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Feeds for the Aquaculture Sector

Current Situation and Alternative Sources

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Contents

Abbreviations

ADC	Apparent digestibility coefficient
ALA	Alpha-linolenic acid
BI	Biomass index
BM	Blood meal
Ca	Calcium
CFP	Common Fisheries Policy
CFU	Colony forming unit
CP	Crude protein
CPO	Crude palm oil
Cr	Chromium
CRS	Catch returned to the sea
Cu	Copper
DHA	Docosahexaenoic acid
DI	Density index
DM	Dry matter
EAA	Essential amino acid
EE	Ether extract
EFA	Essential fatty acids
EFSA	European Food Safety Authority
EOLSS	Encyclopaedia of Life Support Systems
EPA	Eicosapentaenoic acid
EU	European Union
FA	Fatty acids
FAD	Flavin adenine dinucleotide
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed conversion rate
Fe	Iron
FI	Feed intake
FM	Fishmeal
FMN	Flavin mononucleotide

FO	Fish oil
FR	Feeding rate
GFCM	General Fisheries Commission for the Mediterranean Sea
GSA	Geographical sub-area
HI	*Hermetia illucens*
HUFA	Highly unsaturated fatty acid
ISSFAL	International Society for the Study of Fatty Acids and Lipids
LA	Linoleic acid
LAB	Lactic acid bacteria
Lc	Length at capture
LC-HUFA	Long chain-highly unsaturated fatty acids
LC-PUFA	Long chain-polyunsaturated fatty acids
LCRS	Landed catch returned to the sea
Lm50%	Length at sexual maturity
LO	Linseed oil
MBM	Meat and bone meal
MCS	Minimum conservation size
MD	*Musca domestica*
MEDITA	Mediterranean International Bottom Trawl Survey
Mg	Magnesium
Mn	Manganese
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NRC	National Research Council
OA	Oleic acid
P	Phosphorus
PAP	Processed animal proteins
PBM	Poultry by-product meal
PER	Protein efficiency rate
PO	Plant oil
PUFA	Polyunsaturated fatty acid
RO	Rapeseed oil
SAUP	Sea Around Us Project
SBM	Soybean meal
SBO	Soyabean oil
SFA	Saturated fatty acids
SGR	Specific growth rate
SIBM	Società Italiana di Biologia Marina
STECF	Scientific, Technical and Economic Committee for Fisheries
TL	Total length
TM	*Tenebrio molitor*
TW	Total weight
WBC	Whole body composition
WG	Weight gain
Zn	Zinc

Chapter 1
Fishmeal Alternative Protein Sources for Aquaculture Feeds

Laura Gasco, Francesco Gai, Giulia Maricchiolo, Lucrezia Genovese, Sergio Ragonese, Teresa Bottari and Gabriella Caruso

Abstract Aquaculture currently accounts for approximately 50% of fish consumed by humans. The future development of aquaculture will be greatly constrained by the increasing costs of fishmeal and fish oil. To remedy this situation, scientific research and feed manufacturers have made a significant progress by looking for alternative protein sources for use in fish diets in order to develop feeds that provide adequate nutrition for animals' growth, while reducing to minimum the use of traditional sources of protein. This chapter aims at critically reviewing recent studies, carried out worldwide, about the effects of the inclusion of new protein sources as insect, poultry by-products, meat and bone meals and other protein sources alternative to fishmeal in aquafeeds. In particular, the impacts of these protein sources in terms of growth, nutrient digestibility, fillet quality traits and sensorial perception in the most important farmed marine and freshwater fish species are evaluated.

Keywords Alternative proteins · Aquaculture · Fishmeal · Insects
Poultry By-Products · Processed animal proteins

1.1 Introduction

The global demand for fish products is expected to increase significantly in the next 35 years due to the increase in world population that, according to the last Food and Agriculture Organization of the United Nations (FAO) evaluations, will reach 9.5 billion people in 2050 (FAO 2016). Although there is a slight improvement in the state of certain fish stocks due to improved fisheries management, the expected increase will be possible only through aquaculture production that already provides half of all seafood for human consumption (FAO 2016). Aquaculture is claimed to be the fastest growing food production sector in the world. Fish from fisheries and

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aquaculture provide important nutrients (energy, protein, vitamins and minerals) and fish supply reached a new record of about 20 kg in 2014 accounting for about 17% of the global population's intake of animal protein and 6.7% of all consumed proteins (FAO 2014).

While in the past, aquaculture feeds largely used fishmeal (FM) and fish oil (FO), these ingredients are no longer sustainable (Hardy 2010; Oliva-Teles et al. 2015; Tacon and Metian 2008). Moreover, these resources experienced periodic fluctuations in availability and pricing. Nowadays, their inclusion is reduced to the minimum amount able to cover the optimal content of amino acids and other nutrients needed for fish growth and flesh quality; sometimes, aquafeed industry produces fish diets that are completely free of these marine ingredients. Plant feedstuffs are widely used as an alternative to FM as they are available in large quantities when compared to FM (Gatlin et al. 2007; Naylor et al. 2009; Oliva-Teles et al. 2015). Nevertheless, in carnivorous fish species, the complete substitution of FM is still a challenge and its complete elimination is still associated with reduced performances and fish health (Desai et al. 2012; Krogdahl et al. 2010; Oliva-Teles et al. 2015). Moreover, in recent years, plant proteins for fish feeds face problems of increasing price and of competition with other sectors as human consumption, animal husbandry sector or biodiesel production (Moutinho et al. 2017; Pinotti et al. 2014). The use of terrestrial processed animal proteins (PAP) such as blood meal (BM), meat and bone meal (MBM), feather meal and poultry by-product meal (PBM) in aquaculture feeds is a common practice.

PAP have high protein and lipid content which make them very interesting for the formulation of cost-effective aquaculture feeds. PAP are a natural source of several nutrients such as amino acids (lysine, sulphur amino acids, histidine and arginine) or phosphorus. They are relatively free from any anti-nutritional factor, result to be highly palatable to most fish species, and their inclusion in aquafeeds complement very well certain plant protein ingredients (Bureau 2006). Nevertheless, the inclusion levels are limited by fish species, poor digestibility, deficiency of some essential amino acids (EAA) and general nutritional quality that highly depend on the raw material composition, freshness and processing conditions (Bureau et al. 1999, 2000; Goda et al. 2007). The use of PAP is highly depending on the considered region of the world. For instance, following the bovine spongiform encephalopathy emergency, European Union (EU) prohibited the use of PAP (EC No 999/2001). In 2013, this ban was partially lifted (EC No 56/2013), and the use of PAP from non-ruminant animals (poultry and pigs) (category 3) was reintroduced in fish feeds. Recently, the European Commission approved the use of PAP from seven insect species in aquafeed with the Regulation 2017/893/EC.

In the following paragraphs, different alternative protein sources investigated in field trials on cultured fish species as well as their effects on growth, nutrient digestibility, fillet quality traits and sensorial perception are reported and commented.

1.2 Insect Meals

The potential use of insect meal in fish diets has recently attracted much attention (Barroso et al. 2014; Henry et al. 2015). Carnivorous fish already count insects as part of their natural diet (Henry et al. 2015). It seems therefore reasonable to consider insect meals as raw material in fish feeds. Following the European Food Safety Authority (EFSA) scientific opinion on the use of insects as food and feed, the Standing Committee on Plants, Animals, Food and Feed has approved recently the draft of the Regulation amending Annexes I and IV to Regulation (EC) No 999/2001 and of the Council and Annexes X and XV to Commission Regulation (EC) No 142/2011 as regards the provisions on processed animal protein. The use of insect-derived PAP in aquafeeds in Europe is allowed since July 2017 (Commission Regulation (EU) 2017/893 of 24 May 2017).

In EU, the authorized insect meal is only those obtained from: (i) *Hermetia illucens* (HI, Black Soldier Fly) and *Musca domestica* (MD); (ii) *Tenebrio molitor* (TM, Yellow Mealworm) and *Alphitobius diaperinus* (Lesser Mealworm); (iii) *Acheta domesticus* (House cricket), *Gryllodes sigillatus* (Banded cricket) and *Gryllus assimilis* (Field Cricket). Nevertheless, in countries other than EU, rules could be different and other insects are considered as very interesting for fish nutrition (Barroso et al. 2014; Henry et al. 2015; Makkar et al. 2014).

When considering insects and FM ingredients for fish feeds, not only aspects such as energy, protein and EAA, fat or mineral content and many other chemical data (Tables 1.1, 1.2 and 1.3) have to be considered but also the raw material availability. In this sense, only few insect species so far have the potential to be produced in large scale and thus have received much attention as aquaculture feeds namely TM, HI and MD.

The chemical composition and the nutritional value of insect larvae meals (Table 1.1) largely depend on the treatment (i.e. drying methodologies, defatting procedures) and on the substrate used to rear them (Henry et al. 2015). In particular, while the protein content does not vary to a large extent due to the rearing substrate, the lipid fraction is the most susceptible to changes, both from a quantitative and qualitative fatty acid (FA) profile point of view (Henry et al. 2015; Makkar et al. 2014). As far as gross energy is considered, insect larvae meals have contents greater than 21 MJ kg/dry matter (DM). The high insect larvae fat content (15–50%) can sometimes cause problems. In fact, their inclusion as protein source automatically brings also a high fat content that can generate problems both for feed formulation but also for storage and pellet stability. For these reasons, insect producers consider defatting process using various methods (physical or chemical extractions). In this case, the percentage of protein (and consequently of EAA) is greatly increased and the extracted oils may be used for other purposes such as feed inclusion (Schiavone et al. 2017) or biodiesel production (Henry et al. 2015; Li et al. 2016; Surendra et al. 2016). As far as EAA are concerned (Tables 1.1, 1.2 and 1.3), the profiles of HI and MD are considered close to FM profiles while the one of TM closer to that of SBM.

Table 1.1 Nutrient composition and nutritive value of most promising insect larvae meals compared to fishmeal (FM) and soybean meal (SBM). Values are reported as mean of values found in the cited references (in parenthesis minimum and maximum values). The following chemical values are shown here: dry matter (DM), crude fibre, crude protein, lysine, methionine, sum of methionine and cysteine, tryptophan, threonine, leucine, isoleucine, valine, histidine, arginine and the sum of phenylalanine and tyrosine

	Unit	*Tenebrio molitor*[a]	*Hermetia illucens*[b]	*Musca domestica*[c]	FM[d]	SBM[e]
Dry Matter (DM)	% as fed	42.2 (37.1–57.6)	91.3 (90.0–92.5)	92.4 (90.0–94.7)	92.1 (90.0–94.4)	87.9 (85.0–92.1)
Crude fibre	% DM	5.9 (5.0–6.9)	7.0	14.7 (1.6–29.7)	–	6.7 (3.5–10.1)
Crude protein	% DM	51.5 (44.1–60.3)	49.1 (35.5–72.5)	49.9 (37.5–63.8)	75.6 (70.2–80.7)	51.4 (48.3–54.5)
Lysine	% protein	4.5 (1.7–6.1)	6.4 (5.6–8.0)	6.1 (4.4–8.2)	6.1 (5.5–7.5)	6.1 (5.7–6.6)
Methionine	% protein	1.5 (1.2–2.0)	1.8 (1.4–2.4)	2.3 (1.3–3.7)	2.2 (2.0–2.6)	1.4 (1.2–1.6)
Methionine + Cystine	% protein	2.3 (1.8–2.9)	2.2 (1.5–3.1)	3.0 (1.7–4.7)	2.9 (2.6–3.2)	2.9 (2.5–3.3)
Tryptophan	% protein	0.9 (0.0–1.8)	0.8 (0.5–1.1)	1.8 (1.4–3.2)	0.8 (0.7–0.9)	1.3 (1.2–1.4)
Threonine	% protein	3.6 (2.7–4.4)	3.6 (1.3–4.8)	3.8 (2.0–7.6)	3.1 (2.9–4.3)	3.9 (3.5–4.3)
Leucine	% protein	7.6 (4.5–10.6)	7.3 (6.6–8.4)	5.7 (4.5–6.4)	5.9 (5.2–7.3)	7.5 (6.8–8.0)
Isoleucine	% protein	4.1 (2.6–5.0)	4.7 (4.0–5.6)	2.9 (1.7–3.7)	3.7 (3.3–4.4)	4.6 (4.3–5.0)
Valine	% protein	5.5 (3.7–6.6)	6.9 (5.6–9.1)	3.3 (1.3–4.9)	4.2 (3.9–4.8)	4.8 (4.3–5.4)
Histidine	% protein	3.0 (2.1–3.6)	3.1 (2.3–4.5)	3.0 (1.0–5.1)	1.8 (1.7–1.9)	2.6 (2.4–2.9)
Arginine	% protein	4.5 (3.6–5.6)	5.4 (4.8–6.1)	4.9 (3.7–5.8)	4.6 (4.0–6.0)	7.4 (6.8–8.1)
Phenylalanine + tyrosine	% protein	10.7 (8.6–12.1)	11.2 (9.6–13.3)	9.8 (6.2–17.3)	5.5 (5.2–6.5)	8.5 (7.7–9.4)

[a]Data from Barroso et al. (2014), Feedpedia: http://www.feedipedia.org, Gasco et al. (2016), Marono et al. (2015), Sánchez-Muros et al. (2015), Siemianowska et al. (2013)

[b]Data from Diener et al. (2009), Feedpedia: http://www.feedipedia.org, Józefiak et al. (2016), Makkar et al. (2014), Maurer et al. (2016), Marono et al. (2015), Sánchez-Muros et al. (2015), Tschirner and Simon (2015)

[c]Data from Aniebo et al. (2008), Aniebo and Owen (2010), Barroso et al. (2014), Feedpedia: http://www.feedipedia.org; Fasakin et al. (2003), Józefiak et al. (2016), Makkar et al. (2014), Sanchez-Muros et al. (2015), Sogbesan et al. (2006), Tschirner and Simon (2015), Zuidhof et al. (2003)

[d]Data from: Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)

[e]Data from: Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)

Table 1.2 Nutrient composition and nutritive value of most promising insect larvae meals compared to fishmeal (FM) and soybean meal (SBM). Values are reported as mean of values found in the cited references (in parenthesis minimum and maximum values). The following chemical values are shown here: ether extract, saturated fatty acids (FA), monosaturated FA, n6-polyunsaturated FA and n3-polyunsaturated FA

	Unit	*Tenebrio molitor*[a]	*Hermetia illucens*[b]	*Musca domestica*[c]	FM[d]	SBM[e]
Ether extract	% DM	30.2 (16.6–43.1)	20.0 (3.4–38.6)	15.8 (6.3–31.3)	8.1 (2.0–12.0)	2.1 (2.0–2.2)
Saturated fatty acids (FA)	% Total FA	26.4 (22.2–35.1)	33.3	41.3 (33.1–49.2)	19.5 (18.9–19.7)	15.1 (14.9–15.2)
Monosaturated FA	% Total FA	41.7 (35.1–51.5)	43.4	38.9	50.8 (50.5–52.1)	21.1 (20.5–21.7)
n6 Polyunsaturated FA	% Total FA	25.5 (11.5–34.5)	15.0	18.4	1.8 (1.8–2.0)	56.1 (55.9–56.3)
n3 Polyunsaturated FA	% Total FA	1.1 (0.8–1.4)	8.3	–	8.1 (2.0–12.0)	2.1 (2.0–2.2)

[a]Data from Barroso et al. (2014), Feedpedia: http://www.feedipedia.org, Gasco et al. (2016), Marono et al. (2015), Sánchez-Muros et al. (2015), Siemianowska et al. (2013)

[b]Data from Diener et al. (2009), Feedpedia: http://www.feedipedia.org, Józefiak et al. (2016), Makkar et al. (2014), Maurer et al. (2016), Marono et al. (2015), Sánchez-Muros et al. (2015), Tschirner and Simon (2015)

[c]Data from Aniebo et al. (2008), Aniebo and Owen (2010), Barroso et al. (2014), Feedpedia: http://www.feedipedia.org, Fasakin et al. (2003), Józefiak et al. (2016), Makkar et al. (2014), Sanchez-Muros et al. (2015), Sogbesan et al. (2006), Tschirner and Simon (2015), Zuidhof et al. (2003)

