. oryzae* and *A. flavus* genomes revealed 99.5% gene homology (Cleveland et al. 2009).

Comparative analysis of the *A. flavus* NRRL 3357 and *A. parasiticus* SU-1 (39 Mb) genomes also showed similarity between these genomes. A total of 13 290 predicted genes were identified in *A. parasiticus*, based on comparison with the 13 487 predicted annotated genes in the *A. flavus* genome, with a total of 96% of open reading frames (ORFs) observed in *A. flavus* having at least one homologue in *A. parasiticus*. In terms of mapping of the gene clusters responsible for the secondary metabolite biosynthesis, a total of 55 gene clusters have been identified in the *A. flavus* genome. Similar gene clustering has also been observed in *A. parasiticus*, although lacking clusters 4, 22, 28, 33, 36, 38, 40, 45, 48, 49, and 55, which include genes responsible for mediating cyclopi-azonic acid (CPA) synthesis (Linz et al. 2014). The whole genome sequence for *A. nomius* NRRL 13137, another aflatoxigenic species, is also now available. With a genome size of 36 Mb, it contains 11 918 predicted genes. A total of 1124 genes, most coding for proteins with oxidoreductase activity, were identified as unique to *A. nomius* according to Gene Ontology (GO) terms (Moore et al. 2015).

The genome sequence for the ochratoxin-producing species *A. carbonarius* was also completed recently (<http://genome.jgi.doe.gov/Aspca3/Aspca3.home.html>). For the strain *A. carbonarius* ITEM 5010 (Acv3), which is an ochratoxin A producer, its 36 Mb genome contains a large number of genes that encode PKS and NRPS, which are involved in this secondary metabolite biosynthetic pathway. Comparative analysis with the resequenced non-toxigenic strain *A. carbonarius* A-2160 revealed 97.2% similarity with the toxigenic strain. A total of 52 661 single nucleotide polymorphisms (SNPs) and 7567 deletion-insertion polymorphisms (DIPs) were identified, and a total of 43 GO families contained more than 5% of the mutated genes. From a total of 24 NRPS and 25 PKS encoding genes, a high number of nonsense and missense mutations were observed, explaining the lack of OTA production by *A. carbonarius* A-2160 (Cabañes et al. 2015). Of the ochratoxin A-producing fungal species, WGS have also been completed for *Penicillium* species *P. nordicum* and *P. verrucosum*, with genome sizes of 30.42 Mb (JNNR00000000.1) and 31.15 Mb (LAKW00000000.1), respectively (www.ncbi.nlm.nih.gov).

Considerable WGS analysis has been conducted for members of the genus *Fusarium*. For example, for *F. graminearum* strain PH-1, which is a trichothecene and fumonisin producer, a 36.1 Mb genome was reported, with 13 332 predicted genes that span four chromosomes (Cuomo et al. 2007). For *F. verticillioides* strain 7600, by contrast, a

genome of 42 Mb and 14 188 predicted genes spanned 11 chromosomes. In a second comparative analysis between these two genomes, 9034 orthologous genes were identified (Sikhakolli et al. 2012). Differences in chromosome size and number among *Fusarium* species can be explained by horizontal transfer events and gene duplication, with comparative analysis between the *F. graminearum*, *F. verticillioides*, and *F. oxysporum* genomes also revealing compartmentalized regions responsible for primary metabolism, reproduction, secondary metabolism, pathogen virulence, and host specificity (Cuomo et al. 2007; Ma et al. 2010, 2013). Comparative analysis between *Aspergillus* and *Fusarium* genomes has also suggested such mechanisms occurring in both genera, resulting in differences in genome size and gene number, with genome and segmental duplication resulting in genome size differences, and horizontal gene transfer shaping genomes between donor and recipient strains (Gibbons & Rokas 2013; Khaldi & Wolfe 2008; Mallet et al. 2010).

Genomics of mycotoxigenic *Fusarium* spp. has revealed approximately 40 distinct secondary metabolite structural family pathways. Surprisingly, a total of 16 PKSs, 19 NRPSs, and eight terpene synthases (TSs) have been identified in the *F. graminearum* genome, indicating that a single species can have the potential to produce a number of mycotoxin secondary metabolites, including fusarines and trichothecenes (Ma et al. 2013).

17.4 Functional Genomics of Mycotoxigenic Fungi

Mycotoxin biosynthesis in *Aspergillus*, *Fusarium*, and *Penicillium* species is a complex biological process which is influenced by external factors such as temperature, humidity, pH, and nutrient availability. Understanding the mechanisms controlling activation and gene modulation during mycotoxin biosynthesis is of considerable importance for the optimization of molecular approaches for identification and detection mycotoxin-producing and non-producing species.

Functional genomics, or transcriptomics, comprises the sequencing of the transcribed portion of the genome, enabling both identification of genes and comparison of gene expression levels. Comparative gene expression analysis on a transcriptome scale offers potential for isolation of differentially expressed genes or genes that regulate essential characteristics for the development and adaptation of microorganisms to environmental conditions (Ward et al. 2006). Functional genomics of mycotoxin-producing fungi began in the late 1990s with large-scale expressed sequence tags (ESTs) sequencing. Since then, numerous studies have contributed to the number of ESTs now deposited in the NCBI GenBank database, where, for mycotoxin-producing species from the genera *Aspergillus* and *Fusarium*, ESTs total 49 389 for *A. flavus*, 28 973 for *A. parasiticus*, 58 123 for *F. graminearum*, and 87 134 for *F. verticillioides*. With focus on aflatoxigenic *A. parasiticus*, OBrian et al. (2003) developed an Affymetrix microarray GeneChip for analysis of expression of over 700 candidate genes, including the key genes of the aflatoxin biosynthetic pathway *aflD*, *aflP*, and *aflR*. Twenty-four and 18 genes were highly expressed during aflatoxin biosynthesis and prior aflatoxin biosynthesis, respectively. The *aflD*, *aflM*, and *aflP* genes were identified among the highly expressed genes.

Next-generation sequencing (NGS) technologies have been used for transcriptome analysis in filamentous fungi, enabling fast and precise characterization of unigenes and

their expression (Mardis 2008). Illumina RNA-seq is a shotgun-based method for transcriptome sequencing, where cDNA is fragmented mechanically to produce small overlapping fragments, which can cover the entire transcriptome. This approach allows the identification of novel exons, isoforms and genes, with a sensitivity also appropriate for characterization of transcripts with low expression levels (Garber et al. 2011).