[d]Data from: Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)

[e]Data from: Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)

Table 1.3 Nutrient composition and nutritive value of most promising insect larvae meals compared to fishmeal (FM) and soybean meal (SBM). Values are reported as mean of values found in the cited references (in parenthesis minimum and maximum values). The following values are shown here: minerals (ash), calcium, phosphorus, sodium, potassium, magnesium and gross energy

	Unit	*Tenebrio molitor*[a]	*Hermetia illucens*[b]	*Musca domestica*[c]	FM[d]	SBM[e]
Minerals (ash)	% DM	3.8 (1.0–6.5)	13.6 (4.3–28.4)	11.4 (5.0–23.1)	16.6 (12.0–23.3)	6.9 (6.8–7.0)
Calcium	g/kg DM	2.7 (0.3–6.2)	75.6 (50.0–86.3)	4.7 (3.1–8.0)	36.3 (15.4–78.3)	3.9 (2.3–6.3)
Phosphorus	g/kg DM	7.8 (4.4–14.2)	9.0 (6.4–15.0)	16.0 (9.7–24.0)	25.9 (19.0–40.4)	6.9 (5.8–8.6)
Sodium	g/kg DM	0.9	1.3	5.2 (2.8–8.6)	10.0 (5.9–14.4)	0.1 (0.0–0.8)
Potassium	g/kg DM	8.9 (8.5–9.3)	6.9	5.7 (1.0–12.7)	10.2 (5.9–14.4)	23.7 (21.8–26.0)
Magnesium	g/kg DM	2.3 (2.0–2.8)	3.9	3.4 (0.7–11.5)	2.5 (1.6–3.1)	3.1 (2.4–3.6)
Gross energy	MJ/kg DM	26.2 (24.4–28.7)	22.8 (21.2–24.4)	21.7 (19.3–24.4)	21.4 (19.6–23.8)	19.9 (19.8–20.0)

[a]Data from Barroso et al. (2014), Feedpedia: http://www.feedipedia.org, Gasco et al. (2016), Marono et al. (2015), Sánchez-Muros et al. (2015), Siemianowska et al. (2013)

[b]Data from Diener et al. (2009), Feedpedia: http://www.feedipedia.org, Józefiak et al. (2016), Makkar et al. (2014), Maurer et al. (2016), Marono et al. (2015), Sánchez-Muros et al. (2015), Tschirner and Simon (2015)

[c]Data from Aniebo et al. (2008), Aniebo and Owen (2010), Barroso et al. (2014), Feedpedia: http://www.feedipedia.org, Fasakin et al. (2003), Józefiak et al. (2016), Makkar et al. (2014), Sanchez-Muros et al. (2015), Sogbesan et al. (2006), Tschirner and Simon (2015), Zuidhof et al. (2003)

[d]Data from: Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)

[e]Data from: Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)

Deficiencies in lysine or methionine (Barroso et al. 2014; Henry et al. 2015) are reported.

TM larvae meals evaluated so far have a protein content varying from 44 to 60%. The lipid fraction (about 16.6 to 43% DM) is characterized by high levels of oleic (42.18% fatty acid—FA), linoleic (24.70% FA) and palmitic (18.42% FA) acids. The low ash content (about 3.8% DM) is very interesting for the aquaculture sector even if TM larvae are usually low in calcium. Nevertheless, it has been highlighted that their calcium content can be modified through the rearing substrate (Anderson 2000; Klasing et al. 2000), increasing the level of this important mineral in meals.

The protein content of HI larvae meals evaluated in different research varied between 35.30 and 72.50% of DM. This high variation can be justified by the availability on the market of several different defatted HI meals. The processing and extraction of part of the lipid fraction from HI larvae generates protein meals having lipid content that varied from 3.4 to 38.6% of DM. The FA profile of HI meals use to be characterized by high values of lauric acid as, independently from the used substrate, larvae neo-synthesized and accumulated this FA (Spranghers et al. 2016). HI meals are rich in ash, calcium and phosphorus (Makkar et al. 2014; Tschirner and Simon 2015). MD larvae meals have a protein and lipid content ranging from 37.5 to 63.8% of DM and from 6.28 to 31.30% of DM, respectively (Aniebo and Owen 2010; Barroso et al. 2014; Józefiak et al. 2016; Makkar et al. 2014; Sánchez-Muros et al. 2014; Sogbesan et al. 2006; Zuidhof et al. 2003). The main reported FA are palmitic (32.37% FA), oleic (21.96% FA), linolenic (19.70% FA) and palmitoleic (17.10% FA) acids. The calcium content (about 4.7 g/kg DM) is higher than that of the TM larva but lower than that of HI (Makkar et al. 2014).

When compared to conventional protein sources (FM or soybean meal—SBM), the insect larvae protein content is lower than that of the FM (66–72%) but similar or higher than SBM (44–50%). With regard to the lipid content, insect larvae used to have higher values than conventional sources. Larvae contain between 8 and 36% of nitrogen-free extracts (sugars, starch, chitin and fibrous fractions) (Barroso et al. 2014).

As far as the lipid profile is concerned, insect larvae are poor in highly unsaturated fatty acids. The main difference between insect meals and FM is the content of eicosapentaenoic acid (EPA, C20:5 n3) and docosahexaenoic acid (DHA, C22:6 n3) which are present in the marine and freshwater products, but are absent in land-based products (including SBM) and insects. Usually, increasing levels of insect meal inclusion lead to a dramatic change of the FA profile in fish with a decrease in EPA and DHA and a decrease in the n3/n6 fatty acid ratio (Belforti et al. 2015; Gasco et al. 2016; St-Hilaire et al. 2007a, b). Nevertheless, it is possible to increase the insect unsaturated FA content manipulating the rearing substrate (Belforti et al. 2014; St-Hilaire et al. 2007a). In particular, while the fatty acids of HI pre-pupae reared on cow manure were high in saturated fatty acids (SFA) and very low in poly-unsaturated FA n3 (0.2%), when larvae were fed manure cattle enriched with fish by-products, this content increased up to 4% (St-Hilaire et al. 2007a).

Any change in the profile or the lipid composition of the diet directly affects the lipid–volatile component, then the aroma and flavour of fish (Turchini et al. 2007), and can therefore dramatically modify the perception of the product by the consumer.

The inclusion of insect meals could thus influence the sensory properties of fish products even if the information available, till now on this aspect, did not highlight any negative effect. For instance, the results of a panel test (aroma and texture) using fish (catfish and tilapia) fed chopped HI larvae alone or in combination with commercial diets indicated that fish were scored and ranked similarly with regard to control diets (Bondari and Sheppard 1981). In the same way, no significant differences were detected by panellists comparing fish fed with diets containing HI meals and fish fed with FM-based diets (Borgogno et al. 2017; Lock et al. 2016; Sealey et al. 2011a). The influence of dietary inclusion of insect meals on fillet or whole body composition (WBC) lead to controversial results. Some authors reported decreased values of DM and ether extract (EE) with the inclusion of insect meals (Belforti et al. 2015; Dong et al. 2013; Kroeckel et al. 2012; Ogunji et al. 2008a). Concerning crude protein (CP) content, only two trials reported changes (Belforti et al. 2015; Ng et al. 2001) while others did not find any influence of insect meal inclusion on this value (Gasco et al. 2016; Kroeckel et al. 2012; Ogunji et al. 2008a; Sealey et al. 2011a).

A problem often reported using insect meals in fish feeds is their high chitin content. Chitin is a primary component of the insect exoskeletons and it is considered as poorly digestible by fish, due to a reduced enzyme activity (Gasco et al. 2016; Henry et al. 2015; Rust 2002; Sánchez-Muros et al. 2014). The presence of chitinase, chitobiase and lysozyme has been reported in several species (Gasco et al. 2016; Henry et al. 2015). Nevertheless, because of the complex matrix in which chitin is encompassed, the enzyme activity seems to be limited, reducing thus the overall nutrient digestibility (Belforti et al. 2015; Henry et al. 2015; Sealey et al. 2011a).

Insect meals producers can reduce the content of chitin through extraction process (Belluco et al. 2013; Sánchez-Muros et al. 2014) or its digestibility can be increased through dietary enzyme inclusion (Henry et al. 2015) but these technologies are still not fully applied and studies need to be implemented. For instance, the inclusion of exogenous enzymes (carbohydrases or proteases) in diets for European sea bass did not improve the protein and fibre digestibility (Gasco et al. 2016).

It has to be underlined that low levels of chitin have been reported to have immune stimulants (Esteban et al. 2001; Henry et al. 2015, 2018; Hoffman et al. 1997; Lin et al. 2012), bacteriostatic (Vidanarachchi et al. 2010) or antifungal and antimicrobial properties (Faruck et al. 2016; Khoushab and Yamabhai 2010). Insects also contain antimicrobial peptides that have been proved to be active against Gram-positive and Gram-negative bacteria and to have antifungal properties. Great attention is then paid to their possible use as natural antibiotic or antifungal (Yi et al. 2014; Żyłowska et al. 2011).

Positive results on the fish immune status and resistance to diseases were observed with the supplementation of 2.5% of MD meal in black carp (*Mylopharyngodon piceus*) (Ming et al. 2013). Higher survival rates in fish fed with insect meals have been reported when compared to fish with fed other protein sources (Atse et al. 2014).

While several papers reported the possible negative effects of plant proteins on histology of liver and gastrointestinal tract (Oliva-Teles et al. 2015), very few information is available on the consequences of the dietary inclusion of insect meals. First investigations on these aspects are promising with no statistical differences detected for histology or morphometry parameters between fish fed with insect diets and control diets (Lock et al. 2016; Renna et al. 2017).

Results on the use of insect in aquaculture species are dramatically impacted by the type of used larvae, its condition (fresh or dried, whole, ground, defatted) or the method of nutrient isolation and processing (sun drying, thermal treatments, lipid extraction methodologies) and, of course, the fish species object of the experimentation.

As far as whole or cut, live or frozen larvae are concerned, they were mainly tested on warm water fishes and detailed results can be found in Henry and coworkers (Henry et al. 2015).

1.2.1 Tenebrio Molitor

Several experiments have shown that TM meal could be used in partial or total substitution of FM or other conventional protein sources. The level of TM meal inclusion ranged from 8 to 50% substituting up to 100% of FM. Performances results are unequal and bad performances are usually assigned to deficiency of some nutrients when high levels of inclusion were performed. In African catfish fingerlings, no significant differences were found up to 40% of FM substitution while a significant reduction in all parameters was observed when 60% or more of the FM component was replaced by TM (more than 26% of inclusion) (Ng et al. 2001). Roncarati and coworkers performed a pre-fattening trial substituting 50% of FM in common catfish (*Ameiurus melas*) fingerlings diets (Roncarati et al. 2015). Lower final body weight was found in fish fed with TM diet compared to control diet without TM even if results were still considered as acceptable.

Sánchez-Muros and coworkers investigated the nutritive value of a full-fat TM larvae meal as partial protein replacement of FM and SBM in Nile tilapia (*Oreochromis niloticus*) fingerlings (Sánchez-Muros et al. 2015). The dietary inclusion up to 430 g/kg of TM meal worsened performance parameters. Differences on feeding rate (FR), feed conversion rate (FCR), protein efficiency rate (PER) and specific growth rate (SGR) were observed with diets containing increasing levels of TM inclusion (Belforti et al. 2015). Recently, Gasco and colleagues evaluated the effects of dietary inclusion of a full-fat TM larvae meal on European sea bass (*Dicentrarchus labrax* L.) juveniles (Gasco et al. 2016).

Dietary TM inclusion level of 50% led to a worsening of final body weight, weight gain (WG), SGR and FR. Piccolo and coworkers found that TM larvae meal can replace FM up to 25% of inclusion in the diet for *Sparus aurata* without negative effects on weight gain, CP and ether extract digestibility, and marketable indexes after 163 days of feeding (Piccolo et al. 2017). On the contrary, when TM larvae meal was included at 50%, nutrients digestibility and dressed yield were penalized. Moreover in blackspot sea bream, the use of TM meal as an alternative dietary protein source did not show detrimental effects on fish growth performance even if its effects on fillet quality should be considered (Iaconisi et al. 2017).

1.2.2 Hermetia Illucens

Hermetia illucens larvae have been the subject of study for their exceptional ability to grow on organic waste, giving a value of greater sustainability to the obtained meal. As highlighted for TM, the meal preparation methods affect substantially the trial results. In general, the replacement of FM meal with HI meal is higher when a defatted meal is used.

On channel catfish (*Ictalurus punctatus*) decreased WG was observed using diets containing 10% of HI meal inclusion (Bondari and Sheppard 1987) in cage culture, while no statistical differences were reported (Newton et al. 2005) including up to 30% of HI prepupae meal in total FM and partial SBM substitution. Using dried full-fat prepupae meal in rainbow trout diets, St-Hilaire and coworkers were able to obtain inclusion levels of 15% without adverse effect on WG, feed intake (FI) and FCR (St-Hilaire et al. 2007b). Moreover, this diet allowed a 38% reduction in FO (i.e. from 13 to 8%). Highest levels of inclusion (30%) worsened all parameters. Later, Sealey and coworkers evaluated the growth and sensory parameters of trout fed diets having increasing levels (25 and 50%) of FM substituted with normal (NHI) or fish offal-enriched black soldier fly (EHI) prepupae meal (Sealey et al. 2011a). Growth of fish fed with the EHI diets was not significantly different from those fed with the FM-based control diet, while the growth of fish fed with the NHI diets was significantly reduced. The fatty acid profile was influenced by dietary treatments but fish fed with EHI highlighted good EPA and DHA contents. No differences were highlighted in a blind sensory comparison of fish fed with the FM control diet as compared to fish fed with the EHI or NHI diets.

On juvenile turbot (*Psetta maxima*), Kroeckel and coworkers tested partially defatted HI prepupae meal and found a general worsening of performances at the inclusion levels higher than 33% (Kroeckel et al. 2012). Moreover, authors found a decrease of FI with increasing HI meal incorporation, due to low palatability. The authors suggested that the presence of chitin might have influenced the FI, availability and digestibility of the nutrients and therefore growth performance. Nevertheless, as HI was produced on local greenhouse waste streams, the authors concluded that it could be a sustainable alternative protein source in partial substitution of FM (Kroeckel et al. 2012).

In Atlantic salmon, the FM replacement by two different HI larvae meals, varying in their protein and fat contents, led to controversial results (Lock et al. 2016).

A FM-based control diet and a vegetable protein-based diet were tested against two HI larvae diets (18.5 and 37.5 of HI inclusion in substitution of 25 and 50% of FM) for Siberian sturgeon (*Acipenser baerii*) juveniles (Gasco et al. 2017). Preliminary results indicated that the inclusion of HI significantly affected fish performances and condition factor. Generally, up to 25% of FM substitution, fish performed as well as FM- and vegetable protein-based diets.

Results from a trial using a partially defatted HI larvae meal as potential feed ingredient in rainbow trout diet showed that survival, growth performance, condition factor, somatic indexes and dorsal fillet physical quality parameters were not affected by diet (Renna et al. 2017). The use of HI larvae meal induced a decrease of valuable polyunsaturated fatty acids (PUFA) in trout dorsal fillet even if the differences were only reported at the highest level of HI inclusion. The HI worsened the lipids health indexes of the same muscle.

1.2.3 Musca Domestica

In Africa, sustainable local production of insects is conceivable considering the environmental conditions. Moreover, the rise of imported fish feed price has pushed the research for more sustainable source of protein for several livestock production systems, including fish (Makkar et al. 2014). Due to their ubiquitous nature and the short time needed from eggs to suitable larvae for feed purposes, MD have been extensively evaluated in fish diets, primarily with warm water fish species (Henry et al. 2015).

Dietary inclusion levels performed ranged from 7.5 to 100%. Even if performance results were not always positive, MD meals are considered to be sustainable and economically interesting (Adewolu et al. 2010; Aniebo et al. 2009; Fasakin et al. 2003; Makkar et al. 2014; Olele 2011; Sogbesan et al. 2006), as the cheapest and more easily accessible for farmers. Moreover, often maggots are reared on manure helping in the control of the nuisance dung (Sogbesan et al. 2006).

Results on fish are variable in relation to breeding conditions and larvae treatment. In particular, Fasakin and coworkers evaluated drying and processing methods (hydrolysed, defatted, full-fat, sun-dried and oven-dried maggots), on growth and utilization of African catfish (*Clarias gariepinus*) diets highlighting how these influenced the nutrient composition of obtained meal (Fasakin et al. 2003). The authors stated that fish performed better when fed with diets containing defatted MD larvae meals than full-fat MD larvae meal. In diets for *Clarias gariepinus* fingerlings, Idowu and coworkers substituted whole MD larvae meal to FM and SBM without noticeable differences up to 25% of MD inclusion with 50% of replacement (Idowu et al. 2003).

Positive results in terms of growth performances have also been reported (Sogbesan et al. 2006) in hybrid catfish diets, when the replacement of the FM using oven-dried maggot meal reached 25% (dietary inclusion level of 7.5%).

A whole MD larvae meal (oven-dried) has also been studied in the diet of rainbow trout but with little success, as an inclusion of 9.2% (25% of FM substitution) resulted in a decrease of production parameters and to a deterioration in fish quality (lipid FA profile) (St-Hilaire et al. 2007b).

The fish performance, the concentration of plasma glucose, the cortisol and blood characteristics of *Oreochromis niloticus* fingerlings fed with increasing levels of MD meal in diets were evaluated (Ogunji et al. 2008b). The inclusion of MD larvae meal in the diet did not impair fish growth and performance. At the same time, no adverse or stress effect on the haematology and homeostasis was observed. Moreover, no significant difference was observed in liver glycogen reserve and in hepatic catalase, activity did not differ significantly. The authors reported elevated glutathione S-transferases activities when fish received higher dietary magmeal concentration.

Adewolu and colleagues evaluated an animal protein mixture containing MD larva meal in diets for *Clarias gariepinus* fingerlings (Adewolu et al. 2010). The inclusion up to 50% of animal protein mixture did not influence the performances parameters. However, in fish fed with highest FM substitution levels, these indicators were significantly lower.

Dong and coworkers investigated the effect of dietary supplementation with MD maggot meal or SBM on the growth performance and antioxidant responses of gibel carp and dark barbel catfish (Dong et al. 2013). Interestingly, even if MD inclusion lead to a worsening of some performances when compared to the control diet, fish fed with MD diets exhibited equal or better results compared to fish fed with SBM diets. Moreover, the MD supplementation enhanced the antioxidant capacity in gibel carp. Positive results have also been reported (Ming et al. 2013) on the black carp, where the replacement of the FP has reached 25%.

Feeding African catfish (*Heterobranchus longifilis*) larvae with non-isonitrogenous nor isoenergetics diets containing different protein sources, Atse and coworkers reported similar or better performances in fish fed with MD diets if compared to fish fed with *Artemia salina* or fish by-products diets even if the CP was lower (Atse et al. 2014). If compared to other protein sources based diets, (blood, brain or SBM) MD always showed better results. When diets were fortified with minerals, vitamins and amino acids premix, MD-fed fish reported the highest performances. The survival rate was also always higher in fish fed with MD compared to other groups.

Recently, Lin and colleagues reported that MD meal can be included up to 30% without negative effects on growth and feed utilization of barramundi and no major influence on body composition (Lin and Mui 2016). When oxidative status and immune responses are considered, the inclusion rate of 10% corresponding to a FM substitution of about 25% is recommended.

1.3 Poultry by-Product Meals

Poultry by-product meal (PBM) is a high protein source commonly used in domestic animal feeds. The Association of American Feed Control Officials defines PBM as the '*ground, rendered, clean parts of the carcass of slaughtered poultry such as necks, heads, feet, undeveloped eggs, gizzards and intestines (provided their content is removed), exclusive of feathers (except in such amounts as might occur unavoidably in good processing practices)*' (AAFCO 2010).

In recent years, mainly two PBMs are available in the market: feed grade and pet food grade. The former, less expensive, is usually considered as produced from low-quality by-product fractions and contains a higher level of ash and lower protein content (Aldrich 2006; Dozier and Dale 2005). The latter, due to its high price and quality, is mostly used in pet foods.

Poultry by-product meal quality and nutritional value (Tables 1.4 and 1.5) can change from one batch to another depending on the included materials and on the processing (time and temperature of the cooking process) applied for the production (Cruz-Suárez et al. 2007; Dale et al. 1993).

As for all the rendering processes, the PBM production involves the application of heat, the extraction of moisture and the separation of fat (Cruz-Suárez et al. 2007; Meeker and Hamilton 2006). The applied rendering process enables the destruction of pathogenic microorganisms and provided aseptic protein product free of potential biohazards and environmental threats (Hamilton et al. 2006). Technological developments in the production process have significantly improved the quality of PBM (Badillo Zapata et al. 2016; Cruz-Suárez et al. 2007; Sealey et al. 2011b). Nowadays, due to its high nutritional quality, large availability and palatability, PBM has considerable potential as feed ingredient for aquaculture providing substantial feed cost saving (Hernández et al. 2010).

The PBM protein content ranges from 51.6 to 81 (% DM); despite a relatively good amino acid profile, as far as fish nutrition is concerned, lysine and methionine are often reported as the first limiting EAA (Castillo-Lopez et al. 2016; Hertrampf and Piedad-Pascual 2000; Nengas et al. 1999; Rawles et al. 2006; Riche 2015; Rossi and Davis 2012). Its average gross energy content is similar to the one shown by insect meals and varies between 16 and 25 MJ/kg DM. The crude fibre content is very low (about 1%) while the level of ash varies between 5 and 30%. As far as fat content is concerned, publications reported values from 6.7 to 22.5%. PBM has very low content in precious n-3 FA (EPA, DHA) (NRC 1993; Sealey et al. 2011b). This can cause problems at high levels of inclusion, especially in juveniles or marine species. Particular attention during the diet formulation and the inclusion of appropriate quantities of FO in diets can overcome the problem but increase the feed price (Sealey et al. 2011b).

As far as ADC of nutrients is concerned, the majority of the trials were carried out with salmonids. The first one was that of Cho and Slinger who found quite low CP digestibility (about 70%) in rainbow trout (Cho and Slinger 1979). Research performed over the successive years showed different results (Bureau et al. 1999;

Table 1.4 Nutrient composition and nutritive value of poultry by-product meal (PBM) compared to fishmeal (FM) and soybean meal (SBM). Values are reported as mean of values found in the cited references (with minimum and maximum values). The following chemical data are shown here: dry matter (DM), crude protein and essential amino acids, ether extract and fibre

	Unit	PBM[a]	FM[b]	SBM[c]
Dry Matter (DM)	% as fed	93.7 (82.4–97.4)	92.1 (90.0–94.4)	87.9 (85.0–92.1)
Crude protein	% DM	66.1 (51.6–81.0)	75.6 (70.2–80.7)	51.4 (48.3–54.5)
Lysine	% protein	4.4 (3.3–8.2)	6.1 (5.5–7.5)	6.1 (5.7–6.6)
Methionine	% protein	1.4 (1.0–2.0)	2.2 (2.0–2.6)	1.4 (1.2–1.6)
Methionine + Cystine	% protein	–	2.9 (2.6–3.2)	2.9 (2.5–3.3)
Tryptophan	% protein	0.5 (0–0.8)	0.8 (0.7–0.9)	1.3 (1.2–1.4)
Threonine	% protein	2.8 (1.9–3.9)	3.1 (2.9–4.3)	3.9 (3.5–4.3)
Leucine	% protein	5.0 (3.9–9.7)	5.9 (5.2–7.3)	7.5 (6.8–8.0)
Isoleucine	% protein	2.7 (1.8–4.7)	3.7 (3.3–4.4)	4.6 (4.3–5.0)
Valine	% protein	3.1 (2.2–5.2)	4.2 (3.9–4.8)	4.8 (4.3–5.4)
Histidine	% protein	1.9 (1.2–5.6)	1.8 (1.7–1.9)	2.6 (2.4–2.9)
Arginine	% protein	5.1 (3.2–8.8)	4.6 (4.0–6.0)	7.4 (6.8–8.1)
Phenylalanine	% protein	2.8 (2.2–4.0)	5.5 (5.2–6.5)	8.5 (7.7–9.4)
Ether extract	% DM	13.8 (6.7–22.5)	8.1 (2.0–12.0)	2.1 (2.0–2.2)
Crude fibre	% DM	1.1 (0.5–2.1)	–	6.7 (3.5–10.1)

[a]Data from: Barreto-Curiel et al. (2016), Bureau et al. (1999), Castillo-Lopez et al. (2016), Cheng and Hardy (2002), Cruz-Suárez et al. (2007), de Carvalho et al. (2016), Dozier and Dale (2005), El-Haroun et al. (2009), Fasakin et al. (2005), Feedpedia: http://www.feedipedia.org, Goda et al. (2007), Guimarães et al. (2008), Hernandez et al. (2010), Hernandez et al. (2014), Li et al. (2009), Ma and Wang (2014), Nengas et al. (1999), Riche (2015), Sealey et al. (2011b), Shapawi et al (2007), Subhadra et al. (2006), Sugiura et al. (1998), Wang et al. (2006), Wang et al. (2016), Yang et al. (2004)
[b]Data from: Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)
[c]Data from: Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)

Cheng and Hardy 2002; Dong et al. 1993; Guimarães et al. 2008; Hernández et al. 2010; Pfeffer et al. 1995; Sugiura et al. 1998). The ADC improvement obtained over the years reflects the improvement of PBM processing methodologies, but also supported the hypothesis that nutrients ADC is highly dependent on the PBM origin, quality and the faecal collection methodology.

Several researches have shown the high potential of PBM in aquaculture feeds. The dietary inclusion level, as well as the FM or other conventional protein sources substitution, varies among fish species mainly due to the PBM quality and the diet formulation. Reduced performances with high levels of FM substitution are often associated with decreased protein digestibility or deficit in EAA or essential FA (Badillo Zapata et al. 2016; Gaylord and Rawles 2005; Parés-Sierra et al. 2014; Shapawi et al. 2007). Furthermore, increased attention has recently been paid to the lower taurine content of PBM compared to FM. Historically, taurine has not been considered as an EAA (El-Sayed 2014; Salze and Davis 2015). Nevertheless, recently it has been demonstrated that the ability to synthesize taurine widely varies

Table 1.5 Nutrient composition and nutritive value of poultry by-product meal (PBM) compared to fishmeal (FM) and soybean meal (SBM). Values are reported as mean of values found in the cited references (with minimum and maximum values). The following data are shown here: minerals (ash), calcium, phosphorus, sodium, potassium and gross energy

	Unit	PBM[a]	FM[b]	SBM[c]
Minerals (ash)	% DM	15.0 (5.1–29.7)	16.6 (12.0–23.3)	6.9 (6.8–7.0)
Calcium	% DM	5.1 (2.2–9.9)	36.3 (15.4–78.3)	3.9 (2.3–6.3)
Phosphorus	% DM	2.7 (1.6–5.0)	25.9 (19.0–40.4)	6.9 (5.8–8.6)
Sodium	% DM	0.6 (0.5–1.0)	10.0 (5.9–14.4)	0.1 (0.0–0.8)
Potassium	% DM	0.8 (0.4–1.8)	10.2 (5.9–14.4)	23.7(21.8–26.0)
Gross energy	MJ/kg DM	21.2 (16.2–24.9)	21.4 (19.6–23.8)	19.9 (19.8–20.0)

[a]Data from: Barreto-Curiel et al. (2016), Bureau et al. (1999), Castillo-Lopez et al. (2016), Cheng and Hardy (2002), Cruz-Suárez et al. (2007), de Carvalho et al. (2016), Dozier and Dale (2005), El-Haroun et al. (2009), Fasakin et al. (2005), Feedpedia: http://www.feedipedia.org; Goda et al. (2007), Guimarães et al. (2008), Hernandez et al. (2010), Hernandez et al. (2014), Li et al. (2009), Ma and Wang (2014), Nengas et al. (1999), Riche (2015), Sealey et al. (2011b), Shapawi et al (2007), Subhadra et al. (2006), Sugiura et al. (1998), Wang et al. (2006), Wang et al. (2015), Wang et al. (2016), Yang et al. (2004)
[b]Data from Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)
[c]Data from Feedpedia: http://www.feedipedia.org, Guillame et al. (2001)

among fish species because of the different activities of key enzymes in its biosynthesis pathway (El-Sayed 2014; Rossi and Davis 2012; Salze and Davis 2015).

High levels of FM replacement could lead to decrease the taurine content beyond a limit level causing decrease in performances (Rossi and Davis 2012). The dietary supplementation with taurine could allow highest levels of FM substitution, in particular, when plant-based proteins are also present in the diets formulation (Chatzifotis et al. 2008; Rossi and Davis 2012; Salze and Davis 2015).

The minimum level of FM needed in fish fed with PMB to support good performances is species dependent (Table 1.6). Rossi and Davis showed that 5% of FM is sufficient in Florida pompano when 15% of PBM is used (Rossi and Davis 2012). Compared to a control diet containing 35% FM, 10% of PBM, 15% of SBM and 10% of rapeseed meal, Ma and Wang found reduced performances already at 40% of FM substitution (further 15% of PMB dietary addition) in a trial performed with Golden pompano (*Trachinotus ovatus*) juveniles suggesting that levels higher than 21% of FM are needed (Ma and Wang 2014). Using a locally produced PBM (29.2% of inclusion), Nengas and colleagues reduced the level of FM up to 35% in *Sparus aurata* diets with slight (but not statistical) performances reduction (Nengas et al. 1999). Lower levels of FM requirements were found for Japanese sea bass (8%) (Wang et al. 2015), red drum (10%) (Kureshy et al. 2000), Malabar grouper (25% and 13% reported by Wang and coworkers and Li and colleagues, respectively, Li et al. 2009; Wang et al. 2008), and cuneate drum (18%) (Wang et al. 2006). Badillo-Zapata and colleagues found that the total replacement of FM with PBM in diets for *Totoaba macdonaldi* juveniles led to worsened performances and

Table 1.6 Maximum level of FM substitution (and PBM inclusion) with PBM reached without impairing any of the performance parameter evaluated

Fish Species	Max level of FM substitution	% PBM inclusion	% FM in control diet	Other protein source	CP and CL diet content	Observation	Reference
Sparus aurata	50	29.2	72.9	–	45–13	Locally produced PBM	Nengas et al. (1999)
Morone chrysops x M. sexatilis	100	30	30	SBM (30)	40–6	Decreased (but not statistically different) performances	Webster et al. (2000)
Carassius auratus gibelio	15	10.8	46.3	–			Yang et al. (2004)
Oreochromus niloticus x O. mossambicus	66	30.3	44	–		Best productivity values (WG, FCR, SGR, PER) but not statistical differences *vs* FM diet	Fasakin et al. (2005)
Scophthalmus maeoticus	25	21.2	77.3	–	55–15		Turker et al. (2005)
M. chrysops x M. sexatilis	35	17.2	25	PBM (7.73) SBM (25.9)	42.5–11	Further 9.47% PBM inclusion	Rawles et al. (2006)
D. labrax	50	17.5	35	BM (3)	42.5–12.5		Wang et al. (2006)
Carassius auratus gibelio	100	53	53	–	38–9.5	The optimal replacement level of FM by PBM was estimated by second-order polynomial regression to be 66.5% in protein	Yang et al. (2006)
Psetta maeotica	50	43.2	77.3	–			Yigit et al. (2006)
Clarias gariepinus	100	34.5	25	–	25.5–8.5	Trial performed comparing also other protein sources as FM substitute	Goda et al. (2007)
Cromileptes altivelis	50 and 100	36.1 and 74	69.4	–		Pet food grade PBM enable 100% FM replacement while feed-graded PBM allowed only 50% replacement	Shapawi et al. (2007)

(continued)

Table 1.6 (continued)