For better understanding of the genetic mechanisms employed by fungi in mycotoxin production, Linz et al. (2014), conducted a comparative analysis between genomes and gene expression (RNA-seq) in *A. parasiticus* SU-1 and *A. flavus* NRRL 3357 following growth under aflatoxin-inducing (YES medium) and non-inducing (YEP medium) conditions. Differences in the gene cluster and differential gene expression of specific mycotoxigenic genes were observed, which partly explained the different types and concentrations of mycotoxins produced. More than 93 gene clusters related to secondary metabolite synthesis were identified, with over 10% of the genome of these two species devoted to this cellular activity. From a total of 13 290 expressed transcripts, analysis of differential gene expression after growth on YES medium revealed 1.284 and 1.802 upregulated genes in *A. parasiticus* and *A. flavus*, respectively, in relation to gene expression following growth on YEP medium.

RNA-seq analysis of *A. flavus* has also been employed to examine response to external factors such as water activity (a_w). Based on RPKM values, 5362 differentially expressed unigenes ($\geq 1\text{-log}_2\text{Ratio}$) were identified across treatments with water activities that varied between 0.99 and 0.93 a_w . A total of 16 genes related to aflatoxin synthesis were downregulated when water activity was reduced, and 11 genes related to development upregulated after treatment with a higher water activity at 0.99 a_w . This transcriptome data in response to variation a_w agreed with the current model for aflatoxin production in *A. flavus*, where genes responsible for the development and aflatoxin synthesis are both controlled by water activity (Zhang et al. 2014). The interaction of a_w , temperature, and elevated CO_2 is responsible for increasing *A. flavus* aflatoxin production up to 80-fold, which is also associated with an increased expression of the *aflR* and *aflD* genes (Medina et al. 2015).

In order to resolve the OTA biosynthetic pathway more fully, RNA-seq analysis was employed for the identification of differentially expressed genes (DEGs) in *A. carbonarius* under inducing and non-inducing ochratoxin A production conditions. A total of 3705 DEGs were identified among five *pks* (polyketide synthases) genes, with five *nrps* (non-ribosomal peptide synthetases) genes involved in OTA biosynthesis also identified. Additionally, chloroperoxidases, cytochrome P450 monooxygenases, monooxygenases, dehydrogenases, hydrolases, and methyltransferases were identified as being involved in mycotoxin and secondary metabolite synthesis. Oxidoreductases, transporters, and transcription factors were also highly expressed and found to induce OTA biosynthesis (Gerin et al. 2016).

In the case of *F. graminearum*, RNA-seq analysis of a wild-type strain (*F. graminearum* WT) revealed a total of 799 differentially upregulated genes ($\geq 2\text{-fold}$) when compared to *F. graminearum* ΔFgLaeA under trichothecene-inducing conditions. With focus on the function of the *fgLaeA* gene and genes related to mycotoxin production, a total of eight and 17 genes related to transcription factors were identified as down- and upregulated, respectively. Downregulated differentially expressed genes in *F. graminearum* ΔFgLaeA also included seven genes (*tri3-tri14*) from the cluster responsible for trichothecene synthesis. A number of genes belonging to the *pks* and *nrps* clusters were

also downregulated, whilst *pks7* was upregulated (Kim et al. 2013). Chemotype populations of *F. graminearum* 3ADON (3-acetyldeoxynivalenol) and 15ADON (15-acetyldeoxynivalenol), which produce mycotoxins from DON (deoxynivalenol), were analyzed by RNA-seq. A total of 479 upregulated and 801 downregulated genes, involved in C-compound, carbohydrate metabolism and polysaccharide metabolism, were identified in 3ADON populations when compared to gene expression in the 15ADON population. The *tri* genes involved in trichothecene biosynthesis and genes involved in secondary metabolite production were differentially expressed in 3ADON and 15ADON populations when comparing in vitro versus in planta treatments (Puri et al. 2016).

Continued transcriptome analyses of mycotoxin-producing fungi will likely provide important information on novel exons, genes, and isoforms involved in mycotoxin biosynthesis in response to external factors. A better understanding of the regulatory mechanisms in conjunction with knowledge of genetic variability among mycotoxin-producing species will enable further development of accurate identification and detection systems for use in the food and feed industries.

17.5 Conclusions and Future Perspectives

Mycotoxin-producing fungi cause great economic and agricultural losses due to contamination of grains and cereals for human and animal consumption. The development of diagnostic systems based on molecular techniques, which are capable of detecting mycotoxin-producing species, is very important for quality control of agricultural products. DNA markers based on gene regions can enable detection and identification to the species level. For specific detection of potential mycotoxin-producing fungi, however, knowledge of gene clusters and regulatory pathways related to regulation and expression of mycotoxins is necessary. Ongoing characterization of genomes and transcriptomes of mycotoxigenic fungi will no doubt facilitate the development of robust diagnostic methods.

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18

Molecular Identification of Enteric Viruses in Fresh Produce

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18.1 Introduction

Contamination of fresh produce such as berry fruit and leafy green vegetables by foodborne viruses, and the consequent risk to public health, is gaining increased attention (European Food Safety Authority [EFSA] 2013). Several types of pathogenic enteric viruses can cause foodborne disease, but the most significant types associated with outbreaks in which fresh produce has been implicated are norovirus (NoV), which causes gastroenteritis, and hepatitis A virus (HAV). Several outbreaks of hepatitis A and norovirus have been reported in which fresh produce items have been implicated as the vehicle of transmission. Often, they can occur in several countries at once, if internationally traded food is involved. For example, during the so-called “European Berry Outbreak” which occurred in 2013 and 2014, more than 150 cases of hepatitis A were reported in seven countries (EFSA 2014b). Blackberries from Bulgaria and redcurrants from Poland were the most common items eaten by the cases, and HAV was detected in some samples of these foods.

Detection of foodborne viruses is challenging. Enteric viruses are submicroscopically small, and due to their potentially low infectious dose, they only need to be present in very low numbers in a food to pose a risk to the consumer. They do not replicate in foods, and they cannot be increased in number by enrichment of a food sample using artificial growth media. Therefore, in contrast to traditional culture-based techniques used for analysis of bacteria in foods, direct detection methods must be applied in order to ascertain whether a foodstuff is contaminated by viruses.

Detection methods for bacteria in foods are generally composed of two parts – sample treatment and detection assay – and this applies equally to viruses. This chapter will briefly describe processes and techniques which have been applied for detection of viruses in foods, with a critical overview of emerging international standardized methods.

18.2 Sample Treatment

With viruses, most detection assays only work with microliter volumes of sample extract, and therefore sample treatment can be described as the concentration of viruses from a large volume to a very small one. This sample treatment comprises five basic steps:

- sample receipt
- removal of viruses from the food surface
- removal of food solids
- concentration of suspended viruses
- extraction of nucleic acids.

18.3 Sample Receipt

Analysis of a foodstuff must be conceptually considered to begin immediately upon receipt in the laboratory. This is because various aspects of handling and storage may influence the final result.