Fish Species	Max level of FM substitution	% PBM inclusion	% FM in control diet	Other protein source	CP and CL diet content	Observation	Reference
Epinephelus malabaricus	50	11.5	50	–	53–9		Wang et al. (2008)
Oncorhynchus mykiss	30	30	40	CGM (28) BM (6–12)	49–23.5	PBM partially replaced FM and CGM (total replacement of BM)	El-Haroun et al. (2009)
Epinephelus malabaricus	50	22.8	50	BM (5.7)	53–9.6	Fish fed with the 75% PBM diet had a significantly lower FI than fish fed with FM or 25% PBM diets	Li et al. (2009)
O. niloticus	100	26.5	27	SBM (12) CGM (9)	35.5–6.8	High quality PBM	Hernández et al. (2010)
O. mykiss	100	63.8	68.6	–	48.3–18.7	High quality PBM	Sealey et al. (2011b)
Rachycentron canadum	60	30	50	SBM (11.3)	45–11	Improved PER and FER in fish fed with 15 and 22.65 PBM inclusion. A quadratic regression indicated at 30.75% FM the optimal replacement level for PER value.	Zhou et al. (2011)
Trochinotus carolinus	67	9.8	15	SBM (50)	40–8	Fish growth performance reduced when FM was completely removed from the diets.	Rossi and Davis (2012)
Oncorhynchus mykiss	100	59	66	–	43–12.5	Fish fed with the 67% PBM diet had a significantly higher FBW than the rest of the treatments, whereas the 100% PBM had a significantly lower FBW than 67% PBM	Badillo et al. (2014)
Lutjanus guttatus	65	39.4	52.6	SQ (6) K (7.6)	51.5–16	Fish fed with the 87% PBM showed worsened (not always stat. different) values	Hernández et al. (2014)

(continued)

Table 1.6 (continued)

Fish Species	Max level of FM substitution	% PBM inclusion	% FM in control diet	Other protein source	CP and CL diet content	Observation	Reference
O. mykiss	73.5	44	66	–	43.5–12.5		Parés-Sierra et al. (2014)
Trochinotus carolinus	67	from 19.8 to 22.3	32.6	–	48.5–21	Five different types of PBM. FI reduced only with CC66 PBM type	Riche (2015)
Lateolabrax japonicus	80	38.7	40	SBM (20) RSM (8)	47.5–11.5	Improved performances up to 80 substitution *vs* FM diets	Wang et al. (2015)
O. niloticus	100	26	20	SBM (15) CGM (11)	30.2–11.5		Yones and Mewalli (2015)
Totoaba macdonaldi	67	45	65	–	51–8		Badillo-Zapata et al. (2016)
Dicentrarchus labrax	60	18.94	47.4	SBM (11) CG (11)	48–14		Srour et al. (2016)

FM: fishmeal; PBM: Poultry by-product meal; CGM: Corn gluten meal

FM: Fishmeal; PBM: Poultry by-product meal; CGM: corn gluten meal; BM: blood meal; SQ: Squid meal; K: Krill meal; RSM: Rapessed meal; CG: Corn gelatin

WG: Weight gain; FI: Feed intake; FR: feeding rate; PER: Protein efficiency ratio; FCR: Feed conversion ratio; IBW: Initial body weight (g); FBW: Final body weight (g); FER: Feed efficiency ratio

CP: crude protein; CL: crude lipid

increased mortality and that PBM can be used in up to maximum 67% FM replacement (Badillo-Zapata et al. 2016).

Wang and coworkers obtained 80% of FM replacement with diets containing also other protein sources (SBM, rapeseed meal) showing a high capacity of *Lateolabrax japonicus* in utilizing PBP nutrients (Wang et al. 2015). Recently, it was theorized that low performances obtained with high levels of FM substitution by PBM could be due to Selenium deficiency as the content of this essential nutrient is lower in PBM than in FM (Wang et al. 2016). Webster and colleagues indicated that diets without FM can be fed to juveniles sunshine bass without major negative effects on performances (Webster et al. 2000). Similarly, Sealey and coworkers, using a high-quality PBM, were able to substitute completely FM in rainbow trout diets (Sealey et al. 2011b). In addition, Barreto-Curiel and colleagues positively replaced 100% of FM in rainbow trout using a PBM mixed with acid fish silage (Barreto-Curiel et al. 2016).

As far as WBC is concerned, modifications can occur when alternative protein meals are used as FM replacers (Gatlin et al. 2007). The major effects of PBM inclusion on WBC have been reported for the CP and crude lipid content. Goda and colleagues found a reduction of the CP content in fish fed with 100% PBM as FM replacer and these results were supported by the findings of Zhou and coworkers (Goda et al. 2007; Zhou et al. 2011). Conversely, slightly higher CP values were found in fish fed with PBM than those fed with FM-based control diet (Shapawi et al. 2007; Yang et al. 2006). An increase of crude lipid WBC content was reported in rainbow (Alexis et al. 1985; Steffens 1994), in European eel (Gallagher and Degani 1988), in chinook salmon (Fowler 1991), in gilthead seabream (Nengas et al. 1999), in Nile tilapia fingerlings (Hernandez et al. 2010) and in spotted rose snapper (Hernandez et al. 2014).

The whole body fat increases in fish fed with diets having PBM were often justified by high fat content in PBM. Nevertheless, several research reported no differences in WBC even at high levels of PBM inclusion (El-Haroun et al. 2009; Ma and Wang 2014; Riche 2015; Yones and Metwalli 2015). These contradictory results support the theory that differences in utilization and transformation capacity for PBM exist among fish species, and are related to the quality and quantity of PBM used in diets formulation.

PBM has been widely studied and the improvement of quality due to better processing technologies allows high levels of FM replacement. This may allow reduction of feed cost formulation and increase in profitability. Nevertheless, as the technological process of PAP production was revised (EC No. 94/449; temperature over 133 °C, pressure, 3 bar by steam for 20 min; maximum particle size, 50 mm), this could compromise nutritional quality and modify the reference values obtained so far. Therefore, it is necessary to further evaluate these ingredients and research is highly needed (Moutinho et al. 2017).

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Chapter 2
Fishery Discard as a Source of Food for Reared or Wild Fish? The Bottom Trawling in the Mediterranean Sea as a Case Study

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Abstract Fisheries products found in the landing places or markets usually represent a fraction of what has been originally taken out from the sea by fishermen. A more or less variable amount of the gross (or overall or initial) catch, in fact, is returned either dead or alive to the sea for multiple reasons. This phenomenon, generally known as 'the discard problem', has raised more and more global concern especially in the last decades since introducing more selectivity gears and acquiring more reliable catch data (also about the survival rate of the discarded specimens) are considered basic requirements in the current management plans for recovering and rebuilding fish stocks. To this concern, the current Common Fisheries Policy aims to reduce the discard rate in the European waters through the introduction of a landing obligation (better 'discard ban') according to which all the specimens of the 'target' stock caught must be landed. This chapter focuses on the characterization of the catch returned to the sea 'potential' available to be landed after the introduction of landing obligation, and the possible use as a potential food source in aquaculture (the direct use for human consumption is in fact prohibited).

Keywords Biomass index · Catch returned to the sea · Common fisheries policy Density index · Discard · Fish oil · Fishing practice

2.1 Introduction

'The kingdom of heaven is like a net that was let down into the lake and caught all kinds of fish. When it was full, the men pulled it ashore. Then they sat down and sorted the good fish in containers, but threw the bad away. So will it be at the end of the age: The angels will come and separate the wicked from the righteous, and throw them into the fiery furnace …. '*Matthew 13, 47–50*— 'The Parable of the Net'

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Table 2.1 A synoptic list of 'discard' related terms used in this Chapter

Term and acronym	Meaning
Rough catch (RoC):	All the material taken from the Sea by fishermen during commercial fishing; RoC includes litter and stones
Gross (overall or initial) catch (GrC):	All the living or living related material taken from the Sea by fishermen during commercial fishing.
By-catch (ByC)	Catch different from the main species/sought after species, sold or discarded by fishermen according the fishing typology and peculiar conditions.
Catch returned to the sea (CRS):	The portion of the Gross catch finally returned to the sea by fishermen, no matter the reasons (protected species, damaged specimens, undersized, species considered unmarketable, ceremonial species, upgrading, etc.). Discard (see below) is the most consolidated term
Dirty catch (DyC)	The fraction of gross catch (mainly seaweeds, seagrass leaves, invertebrates such gorgoniaceans, etc.) considered a not at all usable by fishermen
Discard (DiC):	Fish and shellfish or other living animals edible or not, or usable or not, but returned to the sea by fishermen.
Target Catch (TtC):	The consolidated term to indicate fish and shellfish or other living beings strongly searched and highly considered by fishermen (Main catch should be more properly used).
Unreported, Unregulated and Illegal fishery items (UUI)	All the catch components not reported in the official statistics for different reasons; for example, retained for the crew use or undersized fish hidden to be landed and sell out of the market.).

As supported by the previously recalled biblical quotation, assessing what portion of the Gross catch will be returned to the sea by fisheries has been in all times a very critical and difficult objective. The first comprehensive report on CRS (herein discards for simplicity) in world fisheries was published in the 80s (Saila 1983), but they have been recognized as a management problem only at the beginning of the twentieth century. The landings from marine capture fisheries in the world are now approximately 85 million tonnes (Rosemberg et al. 2014), whereas it has been estimated that, globally, over more than 7 million tonnes of catch are discarded annually (Kelleher 2005).

Because of the difficulty correlated with some terms, a list of 'discard'-related terms used in this chapter can be found in Table 2.1.

2.2 Discards in the Mediterranean Sea

Fisheries products found in the landing places or markets usually represent a fraction of what has been originally taken out from the sea by fishermen. Not considering the litter and natural inorganic material originally present in the 'rough

catch', a more or less variable amount of the remaining 'gross' (also initial catch), in fact, is returned either dead or alive to the sea for multiple reasons such as laws regulations (protected species or undersized specimens), local markets sale requirements (no or low commercial value or damaged specimens), storage availability on board (upgrading) and fishing practices (i.e. short or long distance). Hence, a rough initial definition of the 'catch returned to the sea' (CRS) might be 'that part of the biological gross catch brought on board but then returned to the sea dead or alive for whatever reason by fishermen' (Alverson et al. 1994; Borges et al. 2001; Hall et al. 2000).

There is, however, no general agreement about the classification and denomination of the different detectable categories. The above reported classification complexity along with the legal aspects, most of CRS fall within the so-called Unreported Unregulated and Illegal fishery items (UUI), might explain both the difficulty in collecting information and the large variability of available figures (Bazigos et al. 1984; Cingolani et al. 1986a, b; Pauly et al. 2014; Piroddi et al. 2015).

In the Mediterranean Sea, over 230 000 tonnes of discards, corresponding to 18.6% of the gross catch, have been estimated (all fishing gears) for the year 2006 (Tsagarakis et al. 2013). Currently, an average amount of 300 kg/km^2 for the Mediterranean Sea (Kelleher 2005) is recognized. For the Italian marine fishery, in the period between 1950 and 2010, the discard fraction (7% of gross catch) corresponds to about 95% of industrial fisheries. In the same temporal range, discards and by-catch from Italian bottom trawling were about 3.8 million tonnes (Piroddi et al. 2015).

This phenomenon, generally known as 'the discard problem', has raised more and more global concern since introducing more selectivity gears and acquiring more reliable catch data (also about the survival rate of the discarded specimens) are considered basic requirements in the current management plans for rebuilding fish stocks (Colloca et al. 2013; Crean and Symes 1994). Discard composition evaluation is also important to understand the impacts at population, trophic and ecosystem levels (Borges et al. 2001; Hall et al. 2000).

Despite the progress in studying discards, the expansion of monitoring schemes and the creation of new objectives for the discards issue are needed under the framework of ecosystem approach to fisheries (Jereb et al. 2016a; Ragonese et al. 2016; Tsagarakis et al. 2013). The relative importance of discards depends largely on the gear, the gear characteristics, fishing strategies, marketing constraints and legislation (Hall 1996).

Generally, bottom trawls are responsible for the bulk of discards (Hall et al. 2000). Several studies on trawl fisheries from Mediterranean Sea report discards (on as total gross or landing catch ratios) lower than 20% (Atar and Malal 2010; El-Mor et al. 2002; Faltas et al. 1998; Kelleher 2005; Sartor et al. 2003; Zengin and Akyol 2009). Other studies show quite high values (about 50%) with maximum values that reached 64% (Carbonell et al. 2003a, b; Machias et al. 2001; Moranta et al. 2000; Sánchez et al. 2004, 2007; Stergiou et al. 1998; Tsagarakis et al. 2008). Mid-water trawls and purse seines, that have clear target species (small pelagic), despite their

less proportion of discards, produce overall high discards quantities, since they are responsible for the majority of the landings (Santojanni et al. 2005; SAUP 2012). These gears have attracted little attention in examining discards, possibly because they produce low discards ratios.

Small-scale fisheries in the Mediterranean use a variety of fishing gears. Most of the existing discards studies analyse trammel nets and gillnets for which discards ratios between 15 and 40% have been reported (Gökçe and Metin 2007; Quetglas et al. 2004; Tzanatos et al. 2007).

Discarding is also characterized by high temporal and spatial variability (Gristina et al. 2002; Kennelly, 1995; Pauly et al. 2014). Environmental characteristics (substrate type, depth, productivity affecting the species composition of the communities), fishing practice (gear type and target species), high or low local abundance (Gristina et al. 2002) and commercial preference can explain geographic differences in discarding practices (Tsagarakis et al. 2013).

Moreover, the fisheries catch statistics of Mediterranean Countries, reported annually to the Food and Agriculture Organization of the United Nations (FAO), are 2.6 times lower in the 1950s and 1.8 times lower since 2000, than reconstructed catches. The non-reported catch was high in all fishing sectors, including industrial, artisanal and recreational fisheries (Pauly et al. 2014).

However, absolute estimates of discarding in the bottom trawl fisheries in the Mediterranean Sea are difficult to estimate with a reasonable degree of certainty. Over the past 70 years, discarding practice has gradually increased and together with a shift in the species composition of the discarded catch. Discarding can occur for different reasons, in the past was mostly driven by market demand, but more recent constraints in terms of regulations or reduced stock abundance have led to changes in fishing strategies and became a reason for discards (Damalas et al. 2015).

2.3 Impact of Discards on Ecosystem

Discarding has been acknowledged globally among the most important issues for fisheries management, since it is considered a waste of fishing effort and fished resources (from a fisheries point of view) and a factor affecting biodiversity and community structure, from a more ecological oriented point of view (Bellido et al. 2011; Hall and Mainprize, 2005; Hall et al. 2000).

From the fishery point, it would be better to maintain on board, land and use for society benefits as much as possible of the gross catch since the large effort (in term of fuel, ship use and manpower) is employed to realize the catch; always within this perspective, it would be better to return to the sea the not usable (Dirty catch) or less valuable (Not main catch) gross catch fraction in order to partially compensate the loss of energy taken from the system (Jereb et al. 2016a).

On the contrary, from an ecological point of view, fisheries should be managed and conducted in order that the gross catch will be composed almost exclusively by

the main (target) species without capturing the other less important species. The main problem with this scenario is that it might be achieved only by using perfect selective gear (at least at the present utopist). Discarding, on one side, might affect biodiversity and community structure, having a considerable impact on species and populations, but on the other side discarded biomass might represent a benefit for opportunistic and scavenger species (Kelleher 2005; Ramsay et al. 1997; Tingley et al. 2000). Discards, in fact, provide food for a wide range of marine species like seabirds, dolphins, fish and many benthic invertebrates, covering different zones or habitats such as sea surface, pelagic and benthic environments (Oro et al. 2013). These species have adapted to this ready source of energy (Bicknell et al. 2013; Bozzano and Sardà 2002; Hill and Wassenberg 1990, 2000; Ramsay et al. 1996; Svane et al. 2008; Wassenberg and Hill 1990). Several studies have demonstrated that discards re-enter the marine food chain becoming a protein source for other marine species, especially fish and crabs (Safran 2009). For this reason, some authors suppose that rules such as the EU landing obligation (i.e. the removal of such food source from the sea) could have a potential negative impact on food web. To date, the effects of landing obligation are not yet known and could be more harmful than beneficial to an ecosystem, through cascading effects in the food web, that has long been exposed to the ready food sources (Fondo et al. 2015). The implementation of the discard ban might still lead to perverse incentives and changes in fishing practices with consequences where the fisheries do not recover but scavengers still suffer from food reduction. Marine species must revert to their original diet causing a population crash in that prey from sudden increases in predation pressure. Under this perspective, a gradual reduction of discarding should be strongly recommended to allow species to habituate to the food reduction (Fondo et al. 2015).