When fresh produce items arrive at the laboratory, a test portion should be immediately taken for analysis. In several published protocols, test portion sizes have varied from 10 g (Stals et al. 2011) to 100 g (Dubois et al. 2002) although it could be advantageous to use portion sizes similar to those consumed in a meal (Anonymous 1993; Cook & Cliver 2014). Samples can on occasion arrive at the laboratory late in the working day or week. The analyst may consequently have insufficient time to commence and complete the complex procedure, and the samples must consequently be stored. To maintain the validity of the analytical result, it will be necessary to consider storage as part of the process, and the analytical procedure as commencing on sample receipt. Therefore, a sample process control virus (SPCV) should be added at the commencement of the analytical procedure (Ruhanya et al. 2015). The detection of the SPCV at the end of the whole method verifies that the analysis has been performed correctly (D'Agostino et al. 2011; Diez-Valcarce et al. 2011a).

18.4 Removal of Viruses from the Food Surfaces

Viruses contaminate fresh produce extrinsically; that is, they do not occur naturally within these commodities, and are introduced from an external source during primary production, processing, or point of sale/consumption. They are not located within plant tissues but on the plant surfaces. Therefore, the first step in sample treatment is to remove them from these surfaces. Electrostatic and hydrophobic attractions can occur between virus particles and surfaces (Gerba 1984), which need to be broken to mediate release of the particles. These attractions can be broken by increasing the ambient pH to >7 , and this is performed by washing or stomaching the food sample with an alkaline solution or buffer to raise the pH (Kurdziel et al. 2001). The addition of protein, such as beef extract or soya protein, to the eluant increases the effectiveness of the removal process by preventing reattachment of the viruses to the food surface. This step results in virus particles in suspension, along with solid or dissolved food substances.

18.5 Removal of Food Substances

Low-speed centrifugation is generally used to remove suspended food solids out of suspension. Addition of a cationic flocculant (Kostenbader & Cliver 1981) can cause flocculation of food solids, making them more susceptible to sedimentation, and consequently more readily removed by centrifugation. When analyzing soft fruit, pectinase should be added to prevent fruit-derived pectin from being extracted and interfering with downstream processes (Rzeżutka et al. 2005).

After this step, viruses are left in suspension free from the presence of gross solids. The volume of the suspension is still several milliliters, and this must now be reduced, by concentration of the viral particles.

18.6 Concentration of Viruses

The most commonly used method of virus concentration is precipitation of particles out of suspension with polyethylene glycol (PEG) (Crocchi et al. 2008). PEG reduces protein solubility, and addition of it to a virus suspension encourages virus particles to bind to each other, and to food-derived proteins coming out of solution. A short slow-speed centrifugation then pellets the virus particles, which are subsequently resuspended in a small volume of liquid, thus achieving concentration. PEG precipitation can result in high efficiencies of recovery, up to 100%, depending on the type of food matrix and target virus (Lewis & Metcalf 1988). PEG can interfere with downstream processes, so must be removed, commonly using chloroform:butanol.

Another technique which can be used to concentrate viruses is ultracentrifugation out of suspension by sedimentation. Applying a centrifugal force of around $230\,000 \times g$ is highly effective in concentrating all viruses into a pellet at the bottom of the centrifuge tube, which can then be resuspended in a small volume of liquid (Rzeżutka et al. 2006). Ultracentrifugation is very efficient, but requires expensive instrumentation and is unlikely to be widely used. The end result of the concentration step is that the viruses which were in the original food sample are now in suspension in a small volume of liquid, and can then be readily subjected to further analysis.

There are several techniques which can be used to identify viruses in a suspension. With regard to foodborne HAV and NoV, however, techniques such as cell culture, electron microscopy, and enzyme-linked immunosorbent assay are not appropriate, being too laborious, insufficiently sensitive, or in the case of cell culture and NoV not effective as there is no cell line available. The most effective and very widely used method for detection of foodborne viruses is nucleic acid amplification. To employ this, nucleic acids must be purified from the concentrated virus suspension.

18.7 Nucleic Acid Extraction

Early methods used in-house reagents, but for many years highly efficient commercial kits have been available for nucleic acid extraction and purification, some specifically marketed for use on viruses. Most of these kits use the principle devised by Boom et al. (1990) of lysing the virus capsid through denaturation of the protein coat by

guanidinium thiocyanate, then binding the released genomic material to silica, and washing impurities away before eluting the purified nucleic acids into a small volume (generally around 50–100 μL) of solution. Impurity removal is highly important when analyzing complex matrices such as foodstuffs, which will contain many substances which are inhibitory to nucleic acid amplification assays (Rossen et al. 1992) and can be extracted and concentrated from the food sample along with viruses. A disadvantage to the use of nucleic acid extraction kits is that they can be expensive and labor-intensive, comprising several pipetting and microcentrifugation steps. An alternative is to release the viral nucleic acids directly by heat (Cook et al. 1999), and this can be done as a first stage of the amplification reaction. However, a highly purified virus suspension is necessary for this, as this approach will not remove inhibitory substances. The end result of this step is a purified extract containing the nucleic acid from the viruses in the food sample.

All the steps described above together comprise the sample treatment. The efficiency of the sample treatment can be determined experimentally by spiking food samples with a known number of virus particles, and measuring the number of virus genome copies in the final nucleic acid extract, then expressing the latter as a percentage of the former.

The next stage of the method is to apply the detection assay.

18.8 Detection Assay

The assay type most widely used for detection of foodborne viruses is nucleic acid amplification based on the polymerase chain reaction (PCR) (Crocchi et al. 2008). PCR amplifies DNA, but HAV, NoV, and most other foodborne viruses possess an RNA genome. In consequence, reverse transcription (RT) to produce a complementary DNA (cDNA) copy of the viral RNA sequence must be used prior to PCR.

Reverse transcription-PCR has the potential for extreme sensitivity, theoretically being capable of detecting one genome copy in a single reaction. Because it can target specific nucleic acid sequences, identification of a target virus from strain to genus level can be mediated by amplification of characteristic genomic sequences.

In the early years of PCR application, detection of the signal was conventionally performed by gel electrophoresis followed by staining the amplicon with a UV-fluorescent dye and visual examination. This approach has been superseded by “real-time” PCR (Heid et al. 1996). The term “real-time,” while in colloquial usage in most laboratories, is not really fully accurate, and its abbreviation can be confused with that of reverse transcription. The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines have recommended that the term qPCR should be used (Bustin et al. 2009), and this will be followed in this chapter.

In qPCR, sequence-specific fluorescent oligonucleotide probes bind to the amplicon and an increase in fluorescence over time can be monitored. The use of the probes confers an extra level of specificity to a qPCR assay. Amplification of a target molecule results in exponential increase in fluorescence. With the majority of thermocyclers, a fluorescence intensity threshold is set, and when the signal reaches that threshold, it signifies positive detection of the target. The reaction duration necessary for the signal to reach the threshold is expressed as a C_t (“threshold cycle”) value (sometimes

C_p – “crossing point”, or “TOP – “take-off point”). In this chapter, C_q (“quantification cycle”) will be used following the MIQE recommendations (Bustin et al. 2009). The C_t value is the number of thermocycles taken until the threshold is reached; if it takes 30 cycles to reach the threshold, the C_t value is 30. The C_q value can correlate with the number of targets present – the greater the number of targets, the lower the C_q. Consequently, a qPCR assay can be calibrated to facilitate a quantitative determination of the original number of target sequences in the volume of nucleic acid extract used in the reaction, and the results can be expressed as number of genome copies (GC).

As previously mentioned, substances can be extracted and concentrated from the food sample along with viruses, and these substances can inhibit an RT-PCR. Such inhibition has the potential to result in a falsely negative interpretation of a reaction which does not produce a target signal. This can be avoided by the use of an amplification control (AC), a molecule added to a nucleic acid amplification reaction and amplified to demonstrate that the reaction has proceeded successfully (Hoorfar et al. 2004). ACs can be internal (included in all test reactions) or external (in a separate reaction to the test), homologous (amplified using the same oligonucleotide primers as the target) or heterologous (amplified using a different set of primers than those used for the target). ACs are considered essential in nucleic acid amplification-based diagnostics (Hoorfar et al. 2003). Several RT-PCR assays which include internal ACs (IACs) have been incorporated into methods to detect foodborne viruses, for example Kokkinos et al. (2012) and Maunula et al. (2013). The draft international standards ISO/TS 15216-1/2 (International Standards Organization [ISO] 2013a, b) for the RT-qPCR-based quantification and qualitative detection of HAV and NoV in food use external ACs (EACs). The ACs of the ISO (ISO 2013a, b), Kokkinos et al. (2012), and Maunula et al. (2013) are homologous; heterologous ACs can be found in some commercial assays for HAV and NoV.

Isothermal methods for detection of foodborne viruses, such as nucleic acid sequence-based amplification assays (NASBA) (Jean et al. 2004; Lamhoujeb et al. 2008) and loop-mediated amplification (LAMP) assays (Luo et al. 2014), have been described in the literature. However, it is unlikely that either a LAMP- or a NASBA-based method for foodborne virus detection will achieve routine-use status, since the RT-qPCR-based ISO/DIS 15216 method will become the standard and is likely to remain so for several years.

18.9 ISO 15216-1/2:2013: The Future “Gold Standard”

In 2004, the European Committee for Standardization (CEN) initiated development of a standard method for detection of norovirus and hepatitis A virus in foodstuffs, based on use of the PCR as the detection assay (Lees 2010). In 2013, the CEN/TC39/WG6/TAG4 working group published two Technical Specifications: ISO/TS 15216-1 (ISO 2013a) for quantitative detection of hepatitis A virus and norovirus, and ISO/TS 15216-2 (ISO 2013b) for qualitative detection of these agents. At the time of writing, these have now been advanced to draft international standards (DIS). Henceforward in this chapter, these methods are referred to as the “CEN methods.”

The two methods are identical in their basic stages; the difference is in the application of the detection assay. Viruses are removed from food surfaces by washing in an alkaline

(pH 9.5) buffer containing 1% beef extract, and food solids removed by centrifugation at $10000 \times g$. Concentration of suspended viruses is performed using PEG. The procedure for nucleic acid extraction is left to the discretion of the user. The detection assay is RT-qPCR; in ISO/TS 15216-1, the assay is used in a quantitative format, while in ISO/TS 15216-2, it is employed to give qualitative (presence/absence) results. The methods have been implemented in our laboratory, and in general work effectively. However, there are points at which modification of the methods would be beneficial. The methods stipulate that the test portion of soft fruits or salad vegetables is 25 (± 0.3) g. This sample size appears to have been arbitrarily chosen; it is similar to that used in several standard methods for detection of bacteria in foods, where 25 g foodstuff is added to 225 mL growth medium and bacteria enriched by subsequent incubation. It seems a small sample size considering that viruses may be present in foods in low numbers, although in some studies viruses have been detected from smaller samples (Baert et al. 2011). A potential modification which could be explored is the use of sample sizes which relate to the average portion sizes eaten by consumers. For example, in the United Kingdom, it is estimated that the average portion size consumed is 30 g lettuce, 60 g raspberries, and 100 g strawberries. This would provide more suitable exposure assessment data for risk assessment purposes, as the number of microorganisms detected in the sample would reflect the number which would be ingested.

The CEN methods stipulate that the SPCV is added along with the alkaline buffer, that is, immediately upon commencement of the virus removal procedure. However, to maintain the validity of the analytical result, it will be necessary to consider the analytical procedure as commencing on sample receipt. On occasion, samples can arrive at the laboratory late in the working day or week and the analyst may have insufficient time to complete the complex procedure, so the samples must consequently be stored. Therefore, it is recommended that immediately upon receipt of a sample, test portions are weighed out and the SPCV added (Ruhanya et al. 2015). This will allow monitoring of the efficiency of the whole procedure commencing from sample receipt.

The CEN methods stipulate the use of an EAC to monitor failure of the RT-qPCR due to inhibitory substances in the sample nucleic acid extract. Two separate RT-qPCRs are performed for each sample – one (the test) reaction contains only the sample nucleic acid, and the other (the control reaction) contains the sample nucleic acid plus the EAC (Costafreda et al. 2006). If the EAC is successfully amplified to produce a signal, any non-production of a target signal in the test reaction is considered to signify that the sample does not contain detectable virus. If, however, no signal is produced in both the test and control reactions, it signifies that the nucleic acid extract contains inhibitory substances and the reaction has failed.

This approach contains a degree of ambiguity, because one can never be completely certain that the test reaction has not individually failed, for example through pipetting error or non-homogeneous contamination by inhibitory substances. There is a significant cost implication too: the necessity of running a separate RT-qPCR for the EAC increases the cost of the analysis. Furthermore, the signals derived from amplification of the CEN method EACs are identical to the signals from their corresponding virus targets, and therefore to avoid false-positive results through contamination of the test reactions with EACs, very stringent diligence must be maintained when setting up the RT-qPCRs. Using an IAC would eliminate these issues, since it is present in the master-mix and a signal will appear when the reaction has not failed, there is no need for an

extra reaction to be performed, and an IAC signal is clearly distinguishable from a target signal. The concern of the proponents of the EAC approach regards the possibility that a low level of target may be outcompeted by the IAC, leading to a false-negative result. However, a thoroughly optimized assay should not present these problems (Cook et al. 2013; Diez-Valcarce et al. 2011b).