The survival rate of discarded fish and invertebrates represents another important item in the discard problem debate. The classic fisheries (more conservative) assumption is that the short- or medium-term destiny of the returned fish to the sea should be the death and hence the CRS should be included in the estimation of the fishing mortality. However, there are evidences that at least for some 'rough' species (such *Scyliorhinus canicula* and *Nephrops norvegicus*), an important fraction of CRS could survive.

In any case, the survival of CRS is highly variable depending on multiple and interacting factors such as the species and fishing gear features, the depth of capture and the season (Bergmann and Moore 2001; Mesnil 1996; Revill et al. 2005). If the stock is characterized by a high survival rate of the specimens returned to the sea the application of best practice of handling and release could contribute to stock maintaining (Gilman et al. 2012). For example, a post-release survival rate of 25–30% has been estimated for the Norway lobster (*Nephrops norvegicus*) in Atlantic waters (Bergmann and Moore 2001; Bergmann et al. 2001). In other words, the fishing mortality of Norway lobster is expected to increase if the undersized juveniles will be not excluded from the landing obligation. However, a quite different (and worse) destiny might expect Norway lobsters returned to the sea during the warm and sunny

Mediterranean summers; these specimens passed from 12–14 °C of the slope bottoms to 20–25 °C and even more of the surface waters. Surviving is quite unlikely in these situations.

2.4 Management Actions and Tools

Management (often quoted as 'Mitigation') tools aim to reduce discard rate mainly comprise legal dissuasive constraints (such the famous minimum landing size), gear selectivity improvement (mostly the mesh in the cod end of trawls) and spatial-temporal closures. Many European fisheries have put in place measures such as minimum mesh sizes, effort regulations including spatial-temporal fishery closures, days at sea quotas, daily hour restrictions and landing quotas in an effort to mitigate the capture of unwanted species/sizes (STECF 2008). All of these tools could be a powerful alternative to improve the management of discards and create more sustainable fisheries, but the efficacy must be checked with the context. For example, Italy introduced in the '60s the closure of the whole national coastal strip (i.e. the grounds within 3 nautical miles from the coastline or with less than 50 m of depth) to bottom trawling to protect the massive recruitment phase of red mullet (*Mullus barbatus*) (Froglia 1984). Unfortunately, with low success due to the chronic weak enforcement capability. However, solutions to by-catch/discards need to be designed for specific fisheries and may differ between regions of the world (Hall and Mainprize 2005; Johnsen and Eliasen 2011). The European Union Common Fisheries Policy (CFP) has addressed the discards issue by banning discarding (EU 2011). The new CFP aims to reduce the discard rate in the European waters through the introduction of a landing obligation according to which almost all the 'target' catch must be landed. This change was introduced gradually, since 2015 and will extend until 2019 for all commercial fisheries. Under the landing obligation, in the Mediterranean Sea, all catches of the species for which a 'Minimum Conservation Size' has been established (regardless of whether they are pelagic or demersal) must be stored on board mixed each other but separately from the oversized catch and landed. The mix of undersized fish landed can be commercialized and used for whatever undefined goals but not for direct human consumption).

The landing obligation is aimed mainly in discouraging fishers to exploit grounds full of undersized fish and collaterally (assuming a positive answer by the fishers) to improve resource use and management of marine stocks through an effective reduction in harvest rates, resulting from changes in fishing practices to reduce or eliminate the capture of unwanted fish.

The fishing industry and part of the scientific community (Garcìa et al. 2015; Jereb et al. 2016a) have rejected this kind of measure and have continually argued that an alternative approach based on avoidance and reduction of discards supported by a range of incentives (fishing industry) or a new paradigm based on effort reduction compensated by the possibility to legally sell small fish, but enforcing the

square mesh size of 40 mm (Jereb et al. 2016a) are the only ways to address the discards problem. Fisheries management and discarding are linked and an overall discards ban will not solve the real issue behind discarding with the risk of introducing further economic inefficiency in an already almost collapsed sector. More actions aimed to rebuild fisheries and implement reforms to sustain them are still necessary.

2.5 Use of 'Recovered' Discards in Aquaculture

A big amount of the total world fisheries retained on board catch is yet destined for non-food uses. This 'not main or low value by-catch' is used mainly for the production of fishmeal and fish oil, within industrially compounded animal feeds, but also in fresh or frozen form as animal feed (Tacon and Metian 2009). Yearly, capture fisheries produces about 90–95 million tonnes of fish, of which 20–25 million tonnes are processed into fishmeal and oil (Wijkstrom 2012).

Fishmeal and oil are electively produced from the harvest of massive schooling small pelagic fish such as herring, menhaden, capelin, anchovy (as the famous Peruvian *Engraulis ringens* stock), pilchard, sardines and mackerel. Fishmeal and oil are also produced from 'rejects' related to fish processed for human consumption.[1]

There is an increasing trend for aquaculture productions to be dependent on feeds (FAO 2011; Jackson 2009). This dependency is particularly strong within compound aquafeeds for farmed carnivorous as finfish species and marine shrimp. Fishmeal and fish oil produced from wild harvest fisheries are among the highest quality proteins and lipids available and for this reason, the replacement of fishmeal with other protein sources is difficult. Since production from wild fisheries is stagnating and competition for fishmeal and fish oil by other sectors is increasing, fishmeal and fish oil are therefore only available in limited quantities (Hannenson 2015; Tacon et al. 2012). This is perceived as a threat to the growth of the aquaculture sector (Diana 2009).

The use of aquafeeds in the aquaculture sector is notable: As an example, the world production in this field has been reported to reach 31.5 million tonnes in 2008 with exclusive reference to processes involving feed as single ingredient, farm-made aquafeeds or compound aquafeeds derived from industrial sources (FAO 2010). This trend has been observed since 1995 at least: the global production of industrial compound aquafeeds is reported to show remarkable increases: +285.5% in 2008 if compared to 1995 (Tacon et al. 2010). Normally, the composition of similar aquafeeds should be considered as a three-component system (De Silva and Hasan 2007): proteins (fishmeal, soybean meals, etc.); carbohydrates (cereals and cereal-based ingredients are generally used); and fats/oils

[1]More information can be found at the following website: http://www.nmfs.noaa.gov

(from fish and vegetable sources). The use of similar compounds concerns many fish productions without market distinction or economic preferences between higher or lower value species. The production of aquafeeds affects significantly the total costs of production, the production of 1 kg of fishmeal and fish oil require 4–6 and 10–50 kg of fish, respectively (Shepherd and Jackson 2013). Annually, it has been estimated that about 60% of fishmeal and 80% of fish oil of the world total production are consumed by the aquaculture field (with an increasing positive trend; Péron et al. 2010). The raw material used in industrial reduction processes consists mainly of low-value fish, obtained from reduction fisheries and as by-catch.

The landing obligation has been welcomed by the fishmeal and fish oil industry. The supply of fishmeal and fish oil is not meeting current demand and raw material that could be used to supply these needs is being dumped at sea.

The International Fishmeal and Fish Oil Organization stated that the demand for fishmeal is greatly increasing. About 4.5 million metric tonnes (MT) of fishmeal and 1 million MT of fish oil are produced each year, but this is not sufficient to satisfy the demand. There is a ready market for the unwanted fish. It could be a reasonable thinking that discards should not be caught in the first place, but if once they are captured they should be used to produce fishmeal and fish oil.[2] However, wild marine stocks should be used only to produce food immediately available to consumers and that can be obtained by maintaining the fishing mortality quite below the point corresponding to maximum sustainable yield; in other words, there are consolidated evidences that a massive use of wild marine stocks such that necessary for the fishmeal/oil production cannot be sustained.

Furthermore, about five million tonnes of 'trash catch' (i.e. very low or no market value fish) are used directly (i.e. as raw ingredients not reduced into fishmeal) as feed in aquaculture (Edwards et al. 2004).

Another important item is that in contrast with the optimistic commercial projections, concerning the use of these fishery products within aquafeeds, made by fishmeal and fish oil manufacturing industry, some authors have estimated that the use of fishmeal and fish oil in compound aquafeeds will decrease in the long term. On the other hand, there are not reliable predictions concerning the availability and the economic stability of similar aquafeeds in the next years. For these reasons also, several concerns have been expressed at present when speaking of the use of trash fish as substitute aquafeed: different effects, including possible environmental damages and the reduction of available 'trash fish' for human consumption in disadvantaged areas, have been reported (Tacon et al. 2006). As a result, recent technological researches have been carried out with a well-defined goal: the possible use of alternative dietary feeds with the exclusion of trash fish and abovementioned solutions (Gatlin et al. 2007; Tacon et al. 2006).

The use of fish as feed for aquaculture has been the object of controversy (Wijkström 2012). Some authors say that feeding fish with fish is a practice that should be at least reduced if not stopped, because it reduces the amount of fish

[2]More information can be found at the following website: http://www.seafoodsource.com

available for consumers. It has been estimated that more than 1 kg of fish (in the form of feed) is needed to grow 1 kg of carnivorous fish or shrimp in captivity. Other authors stated that the use of by-catch as feed has only apparently led to a decrease in the availability of fish as food in some regions of Asia.

Moreover, the pressure for fish as feed could lead to overexploitation of forage species (i.e. Peruvian anchovy collapse). Consequently, a decrease of wild fish inputs in feed has been strongly suggested (Naylor et al. 1998, 2000).

2.6 Case Study: Bottom Trawling off South of Sicily (GSA 16–Central Mediterranean)

The potential effect of the enforcement of the landing obligation within the Mediterranean fisheries deals with one of the most important, diffuse, exploited (also in the eastern Atlantic Ocean) and overfished species, the European hake (*Merluccius merluccius*, L. 1758). It is a gadiform species of medium unit value but of high commercial value considering the overall amount landed (Altheit and Pitcher, 1995; Cohen et al. 1990) The European hake is one of the dominant species exploited by different gear types (trawlers, longliners, gill-netters), but the elective gear remains the bottom trawl.

In several Mediterranean regions, the percentage of specimens discarded from trawl fishery is roughly around 40% (Edelist et al. 2011; Martìnez-Abraìn et al. 2002; Sánchez et al. 2007; Tsagarakis et al. 2008). In most Italian geographical sub-areas (GSA), it is characterized by high rate of discard with percentage ranging between 20 and 50% (SIBM 2003).

In this case study, the possible consequences of landing obligation on *M. merluccius* in terms of the maximum possible expected potential of undersized landed have been analysed, using data coming from experimental trawl surveys (MEDITS project; Anonymous 2016).

The study area is the South of Sicily (Fig. 2.1), the GSA 16 as defined by the General Fisheries Commission for the Mediterranean Sea (GFCM 2001). The GSA 16—representing a portion of the Strait of Sicily sensu *latu* (Jereb et al. 2016b)—is mainly beaten by the Mazara del Vallo trawl fleet (about 180 trawl vessels). South of Sicily has been subjected to traditionally high fishing pressures, only recently mitigated by the displacement in other Mediterranean GSA of many large long-range trawlers (Garofalo et al. 2007).

Data on abundance, maturity condition and length structure of *M. merluccius* coming from 20 experimental trawl surveys carried out between 1994 and 2013 from 10 down to 800-m depth were analysed. It must be remarked that MEDITS gear mounts a very fine diamond mesh (20 mm) slightly smaller than the traditional commercial mesh (31 mm) used by Sicilian fishers till few years ago (Anonymous 2016). First of all, it was assessed the trend in both the occurrence and stock

abundance as a basic step in planning any future utilization of the undersized fish that for hake are represented by specimens below 20 cm of total length (TL).

The frequency of occurrence (Fo, computed as percentage of positive hauls), mean density index (DI: Nm^2) and biomass index (BI: kg/km^2) were estimated according a swept area based standard procedure (Anonymous 2016). The individual lengths were combined (all surveys) in classes of 1 cm; sexes were also combined since no significant difference in growth and mortality parameters are expected up 20 cm TL. Given that some authors have suggested to adopt the length at onset of sexual maturity (as the future minimum conservation size (MCS), a definition which should replace the traditional minimum landing size), the 'length at sexual maturity' (Lm50%) for females was derived according to the logistic approach and used in the analysis.

Both current and possible alternative MCS were used as cut-off thresholds to separate the undersized hake by computing the potential figures as both absolute number and ratio.

The results are summarized in Table 2.2. In addition, Table 2.3 shows density and biomass indices by undersized and oversized cut-off length of *Merluccius merluccius* estimated during the MEDITS surveys carried out (from 1994 to 2013) in the South of Sicily and corresponding under–oversized/total specimens ratio (%). As concerns the frequency of occurrence (Fo, Fig. 2.1), hake appeared in 75% of the valid hauls analysed throughout the depth range surveyed and the mean density index (DI; all years an all depths combined) showed a significant although only slight positive temporal trend (Spearman test, $Rs = 0.47$, $p < 0.05$; Fig. 2.2). The temporal evolution of the mean biomass index (BI, Fig. 2.3) showed a positive

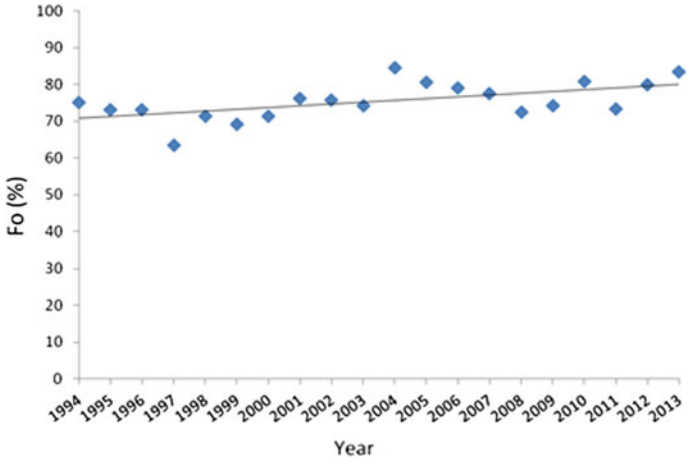

Fig. 2.1 Overall (all depths combined) temporal evolution of the frequency of occurrence (Fo %) of *Merluccius merluccius* from 1994 to 2013 in the GSA16. A simple interpolating line is overimposed to indicate the positive trend (note the flat behaviour of the Fo values from 2004 onwards)

Table 2.2 Parameters employed in the computation of potential Catch Returned to the Sea (CRS) 'recovered' and landed (LCRS) according to the landing obligation for *Merluccius merluccius*. Mean values estimated during the MEDITS surveys carried out from 1994 to 2013 in the South of Sicily

Parameter	Value
Frequency of occurrence (Fo, %)	75
Density index (DI, N/km^2)	872
Biomass index (BI, kg/km^2)	30.5
Length at sexual maturity (Lm50%)	26 cm
Weight–length relationship[a]	TW = 0.0000056 × (TL)$^{3.056}$
Length at capture (Lc) 38 mm square mesh (Sala et al. 2008)	14.2 cm
Current conservation reference size (EU)	20 cm
Possible alternative conservation reference size (EU)	26 cm

[a]TW is for: total weight (g), while TL means: total length (cm)

Table 2.3 Density and Biomass indices by undersized and oversized cut-off length of *Merluccius merluccius* estimated during the MEDITS surveys carried out (from 1994 to 2013) in the South of Sicily and corresponding under–oversized/total specimens ratio (%). The values were estimated in relation to current and alternative reference size

	Merluccius merluccius	
Current conservation reference size	20 cm	
	Estimate (abs)	Ratio (%)
DI undersized (N/km^2)	748	84
DI oversized (N/km^2)	124	16
BI undersized (kg/km^2)	7	36
BI oversize (kg/km^2)	24	64
Possible alternative conservation reference size	26 cm	
	Estimate (abs)	Ratio (%)
DI undersized (N/km^2)	822	94
DI oversized (N/km^2)	49	6
BI undersized (kg/km^2)	10	33
BI oversize (kg/km^2)	20	67

significant trend (Spearman test, R = 0.73, $p < 0.01$) too. However, although the BI Rs (0.73) is higher than that estimated for the DI (Rs = 0.47), in both cases, the percentage of the variance explained might be considered quite low, in other words hake might be considered in a stable or steady-state condition at least in the short term.