18.10 Quantitation

If it is desired that a qPCR assay is used to produce a quantitative determination of the viral load in a sample, it should be calibrated using either an enumerated suspension of virus particles or a quantified (number of GCs present) solution of viral nucleic acid. The correlation between the C_q values and the number of targets is then calculated, and the C_qs resulting from RT-qPCR of the test sample back-calculated to convert to GC number. ISO/TS 15216-1, the standard for the quantitative detection of HAV and NoV in food, recommends that double-stranded (ds) DNA is used to calibrate the RT-PCR assay. However, HAV and NoV have a single-stranded (ssRNA) genome, and using dsDNA will not take into account the reverse transcription step, which may not proceed with the same efficiency as the PCR.

Therefore, there is a likelihood that when using dsDNA calibration standards, the actual number of virus genome copies in a sample will be underestimated. Underestimation of target viral load will have implications, for example if wishing to collect appropriate data to inform development of risk-based microbiological criteria for norovirus in berry fruits, as recommended by the European Food Safety Authority (EFSA 2014a). Furthermore, underestimation of viral load in analyzed foods will impact upon an accurate attribution of the contribution of foodstuffs to the norovirus public health burden. It is therefore highly recommended that only ssRNA calibration standards are used for the purpose of quantitating RT-PCR assays for norovirus and other RNA viruses; this approach has been used in studies to determine the infectious dose of NoV (Teunis et al. 2008).

18.11 What is a Positive?

Very often in our experience of analysis of food samples for viruses, when an RT-qPCR signal is obtained, the C_q value is greater than 35 and sometimes greater than 40. There is ongoing discussion among practitioners of foodborne virus analysis regarding the interpretation of such results, which can be below the limit of quantitation of an RT-qPCR assay. To assist in the interpretation of “high” C_q values, the authors recommend that C_q cut-off points are identified. This can be done by testing quantified virus reference material (some are commercially available) suspensions, diluted beyond the level where 10⁶ GCs are present in the RT-qPCR.

When analyzing a sample, both undiluted sample nucleic acid extract and a 10-fold dilution are subjected to the RT-qPCR; the higher dilution is analyzed to try to dilute out any inhibitory substances which may potentially be present in the nucleic acid extract. Each dilution is analyzed in duplicate. Ideally, if a positive signal is obtained, these will be obtained from each duplicate; however, in our experience, this is not always

the case. On many occasions, it has been found that only one of the duplicate samples returns a positive signal. Another observed scenario is a signal being obtained from a 1:10 dilution but not from the undiluted extract, which can of course be due to inhibitory substances; however, the corresponding EAC has produced a signal therefore inhibition was not occurring. Apparently anomalous results such as these may confuse the analyst – should they be viewed as true positives or not? In the authors' opinion, they should be, or at least considered as presumptive positives until further confirmation (e.g., sequencing) can be obtained.

18.12 Future Developments and Requirements

In the past decade, the role of viruses as major agents of foodborne disease has finally become widely recognized. Consequently, there are currently international efforts aimed at tackling the problem of contamination of foods by pathogenic viruses. For instance, the Codex Alimentarius Commission Committee (CAC) on Food Hygiene has developed guidelines on the control of viruses in food (CAC 2012), and the European Commission supported research towards integrated monitoring and control of viruses in food supply chains (Cook et al. 2015).

As these activities come to fruition, it is timely to consider whether food safety criteria, setting limits for contaminating virus presence in foods for consumption or during food production, can be established and incorporated into regulations. The European Food Safety Authority currently considers that insufficient data exists on virus contamination of fresh produce to develop either food hygiene or process hygiene criteria, and has recommended that efforts be made to obtain information through surveys (EFSA 2014a). The availability of standardized methods should promote these efforts.

There is an issue of whether detected viruses are actually infectious or not. Despite several approaches to modify RT-PCR to give indications of the potential for infectivity (Knight et al. 2012), current technology appears unable to provide a means of unambiguously determining infectivity rapidly. Meanwhile, in the authors' opinion, it should just be accepted that detection of virus GCs at any point within the fresh produce supply chain, indicates that structural vulnerabilities exist in the chain (Kokkinos et al. 2015) and that a risk of infection can exist from consumption of foodstuffs supplied through that chain. Especially with ready-to-eat foods such as leafy green vegetables and berry fruits, which do not naturally harbor viruses pathogenic to humans, it would be prudent for the food industry or regulatory authorities to establish a zero-tolerance approach to virus contamination; in this case, efficient qualitative virus detection methods will be required, with quantitative methods being deployed mainly for risk assessment purposes.

18.13 Conclusions and Future Perspectives

In contrast to bacteriological analysis of foodstuffs, where culture-based methods are the norm and genome-based methods are not widely adopted, molecular methods are essential for foodborne virus detection and are likely to remain so for some considerable time, unless highly robust and efficient cell cultures are developed and implemented.

However, the use of molecular methods for foodborne virus analysis, despite several decades of development, can be seen as an emerging technology, and at present only a few laboratories are capable of their routine use. Questions remain regarding interpretation of results, and the most appropriate ways of controlling the nucleic acid amplification assays. However, it is to be expected that the increased deployment of the standardized methods will lead to a consensus among practitioners, and agreement on standard definitions of data interpretation. The search for rapid methods for infectivity assessment will continue, although until effective cell cultures are developed, these methods will be difficult to validate. Meanwhile, detection of virus genome sequences in a foodstuff is sufficient to reveal a problem in the food supply chain. After all, one should not expect to find a norovirus in one's strawberries!

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Section VI

Future Perspectives

19

Molecular Techniques and Foodstuffs: Innovative Fingerprints, Then What?

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19.1 Introduction

The problem of food adulteration is by no means a contemporary phenomenon and is likely as old as the food processing and production systems themselves (Cordain et al. 2005). In the “modern” scientific era, the first person to address this issue was the German analytical chemist Frederick Accum who completed a treatise on adulteration of food and culinary poisons in 1820 (Accum 1820). This was the first serious attempt to expose both the extent and dangers of food adulteration (Coley 2005). In 2014, the Taiwan food scandal exposed a series of food safety incidents, including the adulteration of cooking oil with recycled waste oil and animal feed oil (Hsu 2014). Such food scares have raised consumer awareness regarding the quality and authenticity of the food they buy and consume.

In the event of frauds or commercial disputes, it is necessary to use sensitive and accurate detection methods as a supplement to the food traceability system to verify the disputed information and fraudulent products. Fingerprinting technology is one method applied in food adulteration detection. Broadly speaking, a food fingerprint refers to the profile, spectrum or image generated by certain analytical tools for the specific food. The term fingerprint is commonly used because the specificity and representative characteristics indicated by these profiles or images are similar to those of human fingerprints and these techniques are collectively called fingerprinting techniques (Zhang et al. 2011). Because the production of counterfeit food is common around the world, food authentication using fingerprinting techniques is undergoing exponential growth, attracting a high level of attention from authorities around the world.

There is a growing need for reliable analytical methods that can give a decisive answer about the authenticity of foodstuffs. Advances in methodological approach and equipment, as well as social needs, dictate, in most cases, the direction in which research is focused. With the maturity of fingerprinting technologies, their application in food authentication will increase.

19.2 Emerging Fingerprinting Technologies

Fingerprinting techniques can be broadly classified into three categories: metabolites-based fingerprinting, proteins-based fingerprinting, and nucleic acid-based fingerprinting (Figure 19.1). Some common tools, equipment, and methods include electrophoresis (e.g., two-dimensional gel electrophoresis (2-DE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)), DNA fingerprints (e.g., DNA sequencing, DNA barcoding, metagenomics, etc.), nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy (IR), ultraviolet and visible spectroscopy (UV), mass spectrometry (MS), gas chromatography (GC), and liquid chromatography (LC).

19.2.1 Elemental Fingerprints

Elemental composition fingerprints have shown great potential for determining geographical origins of food because they reflect the conditions of the local environment (Georgiou & Danezis 2015). Methods such as inductively coupled plasma-mass spectrometry (ICP-MS) can provide ultra-trace level analysis down to parts per trillion (ppt) concentrations. Authenticity issues, where elemental fingerprinting could be deployed, have been applied to discriminate the origins of wine (Rodrigues et al. 2011), honey (Baroni et al. 2015), olive oil (Farmaki et al. 2012), coffee (Barbosa et al. 2014), cheese (Camin et al. 2012; Scampicchio et al. 2012), tea (Ma et al. 2016), fruits

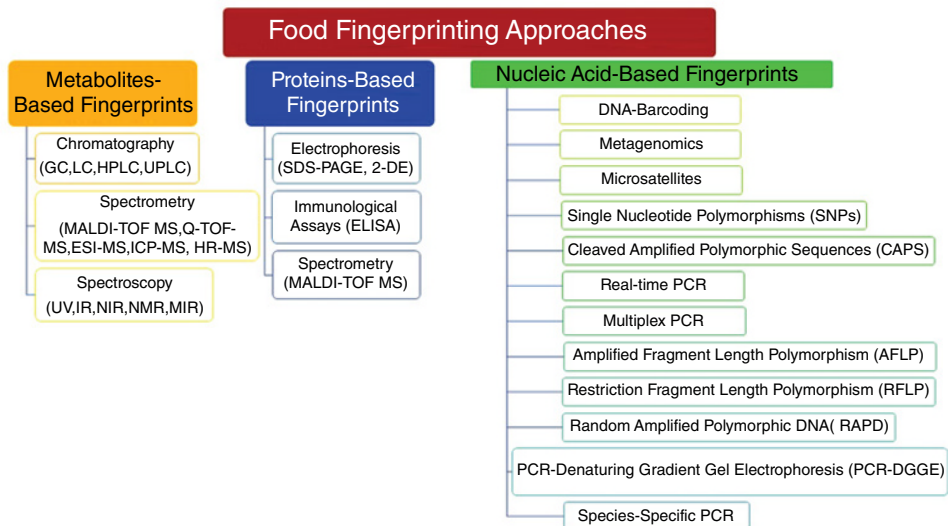


Figure 19.1 A summary of food fingerprinting approaches. ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray ionization mass spectrometry; GC, gas chromatography; HPLC, high-performance liquid chromatography; HR-MS, high-resolution mass spectrometry; ICP-MS, inductively coupled plasma-mass spectrometry; IR, infrared spectroscopy; LC, liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MIR, mid-infrared; NIR, near-infrared; NMR, nuclear magnetic resonance spectroscopy; Q-TOF-MS, quadrupole-time-of-flight mass spectrometry; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; 2-DE, two-dimensional gel electrophoresis; UPLC, ultra-performance liquid chromatography; UV, ultraviolet and visible spectroscopy.

and vegetables (Drivelos et al. 2014; Hu et al. 2014; Laursen et al. 2011), and spices and food additives (Hondrogiannis et al. 2013).

19.2.2 Foodomics Fingerprints

Metabolomics refers to the quantitative analysis of complete metabolite profiles (i.e., the metabolome) or selected subsets of the metabolome. Metabolic fingerprinting technology is rapid but does not necessarily give detailed information about specific metabolites (Cuadros-Rodríguez et al. 2016; Rubert et al. 2014). Gas chromatography mass spectrometry (GC-MS) has been used for detection and quantitation of volatile, semi-volatile, and non-volatile compounds in foods and beverages for decades. Liquid chromatography mass spectrometry (LC-MS) is used to detect secondary plant metabolites and phytochemicals (Johanningsmeier et al. 2016). Improved techniques such as ultra-performance liquid chromatography (UPLC) increase efficiency since they are faster and use fewer reagents than other LC techniques. To maximize the potential obtained by the UPLC high separation capacity, the use of mass detectors is recommended for food authentication (Jandrić et al. 2014). Electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) have also been used for studying the geographical classification of food and adulteration. ESI and MALDI can provide results with higher sensitivity and in a shorter time than most other methods. Therefore, these techniques are increasingly considered to be useful fingerprints for quality and authenticity control. However, the lack of homogeneity in the matrix sample and different sample preparation protocols are regarded as some of the intrinsic disadvantages of these approaches (Cozzolino & de Giulio 2011).

At present, quadrupole-time-of-flight mass spectrometry (Q-TOF-MS) and Orbitrap-based technologies are most commonly used to facilitate the identification and quantification of metabolites in complex food matrices at extremely low concentrations (ng/kg or 10^{-12}). These methods offer adequate reproducibility but there are limitations for cost-effective application when a large number of compounds are analyzed in a single run. High-resolution MS (HR-MS) systems provide high resolution, accurate mass, and high full scan sensitivity and selectivity, making them attractive for food authentication studies (Danezis et al. 2016). Coupled with appropriate statistical analyses, NMR metabolomics provides information that can be used to assess food quality, geographical origin, processing methods, and safety of raw material and digestion processes, among others (Mannina et al. 2012; Marchese et al. 2014). The main advantage of NMR is that it can be applied to studies of complex mixtures without the need for prior separation of components. Vibrational spectroscopic techniques (near-infrared or NIR, mid-infrared or MIR, and Raman) are also used for food authentication, particularly for discriminating closely related foods (Ellis et al. 2005).

Figure 19.2 depicts the frequencies of various analytical techniques that have been used to analyze specific food commodities.