As regards the length structure, the hake specimens (sex combined) ranged between 4 and 83 cm (TL), whereas the length frequency distribution of hake from the South of Sicily (Fig. 2.4) shows the shape typical of both a current overfishing (unimodal at low size class with a skewed long tail, i.e. very high fishing mortality) and overexploited (very low large specimens scattered in many contiguous length

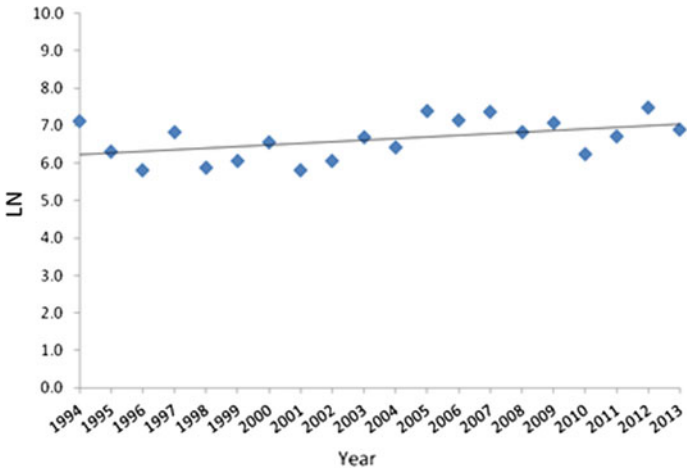

Fig. 2.2 Overall (all strata combined) temporal evolution of the mean density index DI ($N*km^{-2}$; after log transformation) of *Merluccius merluccius* from 1994 to 2013 in the GSA 16. Note the flat behaviour of the DI values from 2004 onwards

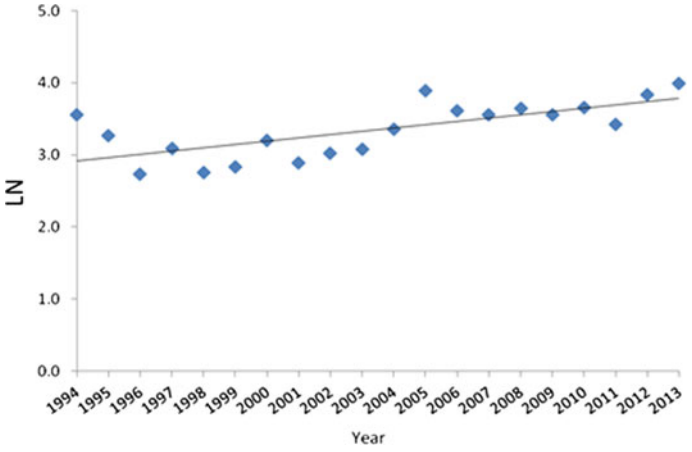

Fig. 2.3 Overall (all strata combined) temporal evolution of the mean biomass index (after log transformation) of *Merluccius merluccius* from 1994 to 2013 in the GSA 16

classes) condition. On the base of the current minimum size/conservation size (20 cm), it is impressive to note that the bulk of the standing stock is represented by undersized specimens, which once caught, should be treated according to the landing obligation.

Theoretically, fishermen were using the current mandatory EU mesh size (40 mm squared or 50 mm diamond) in their cod ends, the amount of potential

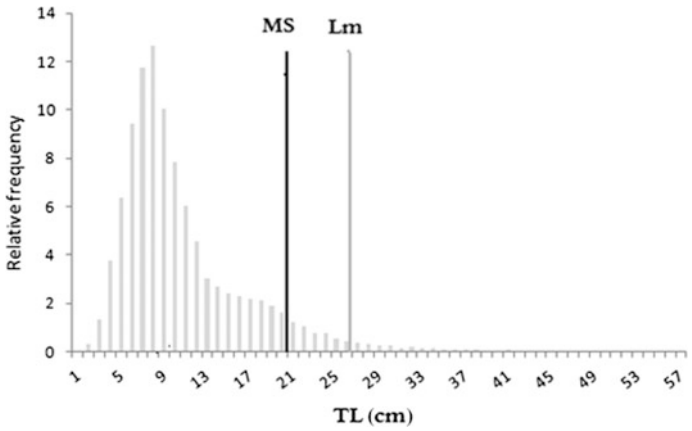

Fig. 2.4 Total length distribution (%) of *Merluccius merluccius* with over imposed the Minimum Size (MS, Council Regulation (EC) No 1967/2006) and the length at sexual maturity (Lm50%). Length class between 58 and 83 cm is not reported for clarity

undersized will be lower. Sala and coworkers have reported a size at capture at 50% of 14.1 cm total length (TL) with a 38 mm square mesh (Sala et al. 2008). Unfortunately, the hake commercial landed length structure (Gancitano et al. 2014) shows a large presence of undersized below the 14.1 cm estimated (Sala et al. 2008) indicating (a) infringements, or (b) a different selectivity pattern in commercial gears.

Considering that most of the short-range trawlers land and sell undersized specimens (Gristina et al. 2002), the landing obligation if enforced will determine a substantial immediate economic loss.

In case the current size will be increased up to the estimated size at maturity Lm50% of 26 cm, the fisheries should be stopped at least in the short term since a very scanty part of the oversized stock will be available (consequently no landed discard will be available for the Industry).

In the case of application of landing obligation, the expanded to the GSA 16 area (37,500 km^2) expected recovered 'landed catch returned to the sea' (LCRS, undersized landed hakes) would be about 28 million specimens corresponding to 262 t. The landed fraction (oversized hakes) would be about 5 million corresponding to 900 t. In the case of future adoption of length at sexual maturity as minimum conservation size, the maximum possible expected potential of undersized landed would become about 31 million corresponding to 375 t. The landed fraction (oversized hakes) would be about 2 million corresponding to 750 t.

2.7 Discussion of the Case Study

The first regional study addresses the magnitude of discards in the South of Sicily which involved the monitoring of trawl fleet. Total annual discards during the 1980s were estimated at around 70,000 tonnes, corresponding to 44–72% of catches (Charbonnier 1990). The discards situation in Sicily reflects the Italian picture: complex issues, high variability in relationship with seasons, the features of the fleet and the fishing tools and local habits (Castriota et al. 2001, 2004; SIBM 2003). A review of the various experiences on assessing discard within the GSA 16 and related problematic is available (Fiorentino et al. 2005).

In some North Atlantic countries, different measures have been introduced to reduce discards, including also the landing obligation. In these countries, however, discard management is less complex than in the Mediterranean context and significant differences between the Atlantic and Mediterranean regions exist (European Commission 2009).

Serious doubts on the viability, efficacy, and consequences of landing obligation have been evidenced for the Mediterranean Sea (Garcìa et al. 2015; Jereb et al. 2016a; Sardà et al. 2014). Returning discards to the sea could be better than subtracting energy from it by landing them (Sardà et al. 2014). Moreover, surveillance, monitoring and ecological costs of landing obligation could produce negative effects.

Fisheries management and discarding are linked and an overall discards ban will not solve the real issue behind discarding with the risk of introducing further economic inefficiency in an already almost collapsed sector. The landing obligation cannot be applied without creating confusion and increasing the inefficiency and the crisis of the sector.

More efforts should thus be directed towards reducing catches of unwanted species or undersized specimens (Garcìa et al. 2015; Jereb et al. 2016a; Sardà et al. 2014).

New actions aimed to rebuild fisheries and implement reforms to sustain them are still necessary: evaluation of discard magnitude, different regulations for Atlantic and Mediterranean contexts, improving the gear selectivity, spatio-temporal fishing restrictions for vulnerable sizes and/or areas, effort reduction and local action plans to integrate stakeholders and the fishing sector in the discards management, may be effective ways to address the discards problem. These suggestions could be proposed as a powerful alternative to improve the management of CRS and create more sustainable fisheries.

As concerns the use of ‘recovered’ as feed, there is uncertainty on the future availability and use of these products due to several factors. Some authors say that feeding fish with fish is a practice that should be at least reduced if not stopped, because it reduces the amount of fish available for consumers. The increasing demand for these products by the aquaculture sector may result in increased fishing pressure on available fish stocks. This situation is observed when speaking of Peruvian anchovy collapse (Naylor et al. 1998, 2000).

The demand for fishmeal and fish oil is strong and the prices of these products will increase in the long run (Tacon et al. 2006). Further, the use of trash fish as direct feeding in aquaculture is problematic for the potential disease risks and environmental/polluting effect.

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Chapter 3
Sustainable Alternatives for Dietary Fish Oil in Aquafeeds: Actual Situation and Future Perspectives

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Abstract Fishmeal and fish oil have been for a long time the dominant raw materials for aquaculture feeds but these are limited and finite resources. Plant oils extracted from oilseeds are the main candidates as sustainable substitutes for fish oil in fish feeds and many studies have demonstrated the feasibility of their use. Plant oils are rich in linoleic and α-linolenic acids but lack in eicosapentaenoic and docosahexaenoic acids that belong to omega-3 long-chain polyunsaturated fatty acids and whose food consumption has been shown to have beneficial effects on human health. In the light of these considerations, it is clear that the most important problem of the replacement of dietary fish oil with plant oils is a reduction of the nutritional value of the flesh of farmed fish. This review attempts to provide an overview of the effects of the major plant lipid sources alternative to fish oil tested in studies carried out on farmed fish species.

Keywords Aquaculture · Fish oil · Oilseed · Omega-3 fatty acid
Polyunsaturated fatty acid · Sustainability

3.1 Introduction

Nowadays, aquaculture or farmed seafood provided half of the seafood consumed globally (Tacon and Metian 2013). Aquaculture, according to the latest statistics, currently supplies around 50% of the world's fish and seafood for human consumption, and therefore plays an increasingly important role in the global food system within an impact on the environment (Fry et al. 2016) and human health (FAO 2014). Aquafeeds, used to farm fish carnivorous species, have been for a long time based on fishmeal (FM) and fish oil (FO) as protein and lipid sources, respectively. These ingredients, mainly derived from capture fisheries, are limited and finite resources (Naylor et al. 2009; Shepherd and Jackson 2013; Tacon and

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Table 3.1 Fatty acid compositions (% total fatty acids) of marine and plant origin oil sources. Adapted from Turchini et al. (2009). LA, linoleic acid, 18:2 n-6; ALA, alpha-linolenic acid, 18:3 n-3; AA, arachidonic acid, 20:4 n-6; EPA, eicosapentaenoic acid, 20:5 n-3; DHA, docosahexaenoic acid, 22:6 n-3LC, long chain; HUFA, highly unsaturated fatty acid; PUFA, polyunsaturated fatty acid

	Saturated	Monounsaturated	HUFA			LC-HUFA		n-6 PUFA	n-3 PUFA
Fatty acid			LA	ALA	AA	EPA	DHA		
Marine origin									
Algal oil[a]	20.2	28.0	1.9	0.1	1.1	16.5	27.1	4.2	47.7
Anchovy	28.8	24.9	1.2	0.8	0.1	17.0	8.8	1.3	26.6
Capelin	20.0	61.7	1.7	0.4	0.1	4.6	3.0	1.8	8.0
Cod liver	19.4	46.0	1.4	0.6	1.6	11.2	12.6	3.0	24.4
Herring	20.0	56.4	1.1	0.6	0.3	8.4	4.9	1.4	13.9
Menhaden	30.5	24.8	1.3	0.3	0.2	11.0	9.1	1.5	20.4
Plant origin									
Camelina[a]	9.9	36.4	19.3	31.9	0.1	–	–	20.9	32.9
Corn	12.7	24.2	58.0	0.7	–	–	–	58.0	0.7
Linseed	9.4	20.2	12.7	53.3	–	–	–	12.7	53.3
Palm	48.8	37.0	9.1	0.2	–	–	–	9.1	0.2
Rapeseed	4.6	62.3	20.2	12.0	–	–	–	20.2	12.0
Soybean	14.2	23.2	51.0	6.8	–	–	–	51.0	6.8
Sunflower	10.4	19.5	65.7	–	–	–	–	65.7	–

[a] Data from Sprague et al. (2015)

Metian 2009); therefore, aquaculture industry has made great efforts towards developing diets for finfish using more sustainable feeds derived from ingredients of the terrestrial agriculture (Bendiksen et al. 2011; Tocher 2009; Turchini et al. 2011). Among terrestrial ingredients, different plant oils (PO) extracted from oilseeds, suitable as lipid sources for FO replacement, showed to be cheaper than FO (Turchini et al. 2009) and, for this reason, were increasingly used in salmon feeds where they did not evidence any detriment to fish health or growth (Bell et al. 2004).

On the other hand, even if PO are suitable and less expensive energy sources for carnivorous fish without negative effects on growth performance (Glencross 2009; Tocher et al. 2010), their quality in terms of fatty acid (FA) profiles differs from those of FO as shown in Table 3.1. In fact, comparing FA compositions of marine and plant origin oil sources is evident that PO resulting richer in n-6 polyunsaturated fatty acids (PUFA) and devoid of n-3 long chain—polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Fillet FA profile of farmed fish reflects diet composition; therefore, the flesh of fish fed with aquafeeds containing PO instead of FO resulting in a modified FA

composition has already been demonstrated in several cultured fish species (Tocher et al. 2010; Torstensen and Tocher 2010).

The consequence of these FA changes, due to altered feed composition, could affect the nutritional content of cultured fish products reducing their well-known nutritional benefits for human nutrition, as observed in a study carried out on salmon products available from United Kingdom retailers (Henriques et al. 2014). This trend was also confirmed by another study carried out comparing the FA composition of fillets of Scottish Atlantic salmon farmed between 2006 and 2015 (Sprague et al. 2016). These authors, in the considered period, recorded a significant increase of terrestrial FA in contrast with a decrease in EPA and DHA flesh levels. Authors, therefore, concluded that, in order to satisfy the EPA + DHA intake levels recommended by the different health organizations (EFSA 2010; ISSFAL 2004), a double portion size of Scottish Atlantic salmon fillet should be consumed respect to those farmed in 2006.

The different PO sources investigated in field trials on cultured salmonids and Mediterranean marine species as well their effects on fish flesh nutritional quality are reported and commented in the following paragraphs.

3.2 Effects of Dietary Plant Oils on the Nutritional Quality of Farmed Salmonids

Atlantic salmon and rainbow trout are the most important cultured salmonids in terms of world production and economical value (Torrissen et al. 2011; Ytrestøyl et al. 2015). Due to this business interest, the effects of a complete or partial replacement of FO with different PO or PO blend for all or part of the production cycle of these fish have been investigated in several studies (Regost et al. 2004; Torstensen et al. 2004, 2005). Different PO sources such as crude palm oil (CPO), soybean oil (SBO), rapeseed oil (RO) and linseed oil (LO) were tested, and even if no significant negative effects were reported on fish growth, the FA composition of the edible portion was affected by the PO dietary treatment (Bell et al. 2002, 2003, 2004; Caballero et al. 2002; Drobná et al. 2006; Rollin et al. 2003; Rosenlund et al. 2001).

On the basis of data displayed in Table 3.1 (FA profiles of PO), it is clear that the FA make-up of alternative lipid sources is highly variable compared to the FO. In fact, someone PO like CPO are extremely rich (>50%) in saturated FA (SFA); other preferences concern RO in monounsaturated FA (MUFA), or SBO rich in n-6 polyunsaturated FA (PUFA) or in C18 n-3 PUFA like LO. In order to obtain, in alternative feeds, a more similar FA class make-up for FO, aquaculture feed industry have studied a formulated PO blend composed of one-third SFA, one-third MUFA and one-third n-3 PUFA.