19.2.3 Microbial Fingerprints

19.2.3.1 Why are Microbial Fingerprinting Methods Relevant?

Microbial fingerprinting can be achieved through analyzing the diversity of microbes in both fresh and fermented foodstuffs (Arcuri et al. 2013; El Sheikha 2010; El Sheikha et al. 2009). The external surface of fresh foods is not sterile and can carry microorganisms

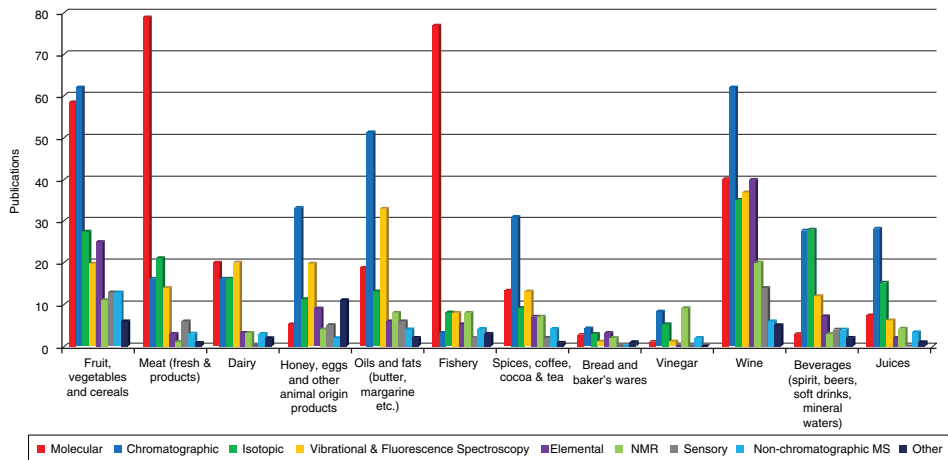


Figure 19.2 Food fingerprinting: analytical approaches and foodstuffs. *Source:* Danezis et al. (2016). Reproduced with permission of Elsevier.

or products of their metabolic processes (Danezis et al. 2016). The occurrence and composition of microbiota depend mainly on the environment where the food was grown and processed, including soil, air, water, insects, humans, and disease agents (Sodeko et al. 1987). Fingerprinting methods can be used to provide an overall view of the microbial community. The overall microbial composition and specific indicator organisms can be used to characterize food samples. This capacity is important because biodegradation inherently depends on the types and abundance of microorganisms present in the surface and subsurface of food (Interstate Technology & Regulatory Council-Environmental Molecular Diagnostics 2011).

19.2.3.2 What Does Microbial Fingerprinting Do?

Microbial fingerprinting methods are techniques that differentiate microorganisms or groups of microorganisms based on unique characteristics of a universal component or section of a biomolecule (e.g., phospholipids, protein, DNA, or RNA). Microbial flora of specific food products such as cheeses (Arcuri et al. 2013; Ercolini et al. 2008; Rychlik et al. 2017), milk (Garofalo et al. 2015), and wines (Marques et al. 2011; Petri et al. 2015; Sánchez et al. 2012) have been shown to provide excellent authenticity markers because of the exclusive starter cultures used in these products.

19.3 DNA Fingerprints

Three characteristics of DNA make it an extremely useful tool for food fingerprinting. First, it is an extremely stable and durable biomolecule that can be recovered from a variety of biological materials, including those that have been subject to non-optimal storage conditions. Second, it is found in all biological substances that contain nucleated or non-nucleated cells (with respect to mitochondria or plastids). And third, DNA

can provide more information than other biomolecules (e.g., proteins) owing to the degeneration of the genetic code and the presence of significant stretches of non-coding regions (Wilkes et al. 2016).

Numerous technical advances within the field of molecular genetics have enabled the routine analysis of genetic variation to be performed in many accredited food testing laboratories. We focus on some of these techniques as follows.

19.3.1 DNA Sequencing

The DNA sequencing of an organism's genome is theoretically the definitive means of identification. Given their rapid decline in cost and increasing availability, next-generation sequencing (NGS) platforms (Xu 2014; Zhou et al. 2015) have enabled the high-resolution sequencing of an individual or population of organisms (Lindeque et al. 2013; Tillmar et al. 2013). Additionally, the adoption of alternative sequencing strategies without pure cultures at one or multiple loci (DNA barcoding) (Murugaiah et al. 2015; Xu 2016), or of microbial communities present on the surfaces of food samples, could prove valuable in food authenticity studies.

19.3.2 DNA Barcoding

DNA barcoding employs the sequencing of a short, standardized DNA sequence which can then function as a molecular fingerprint in the classification of an organism (Hebert et al. 2003). However, DNA integrity is a critical factor in predicting the probability of a successful outcome, which becomes more of an issue when working with highly degraded DNA, such as that isolated from highly processed foods (Wilkes et al. 2016). Currently, use of the *COI* gene is nearly universal for determining the species of animals, birds, and fish in raw and processed food products (Murugaiah et al. 2015). The *COI* gene is located on mitochondrial DNA (mtDNA) and thus has two advantages (Woolfe & Primrose 2004):

- mtDNA is present in multiple copies in every cell compared to nucleus DNA (nDNA), thus making its detection easier
- the mitochondria are likely to remain intact during processing, thereby minimizing DNA degradation.

However, for a variety of reasons, the DNA barcodes for plants and fungi are different (Xu 2016). For plants, the recommended DNA barcode loci are a combination of two chloroplast genes, *rbcL* + *matK* (CBOL Plant Working Group 2009). For fungi, the universal DNA barcode is ITS (Schoch et al. 2012). However, secondary barcodes are often needed in order to discriminate many closely related fungal species (Xu 2016).

19.3.3 DNA Sequencing of Microbial Flora (Metagenomics)

With the introduction of NGS services in multiple fields of investigation and application, microbial community profiling is currently being evaluated across many areas of interest, including food microbial ecology (Ercolini 2013). Sequencing of the entire or specific genomic regions of these microbial genomes (often referred to as metagenomics sequencing) can be used to give a fingerprint to each food sample. However,

adoption of the technique for food authentication would require the establishment of a global microbial distribution database for the target food as well as broad availability and use of NGS technology in most countries (Wilkes et al. 2016).

19.4 Conclusions and Future Perspectives

Food fingerprinting relies on the use of a variety of chemical and physical techniques. Table 19.1 summarizes the intrinsic strengths and limitations of the major approaches.

Table 19.1 Advantages and limitations of different fingerprints.