The main FA composition of the diets and fish tissues, arising from different nutritional trials, of Atlantic salmon and rainbow trout fed with a control diet FO or

a plant oil PO-based diet are reported in Tables 3.2 and 3.3, respectively. Fish were fed with a single PO or a blend of them containing a mixture of canola/rapeseed, palm and linseed oil. Taking a look at Tables 3.2 and 3.3, it is evident that the differences of FA classes or individual FA are most marked than the difference between the fillets of fish fed with the respective diets. Observing data from Table 3.2, in a study on Atlantic salmon post-smolt fed with an FO-based diet or a PO diet based on 100%-linseed oil, some differences (+43, +260, −83 and −70%) between dietary percentages of oleic acid (OA, 18:1n-9), linoleic acid (LA, 18:2 n-6), EPA and DHA were observed, while for the same FA, the differences between the fillets of fish fed with the two diets were +16, +236, −70 and −62%, respectively (Bell et al. 2004). Similarly, Caballero and co-workers in a study on rainbow trout fed with an FO-based diet or a mixture of 40% rapeseed oil and 40% palm oil found that diet percentage differences of OA, LA, ALA and DHA were +227, +191, +255 and −55% (Caballero et al. 2002), while the differences between the fillets of fish fed with the two diets were +102, +141, +156 and −30%, respectively (Table 3.3). It is clear that fish, even if there is a dietary-induced modification of FA composition, are able to contrast the extent of FA modification towards the maintenance of a general fish FA make-up.

3.3 Effects of Dietary Plant Oils on the Nutritional Quality of Farmed Mediterranean Marine Fish

Among Mediterranean marine fish, European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) are the two main species reared in Europe (Ballester-Lozano et al. 2011; Benedito-Palos et al. 2011).

Similarly to as described previously in the 3.2 paragraph, also in European sea bass and gilthead sea bream different studies utilizing different PO sources as FO replacers were carried out. The most investigated PO sources, such as SBO, RO, LO or a blended PO (Izquierdo et al. 2005; Richard et al. 2006), were similar to that tested in the salmonids, even if in some long-term feeding experiment, both species showed a significant reduction in fish growth and a lower feed conversion ratio values when a partial or total replacement of FO was attempted (Montero et al. 2005, 2008). This effect is due because marine fish has an absolute requirement for LC-PUFA that cannot be satisfied by PO respect to freshwater fish where PO may provide all the EFA required by these fish (NRC 2011).

Examining Tables 3.4 and 3.5, where main FA composition of the diets and fish tissues of gilthead sea bream and European sea bass fed with an FO diet or a PO-based diet are reported, similar considerations made for salmonids could be done. In fact, in both tables, it is evident that FA dietary differences between FO and PO groups are higher than FA fillet differences. Fountoulaki and colleagues, in a long-term feeding (6 months) study on the effects of commercial diets with 69%-FO substitution level with CPO, found that diet percentage differences of OA, AA,

Table 3.2 Main fatty acid composition of the diets and fish tissues of Atlantic salmon (*Salmo salar*) fed with a control diet (FO) or a plant oil (PO) based diet. The percentage of substituted fish oil is in parentheses. The percentage difference of the fatty acid percentage between diets and fish tissues is also reported. OA, oleic acid 18:1 n-9; LA, linoleic acid, 18:2 n-6; ALA, alpha-linolenic acid, 18:3 n-3; AA, arachidonic acid, 20:4 n-6; EPA, eicosapentaenoic acid, 20:5 n-3; DHA, docosahexaenoic acid, 22:6 n-3; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; n-6 PUFA, polyunsaturated fatty acids with the first double bond at the sixth carbon atom; n-3 PUFA, polyunsaturated fatty acids with the first double bond at the third carbon atom

	OA	LA	ALA	AA	EPA	DHA	SFA	MUFA	n-6 PUFA	n-3 PUFA	Reference
Diet FO	11.9	3.9	0.6	0.2	5.8	5.9	20.3	59.4	4.4	14.9	Torstensen et al. (2005)
Diet PO[a] blend (100%)	40.4	13.5	8.0	0.2	2.1	3.4	21.8	50.2	13.7	14.3	
Fillet FO	14.0	3.0	0.6	0.5	3.5	13.0	21.0	51.0	4.0	21.0	
Fillet PO blend (100%)	33.5	10.4	5.4	0.8	2.0	11.2	21.0	45.0	13.0	21.0	
Diet difference %[b]	239	246	1233	0	−64	−42	7	−15	211	−4	
Fish difference %[c]	139	247	800	60	−43	−14	−5	−12	225	0	
Diet FO	11.9	4.2	0.9	0.2	5.9	5.0	19.9	58.4	5.0	15.7	Bell et al. (2004)
Diet LO[d] (100%)	17.0	15.1	50.4	0.1	1.0	1.5	10.5	21.1	15.2	53.3	
Fillet FO	16.0	3.9	0.8	0.3	4.3	8.1	19.9	57.1	4.9	17.5	
Fillet LO (100%)	18.6	13.1	38.7	0.1	1.3	3.1	12.7	24.2	14	49.1	
Diet difference %[b]	43	260	5500	−50	−83	−70	−47	−64	204	239	
Fish difference %[c]	16	236	4738	−67	−70	−62	−36	−58	186	181	

[a] PO: mixture of 55% rapeseed oil, 30% palm oil and 15% linseed oil
[b] Diet difference % = (%FA diet PO—% FA diet FO)/(%FA diet FO) × 100
[c] Fish difference % = (%FA fish PO—% FA fish FO)/(%FA fish FO) × 100
[d] LO: linseed oil

Table 3.3 Main fatty acid composition of the diets and fish tissues of rainbow trout (*Oncorhynchus mykiss*) fed with a control diet (FO) or a plant oil (PO) based diet. The percentage of fish oil substituted is in parentheses. The percentage difference of the fatty acid percentage between diets and fish tissues is also reported. OA, oleic acid 18:1 n-9; LA, linoleic acid, 18:2 n-6; ALA, alpha-linolenic acid, 18:3 n-3; AA, arachidonic acid, 20:4 n-6; EPA, eicosapentaenoic acid, 20:5 n-3; DHA, docosahexaenoic acid, 22:6 n-3; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; n-6 PUFA, polyunsaturated fatty acids with the first double bond at the sixth carbon atom; n-3 PUFA, polyunsaturated fatty acids with the first double bond at the third carbon atom

	OA	LA	ALA	AA	EPA	DHA	SFA	MUFA	n-6 PUFA	n-3 PUFA	Reference
Diet FO	10.9	4.6	1.1	0.2	7.6	6.4	21.6	47.4	6.0	19.4	Caballero et al. (2002)
Diet PO[a] blend (80%)	35.6	13.4	3.9	0.1	2.9	2.9	24.4	45.9	13.8	11.1	
Fillet FO	16.1	3.9	0.9	0.3	4.6	9.4	22.7	47.1	5.1	18.6	
Fillet PO blend (80%)	32.6	9.4	2.3	0.3	2.2	6.6	23.3	46.1	11.0	13.3	
Diet difference %[b]	227	191	255	−50	−62	−55	13	−3	130	−43	
Fish difference %[c]	103	141	156	0	−52	−30	3	−2	116	−28	
Diet FO	12.0	4.0	1.7	–	10.0	7.2	45.1	25.1	6.9	23.0	Drew et al. (2007)
Diet PO[d] blend (100%)	39.1	17.0	20.9	–	1.8	1.7	14.6	43.4	17.7	25.2	
Fillet FO	16.3	4.7	1.6	–	8.2	13.0	34.8	29.5	7.9	27.7	
Fillet PO blend (100%)	39.0	14.8	13.4	–	1.6	5.2	17.1	42.8	16.3	23.7	
Diet difference %[b]	226	325	1129	–	−82	−76	−67	73	157	10	
Fish difference %[c]	139	215	738	–	−81	−60	−51	45	106	−14	

[a] PO: mixture of 40%- rapeseed oil and 40% palm oil
[b] Diet difference % = (%FA diet PO—% FA diet FO)/(%FA diet FO) × 100
[c] Fish difference % = (%FA fish PO—% FA fish FO)/(%FA fish FO) × 100
[d] PO: mixture of 65%- canola oil and 35% linseed oil

EPA and DHA were +182%, −75%, −74% and −69% (Fountoulaki et al. 2009), while the differences between the gilthead sea bream fillets of fish fed with the two diets were +82%, −57%, −61% and −41%, respectively (Table 3.4). In another study carried out on European sea bass fed for 8 months with a diet where FO was substituted by 60%RO, diet percentage differences of OA, LA, EPA and DHA were +243%, 251%, −55% and −48% (Montero et al. 2005), while the differences between the sea bass fillets of fish fed with control and PO diets were +69%, +98%, −48% and −38%, respectively (Table 3.5).

3.4 Future Perspectives

As described in the previous paragraphs, the feasibility of PO use as dietary fish oil replacer in aquafeeds has been widely demonstrated although they are devoid of omega-3 LC-PUFA responsible of well-established health human benefits (Calder 2013). In the future, PO enriched in omega-3 LC-PUFA could be obtained by the application of modern biotechnology techniques consisting in the genetic modification of oilseed crops through the introduction in their genome of microalgal genes codifying for the LC-PUFA biosynthetic pathways, as showed by several recent works (Napier et al. 2015; Nichols et al. 2010; Petrie and Singh 2011; Sayanova and Napier 2011; Venegas-Calerón et al. 2010) even if at the moment production costs are relatively high (Olsen 2011).

Among oilseeds, the false flax (*Camelina sativa*), naturally rich in ALA (∼45%) the fatty acid substrate for the biosynthesis of n-3 LC-PUFA, is a good plant model for such metabolic engineering considering its low input cost and ease of transformation (Ruiz-Lopez et al. 2014).

The use of a metabolically engineered EPA-rich camelina oil (ECO) was tested in a field trial carried out on juvenile Atlantic salmon fed 7 weeks with experimental diets containing FO, wild-type camelina oil (WCO) or ECO as the sole lipid sources (Betancor et al. 2015a). Results showed that the inclusion of ECO has no detrimental effects on fish growth or performance parameters studied while enhanced apparent digestibility of individual n-6 and n-3 PUFA compared to dietary WCO. In a second study (Betancor et al. 2015b), analysing the nutritional quality, in terms of EPA to DHA for the human consumer, of the flesh of salmon fed ECO diets observed that replacing FO with ECO did not affect its quality. In the light of these results, authors concluded that n-3-LC-PUFA enriched oils from transgenic camelina are capable of maintaining n-3 LC-PUFA levels in farmed fish and therefore can be an effective substitute for FO in feeds for Atlantic salmon.

Table 3.4 Main fatty acid composition of the diets and fish tissues of gilthead sea bream (*Sparus aurata*) fed with a control diet (FO) or a plant oil (PO) based diet. The percentage of fish oil substituted is in parentheses. The percentage difference of the fatty acid percentage between diets and fish tissues is also reported. OA, oleic acid 18:1 n-9; LA, linoleic acid, 18:2 n-6; ALA, alpha-linolenic acid, 18:3 n-3; AA, arachidonic acid, 20:4 n-6; EPA, eicosapentaenoic acid, 20:5 n-3; DHA, docosahexaenoic acid, 22:6 n-3; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; n-6 PUFA, polyunsaturated fatty acids with the first double bond at the sixth carbon atom; n-3 PUFA, polyunsaturated fatty acids with the first double bond at the third carbon atom

	OA	LA	ALA	AA	EPA	DHA	SFA	MUFA	n-6 PUFA	n-3 PUFA	Reference
Diet FO	11.1	11.1	1.0	0.8	16.7	9.4	30.6	22	12.9	33.7	Fountoulaki et al. (2009)
Diet CPO (69%)[a]	31.3	16.5	0.8	0.2	4.4	2.9	37.9	35.6	16.7	9.7	
Fillet FO	16.2	9.7	–	0.9	9.9	11.8	26.9	30.3	11.3	30.8	
Fillet CPO(69%)	29.5	16	–	0.4	3.9	7	27.7	39.6	16.5	15.7	
Diet difference %[b]	182	49	−20	−75	−74	−69	24	62	29.5	−71.2	
Fish difference %[c]	82	65	–	−57	−61	−41	3	31	46.0	−49.0	
Diet FO	12.5	12.1	1.58	0.3	6.9	8.3	25.3	37.5	–	–	Benedito Palazos et al. (2008)
Diet PO (100%)[d]	25.9	21.3	23.2	0	0.9	1.1	21.5	29.8	–	–	
Fillet FO	16.8	11.8	1.1	0.4	5.0	10.6	26.2	35.8	–	–	
Fillet PO (100%)	27.3	20.5	15.8	0.2	1.6	3.52	21.9	32.4	–	–	
Diet difference %[b]	107	76	1368	−100	−86	−87	−15	−20	–	–	
Fish difference %[c]	63	74	1377	−55	−69	−67	−16	−9	–	–	

[a] CPO; 69% crude palm oil

[b] Diet difference % = (%FA diet PO—% FA diet FO)/(%FA diet FO) × 100

[c] Fish difference % = (%FA fish PO—% FA fish FO)/(%FA fish FO) × 100

[d] PO; mixture of 17% rapeseed oil, 58% linseed oil and 25% palm oil

Table 3.5 Main fatty acid composition of the diets and fish tissues of European sea bass (*Dicentrarchus labrax*) fed with a control diet (FO) or a plant oil (PO) based diet. The percentage of fish oil substituted is in parentheses. The percentage difference of the fatty acid percentage between diets and fish tissues is also reported. OA, oleic acid 18:1 n-9; LA, linoleic acid, 18:2 n-6; ALA, a-linolenic acid, 18:3 n-3; AA, arachidonic acid, 20:4 n-6; EPA, eicosapentaenoic acid, 20:5 n-3; DHA, docosahexaenoic acid, 22:6 n-3; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; n-6 PUFA, polyunsaturated fatty acids with the first double bond at the sixth carbon atom; n-3 PUFA, polyunsaturated fatty acids with the first double bond at the third carbon atom

	OA	LA	ALA	AA	EPA	DHA	SFA	MUFA	n-6 PUFA	n-3 PUFA	Reference
Diet FO	11.3	3.9	0.88	0.88	13.5	11.7	31.7	28.1	6.3	31.9	Montero et al. (2005)
Diet RO (60%)[a]	38.7	13.7	5	0.4	6.1	6.1	17	47.2	14.8	20	
Fillet FO	18.5	4.6	1.2	0.8	9.2	14.1	31.3	31.9	6.8	28.4	
Fillet RO(60%)	31.2	9.1	2.9	0.5	4.8	8.8	26.6	43.3	10.6	18.6	
Diet difference %[b]	243	251	468	−55	−55	−48	−46	68	135	−37	
Fish difference %[c]	69	98	142	−38	−48	−38	−15	36	56	−35	
Diet FO	13.4	5.7	0.8	0.8	10.9	9.2	27.3	33.7	8.4	26.7	Mourente et al. (2005)
Diet LO(60%)[d]	15.6	10.3	25.9	0.4	5.2	4.4	17.6	29.4	11.4	38.5	
Fillet FO	15.5	3	1	1.5	9.6	20.2	25.3	26.5	6	35	
Fillet LO (60%)	18.8	5.7	8.4	0.9	5.7	14.4	21.5	31.9	7.8	31.9	
Diet difference %[b]	16	81	3138	−50	−52	−52	−36	−13	36	44	
Fish difference %[c]	21	90	740	−40	−41	−29	−15	20	30	−9	

[a] RO: rapeseed oil
[b] Diet difference % = (%FA diet PO—% FA diet FO)/(%FA diet FO) × 100
[c] Fish difference % = (%FA fish PO—% FA fish FO)/(%FA fish FO) × 100
[d] LO: linseed oil

3.5 Closing Remarks

In light of the considerations made in the previous paragraphs, the most significant modifications to the FA composition of fish tissues, when FO is replaced, could be summarized as a relative increase in the content of C18 PUFA series, and in particularly LA (18:2 n-6), corresponding to a decrease in the content of n-3 HUFA. Considering these reasons, therefore, the content of LA was the most important parameter that should be considered, when an FO alternative lipid source is searched, because this FA determined the most negative modifications to the FA composition of farmed fish fillets from the nutritional viewpoint. In conclusion, the best potential substitutes for FO should be identified in PO rich in SFA, or to a lesser extent oils rich in MUFA such as CPO, while the use of PO with high LA content like SBO and RO should be avoided.