Fingerprinting approach	Advantages	Limitations
<i>Chromatography fingerprinting</i>		
GC	<ul style="list-style-type: none"> ● Short separating time ● High resolution ● High sensitivity 	<ul style="list-style-type: none"> ● Small analysis scope ● Complex sample preparation
LC	<ul style="list-style-type: none"> ● High separation efficiency ● Good sensitivity ● Wide application 	<ul style="list-style-type: none"> ● Large consumption of solvent ● Inevitable use of toxic reagents
HPLC	<ul style="list-style-type: none"> ● Rapid and sensitive ● Tolerable cost 	<ul style="list-style-type: none"> ● Labor intensive ● Cannot provide quantitative data ● Often require statistical analysis
<i>Electrophoresis fingerprinting</i>		
Protein electrophoresis	<ul style="list-style-type: none"> ● Accurate distinguishing of different species 	<ul style="list-style-type: none"> ● Difficult to identify varieties of near genetic relationship
Isozyme electrophoresis	<ul style="list-style-type: none"> ● Simple ● Stable ● Good repeatability and applicability 	<ul style="list-style-type: none"> ● Small number of available enzymes ● Poor polymorphism
SDS-PAGE	<ul style="list-style-type: none"> ● Simple and easy ● Sensitive ● Less costly 	<ul style="list-style-type: none"> ● Degradation profile of peptide marker ● Need reference sample preparation ● Non-quantitative
2-DE	<ul style="list-style-type: none"> ● Simplistic ● Robust 	<ul style="list-style-type: none"> ● Involves large amount of sample ● Low throughput ● High inter-gel variability ● Poor recovery of hydrophobic proteins
<i>Immunological fingerprinting</i>		
ELISA	<ul style="list-style-type: none"> ● Less costly ● Simple and easy ● Sensitive ● Provides both qualitative and quantitative data 	<ul style="list-style-type: none"> ● Not suitable for extremely processed samples because of the denaturation of the protein-based biomarkers

Table 19.1 (Continued)

Fingerprinting approach	Advantages	Limitations
<i>Spectrum fingerprinting</i>		
UV	<ul style="list-style-type: none"> ● Simple spectrum ● Broad spectrum peak ● Low detection limits 	<ul style="list-style-type: none"> ● Less spectrum signal ● Cumbersome sample pretreatment required
IR	<ul style="list-style-type: none"> ● Each compound has its specific infrared spectroscopy 	<ul style="list-style-type: none"> ● Complex spectrum ● Poor reliability and accuracy
NMR	<ul style="list-style-type: none"> ● Non-destructive testing ● High accuracy ● Rapid detection 	<ul style="list-style-type: none"> ● Expensive equipment ● Complex testing data
MS	<ul style="list-style-type: none"> ● High sensitivity ● Little sample consumption ● Rapid analysis 	<ul style="list-style-type: none"> ● Requires a higher content of main components ● Expensive equipment
MALDI-TOF	<ul style="list-style-type: none"> ● Accurate and sensitive ● High throughput ● Fast 	<ul style="list-style-type: none"> ● High initial cost of the MALDI-TOF equipment
<i>Nucleic acid-based fingerprints</i>		
DNA barcoding	<ul style="list-style-type: none"> ● Highly informative ● Reproducible 	<ul style="list-style-type: none"> ● Requires careful primer design ● Moderate throughput
Metagenomics	<ul style="list-style-type: none"> ● Highly informative ● Adaptable ● Reproducible 	<ul style="list-style-type: none"> ● Technically challenging ● Current high cost ● Resource intensive
Microsatellites	<ul style="list-style-type: none"> ● High specificity ● High reproducibility ● Highly informative 	<ul style="list-style-type: none"> ● Large consumables requirement ● Moderate throughput ● Limited targets ● Technically challenging
SNPs	<ul style="list-style-type: none"> ● Highly informative ● Adaptable method 	<ul style="list-style-type: none"> ● Moderate throughput ● Relatively expensive ● Technically challenging
CAPS	<ul style="list-style-type: none"> ● Simple technique ● High specificity ● High reproducibility 	<ul style="list-style-type: none"> ● Moderate throughput ● Erroneous results from partial digestion
Real-time PCR	<ul style="list-style-type: none"> ● Highly stable DNA biomarkers ● Highly sensitive and can be used with degraded sample ● Robust, reproducible, and efficient 	<ul style="list-style-type: none"> ● Expensive and needs expertise
Multiplex PCR	<ul style="list-style-type: none"> ● Reduced cost and time since many species are detected in a single assay ● Possibility for total analysis 	<ul style="list-style-type: none"> ● Variable sensitivity for different species ● Cannot provide quantitative data

(Continued)

Table 19.1 (Continued)

Fingerprinting approach	Advantages	Limitations
AFLP	<ul style="list-style-type: none"> ● Safe, efficient, convenient ● Reproducible ● Small DNA template 	<ul style="list-style-type: none"> ● High cost ● High requirements for DNA quality and enzyme purity
RFLP	<ul style="list-style-type: none"> ● Mature technology ● Low detection limit ● Good repeatability 	<ul style="list-style-type: none"> ● Vulnerable to individual mutations and false negatives
RAPD	<ul style="list-style-type: none"> ● Fast, easy to operate ● Small sample 	<ul style="list-style-type: none"> ● Poor stability and repeatability ● Method not standardized ● Less information
PCR-DGGE	<ul style="list-style-type: none"> ● Ultra-sensitive since it can distinguish two DNA molecules that differ by as little as a single base ● Reliable, reproducible, rapid, inexpensive, and can analyze samples in a high-throughput fashion 	<ul style="list-style-type: none"> ● Optimization of migration on polyacrylamide gel required for any new gene used ● One band can represent more than one species (co-migration)
Species-specific PCR	<ul style="list-style-type: none"> ● High reproducibility ● Relatively simple technique 	<ul style="list-style-type: none"> ● Limited target availability ● Technically challenging

AFLP, amplified fragment length polymorphism; CAPS, cleaved amplified polymorphisms; ELISA, enzyme-linked immunosorbent assay; GC, gas chromatography; HPLC, high-performance liquid chromatography; IR, infrared spectroscopy; LC, liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PCR-DGGE, polymerase chain reaction-denaturing gradient gel electrophoresis; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNPs, single nucleotide polymorphisms; 2-DE, two-dimensional gel electrophoresis; UV, ultraviolet and visible spectroscopy. *Source:* Adapted from Zhang et al. (2011), Reproduced with permission of Elsevier, and Wilkes et al. (2016), Reproduced under Open Government Licence v3.0.

Over the last decade, the number of publications concerning analytical methods for food authentication has grown exponentially, mainly stimulated by the interests of consumers, regulatory bodies, and relevant food and biotech industries. The results indicate that fingerprinting technologies have been adopted by many industries covering a diversity of foods. They also suggest that fingerprinting technologies (elemental, foodomics, molecular biology methods) have advantages in food detection: fast, accurate, low detection limit, good reproducibility, and so on. Indeed, fingerprinting has become an important technology in food detection and as it is developing rapidly, its application will likely be extended to all types of foods. At the same time, fingerprinting technologies can be improved in instrumentation, running costs, and training analysts. With decreasing costs and an increasing number of nucleic acid-based approaches becoming available, these techniques (especially NGS) will witness tangible development in the next few years in food authentication.

Regarding the future, with recent developments in the field of DNA sequencing concurrently with the technical advances in the next-generation sequencing (NGS), we will see a large leap in the means of studying food microbial flora (the metagenome). This approach is likely to become a practical technical reality in the food fingerprinting field of the future (Wilkes et al. 2016).

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