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Chapter 4
Supplementation of Vitamins, Minerals, Enzymes and Antioxidants in Fish Feeds

Laura Gasco, Francesco Gai, Giulia Maricchiolo, Lucrezia Genovese, Sergio Ragonese, Teresa Bottari and Gabriella Caruso

Abstract The fast increase in marine aquaculture productions has generated some problems related to optimization of fish feed. Moreover, the search for alternative raw materials to supplement or replace fishmeal and fish oil has made the introduction of additives into the diet necessary. Diet additives have recently attracted attention and investments of the aquaculture industry and, consequently, researches aimed at evaluating the costs/benefits ratio and the effects on organisms and environments are increasing. In general, additives are nutritive or nonnutritive ingredients that are added to the diet alone or in combination with each other and that are present in small quantities. Their functions are different: preserve the physical and chemical features of the diet, enhance animal health and welfare, improve the quality of final product and maintain the quality of aquatic environment. This chapter provides an overview of the use of vitamins, minerals and antioxidants in fish feeds. In addition to these natural substances, also enzymes are proposed as new alternative aquafeed additives. The effects on fish growth, welfare, reproduction and health status of the above-reported additives are discussed.

Keywords Antioxidants · Aquaculture · Enzymes · Fish feed Metabolism · Minerals · Vitamins

4.1 Introduction

Fish nutrition—considering with this word the biochemical processes related to the use of food substances by the organisms—is recognized to be one of the most important topics concerning the management of fish farming (Guillaume et al. 1999; Tibaldi et al. 2001). It is well known that proper nutrition is essential for maintenance of normal growth and health of the reared species and, in recent years, increasing attention has been given to the development of nutritional strategies that

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positively influence immunity and disease resistance of cultured organisms to reduce disease-related economic losses (Hixson 2014; Kiron 2012; Oliva-Teles 2012; Pohlenz and Gatlin 2014).

Nutrition practices play a key role both on a technical and economical point of view, since in the overall management of a fish farm, the costs related to nutrition account for 25–50% of total operating costs. Therefore, the choice of the type of feed, the mode of feeding and a right management protocol are the main factors which determine the growth performance of the reared species and the final profitability of the farming system.

The development of feeding regimes for fish under intensive or semi-intensive rearing requires a basic understanding of the nutrition and dietary nutrient requirements of the reared organisms. Food administration must satisfy the nutritional needs of the farmed species, allowing to obtain high-quality final products and environmental sustainability.

The main purpose of aquaculture is to obtain an optimal growth of farmed fish in reduced times, through the formulation of specific diets. Fish use foods as sources of both nutrients and energy. The main dietary requirements of all aquaculture species concern five different nutrient groups: proteins, lipids, carbohydrates, vitamins and minerals (FAO 1987a, b). For most of the species commercially reared, the nutritional needs in terms of proteins, amino acids, vitamins, essential lipids and minerals have been largely studied and established.

Studies on the nutrition of fish have contributed to elucidate several aspects of nutritional requirements and digestive physiology of many commonly farmed fish and of new species that may have a potential interest for aquaculture (Halver 1989, 2002; NRC 1983, 1993).

The nutrients and essential minerals required by fish are the same as those by crustaceans and high vertebrates. However, being poikilotherms, fish do not have to spend energy obtained from diet to maintain their body temperature (De Silva and Anderson 1995) and therefore their nutritional needs are different from homeotherms.

Although knowledge on the fish needs has not yet reached a level comparable to that achieved for homeotherms, current knowledge on fish nutrition and digestive physiology has allowed the achievement of very satisfactory technical and economical results, ensuring excellent performance, high resistance of the organisms against stress and diseases, reducing environmental impact and resulting in high-quality products.

The growth of the aquaculture industry has been accompanied and supported by technological innovation in feeding industry. Indeed, the continuous technological progress made in the field of feeding industry has resulted in progressive improvements in the formulation of animal feeds from a nutritional point of view.

The dietary composition must have a right balance of nutrients, an adequate energetic content, and each nutrient must be bioavailable. The dietary nutrients should be balanced in order to satisfy the energetic needs and to allow optimal fish growth. Unless of the fish species, feeds for fish must be balanced in terms of proteins (essential amino acids), lipids (essential fatty acids), energy, vitamins and

minerals. Nutritional balances have a profound effect on the growth, disease resistance and survival of fish, particularly during the larval and juvenile stages.

The choice of the optimal feeding strategy must comply with the need of obtaining the maximum increase of the biomass together with the minimum consumption of food, which is defined as the highest conversion efficiency of feed into the final fish biomass (Food conversion ratio).

In fish, several evidences of the role of diets and nutritional factors in affecting incidence and severity of a variety of infectious diseases have been reported (Kiron 2012; Landolt 1989; Oliva-Teles 2012; Waagbø 1994). Some studies have focused on the relationship between the immune system and dietary supplements above the minimum levels established for such micronutrients (Montero et al. 1999). Blazer reviewed the close relationship between nutrition and the disease resistance, showing that vitamins A, C, B_6 and E, iron and fluoride are actively involved in fish health (Blazer 1992). Vitamins and minerals enhance the immune system by providing substrates and cofactors necessary for the immune system to work properly. As the deficiency of some micronutrients such as vitamins and minerals is known to cause pathological signs and immunodepression, these compounds are usually included in farmed fish food to promote optimal growth and health, although the effects of these micronutrients on the fish immune system are not always predictable.

4.2 Vitamins

Vitamins are organic compounds essential for animal life. They are required in trace amounts for normal growth, reproduction and health. Since most of vitamins are not synthesized by animals, they are taken from diet to meet their needs. Depending on their solubility, vitamins are classified into water-soluble vitamins and fat-soluble vitamins (Table 4.1).

Table 4.1 General classification of vitamins. These molecules are metabolically essential for most fish species, except for vitamin B_{12} and myo-inositol that are not essential for tilapia and hybrid striped bass

Water-soluble vitamins	Fat-soluble vitamins
Thiamine (vitamin B_1)	Retinol (vitamin A)
Riboflavin (vitamin B_2)	Cholecalciferol (vitamin D_3)
Pyridoxine (vitamin B_6)	Tocopherol (vitamin E)
Cyanocobalamin (vitamin B_{12})	Phylloquinone (vitamin K)
Folic acid	
Nicotinic acid (niacin)	
Biotin	
Pantothenic acid	
Choline	
Inositol	
Ascorbic acid (vitamin C)	

All of the above-reported vitamins are metabolically essential for most fish species, except for vitamin B_{12} and myo-inositol that are not essential for tilapia and hybrid striped bass. The absence from the diet of some of them can result in typical morphological and physiological deficiency signs.

The vitamins belonging to the vitamin B complex (thiamine, riboflavin, niacin, biotin, folic acid, pantothenic acid and vitamins B_6 and B_{12}) are water-soluble vitamins that are required in relatively small amounts and play a role as coenzymes. Conversely, other water-soluble vitamins such as choline, myo-inositol and vitamin C are essential nutritional factors required in larger quantities and cover functions different from that of coenzymes.

Vitamins A, D, E and K are fat-soluble vitamins that function independently on enzymes; vitamin K may sometimes play the role of coenzyme (NRC 2011). Fat-soluble vitamins are absorbed from the gastrointestinal tract in the presence of fats and can be stored within the fat reserves of the body when their dietary intake exceeds their metabolic demands; in this case, a toxic condition called hypervitaminosis may be produced. Conversely, water-soluble vitamins undergo quick elimination and this prevents their storage with consequent vitamins toxicity.

Studies in fish nutrition are currently trying to determine the optimal dose at which vitamins must be introduced with diet to satisfy their needs and increase fish growth performance. Therefore, increasing interest is addressed to the relationships between dietary formulation and health status and particularly to the effects of nutrition on the immune response of fish (Landolt 1989; Shiau and Lin 2015; Waagbø 1994). Vitamin requirements of fish depend on their size, age, growth rate, feed composition and ability of intestinal microflora to synthesize them; also environmental factors may affect their needs, and therefore there are wide ranges in the required values of vitamins also in the same fish species. Several parameters are generally taken into account to quantify vitamin requirements of fish, such as growth performance, survival, activity of enzymes, lipid accumulation, oxidation and tissue vitamin storage.

In several fish species, vitamin A, D, thiamine, vitamin B_6, folic acid, pantothenic acid and myo-inositol have been shown to enhance immune responses and/or disease resistance. Vitamins have been found to increase resistance to infection by increasing migration and proliferation of phagocytic cell. Vitamins C and E, which are vitamins with antioxidant properties, have been shown to possess also immunomodulatory properties when used at high doses.

4.2.1 Water-Soluble Vitamins

The water-soluble vitamins include the vitamin B complex, vitamin C and the essential nutritional factors myo-choline and inositol. Fish require small amounts of the vitamin B complex, which play major roles in growth, physiology and cellular metabolism. They include thiamine—a coenzyme in the carbohydrate metabolism—riboflavin and pantothenic acid (involved in the energetic metabolism),

pyridoxine—which plays a key role in the protein metabolism—and vitamin C which has an antioxidant and anti-stress action. Inositol plays a role in the cell growth, while choline is an essential component of phospholipids. Since essential water-soluble vitamins are not stored in body tissues, they must be supplied to prevent deficiency.

4.2.1.1 Vitamin B Complex

Vitamin B_1 (Thiamine)

Thiamine (Fig. 4.1) is commonly found in the form of its di-phosphate ester thiamine pyrophosphate, which is an essential cofactor for important enzymatic steps in energy production. Thiamine pyrophosphate is a coenzyme in carbohydrate metabolism, involved in the oxidative decarboxylation of pyruvate and α-ketoglutarate to acetyl-coenzyme A and succinyl coenzyme A, respectively; it is also an activator of the enzyme transketolase, which is involved in the oxidation of glucose via the pentose phosphate pathway.

Thiamine hydrochloride is a crystalline compound stable to heat, which is broken down in neutral or alkaline solutions, and is split by sulphites into pyrimidine and thiazole; the pyrimidine ring is relatively stable, while thiazole one is easily opened by hydrolysis. Several derivatives of thiamine are more soluble in weak alkaline solutions than thiamine itself, and still show biological activity in animals. Both thiamine hydrochloride and thiamine mononitrate have been successfully used in fish nutrition.

Thiamine is contained in several dietary sources, such as dried brewer's yeast (100–50 mg/kg); and rice bran, wheat bran, oats, barley, cottonseed meal, soybean meal, linseed meal, dried distillers solubles and the outer coat or germ of cereals.

Riboflavin (Vitamin B_2)

Riboflavin (Fig. 4.2) is essential for the metabolism of carbohydrates, fats and proteins. It plays a role as a coenzyme for many metabolic oxidation–reduction reactions, being a component of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). FMN and FAD favour the enzymatic break-down of energy-yielding nutrients such as fatty acids, amino acids and pyruvic acid,

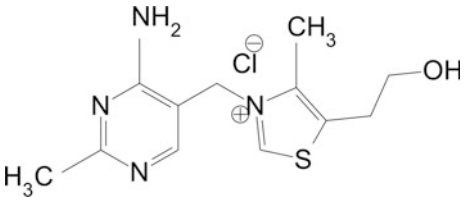

Fig. 4.1 The biochemical structure of thiamine (vitamin B_1). BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

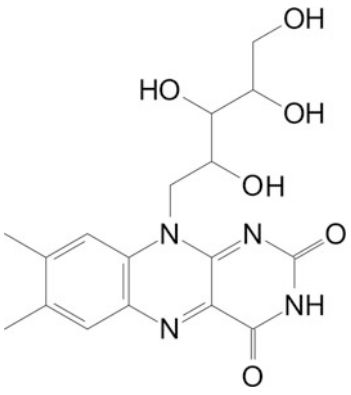

Fig. 4.2 The biochemical structure of riboflavin (vitamin B_2). BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

and therefore riboflavin has an important role in energy metabolism (NRC 2011). Moreover, it is particularly important for the conversion of tryptophan to nicotinic acid, together with pyridoxine (vitamin B_6). Riboflavin is a yellow-brown crystalline pigment soluble in water and alkali, insoluble in most organic solvents with the exception of alcohol. It is stable to heat in dry form and to oxidizing agents in neutral aqueous solutions. It is irreversibly decomposed by visible light or irradiation with ultraviolet rays.

Riboflavin is contained in dried torula yeast, dried brewer's yeast, liver and lung meal (50–30 mg/kg); chicken egg white, dried skim milk, dried distillers solubles, dried fish solubles and alfalfa meal (30–10 mg/kg); poultry by-product meal, fishmeal, meat meal, meat and bone meal, rapeseed meal (10–5 mg/kg), green vegetables and germinated cereal grains.

Vitamin B_6

Vitamin B_6 (pyridoxine) can be present under three forms: pyridoxine, pyridoxal and pyridoxamine, which have similar activity and are readily converted in animal tissues into the coenzyme forms, pyridoxal phosphate (vitamin B_6 phosphate ester) and pyridoxamine phosphate.

Pyridoxal phosphate acts as a coenzyme in a number of enzyme systems; it plays a key role in the metabolism of proteins, as it is a coenzyme for the non-oxidative degradation of amino acid, as well as in the metabolism of carbohydrates, favouring the release of glycogen from muscle and liver (NRC 2011). It is also required for the breakdown of tryptophan to nicotinic acid, the synthesis of haemoglobin, acetyl coenzyme A and *m*-RNA.

Pyridoxine hydrochloride is heat stable in either acid or alkaline solution and sensitive to ultraviolet light in neutral or alkaline solutions. Pyridoxamine and pyridoxal are rapidly destroyed by exposure to air, heat or light; therefore, pyridoxine hydrochloride is used for dietary supplementation.

Rich dietary sources of pyridoxine are dried brewer's yeast and dried torula yeast (50–30 mg/kg); dried fish solubles (30–20 mg/kg); sunflower seed meal (20–10 mg/kg); and dried distillers solubles, rapeseed meal, fishmeal, corn, alfalfa meal, cottonseed meal, rice and soybean meal (10–5 mg/kg).

Vitamin B_{12}

Vitamin B_{12} (cyanocobalamin) is a large molecule (molecular weight: 1,355) that contains a cobalt (Co) atom. The biological function of cyanocobalamin, whose metabolism is closely linked with that of folic acid, consists in its role for normal maturation and development of erythrocytes and maintenance of nerve tissue, in the form of cobamide coenzymes. These are involved in the synthesis of nucleic acid (i.e. synthesis of thymine and deoxyribose) and the recycling of tetrahydrofolic acid, carbohydrate, amino acid and fat metabolism. Since animals cannot synthesize vitamin B_{12}, it must be supplied in trace amounts; a deficiency of this vitamin can result in symptoms similar to those of folate deficiency (NRC 2011).

Dietary sources rich in cyanocobalamin are liver, kidney, fishmeals, animal by-products, meat and bone meal, condensed fish solubles, and poultry by-product meal (1–0.1 mg/kg).

4.2.1.2 Folic Acid

The term 'folate' is used to describe folic acid and related compounds having the biological activity of folic acid. Folic acid is composed of a pteridine ring linked through a methylene bridge to *p*-aminobenzoic acid to form pteroic acid, which is in turn linked to glutamic acid as an amide. Folic acid undergoes enzymatic reduction in the tissues to its active coenzyme form, tetrahydrofolic acid. It is involved as an intermediate carrier of one-carbon groups in many enzymatic reactions where methyl, methylene and other one-carbon groups are transferred from one molecule to another. Reactions involving folate are found in the metabolism of some amino acids and the biosynthesis of purines and pyrimidines, as well as of nucleotides (NRC 2011). It is altered by sunlight or during prolonged storage.

4.2.1.3 Niacin

Niacin (known as nicotinic acid) is the generic name of pyridine3-carboxylic acids and their derivatives, which possess the biological activity of nicotinamide, the amide of nicotinic acid (Fig. 4.3). Niacin is a component of two coenzymes: nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), which are involved in several oxidation–reduction reactions where the transfer of hydrogen and electrons occurs, and in the carbohydrate, lipid

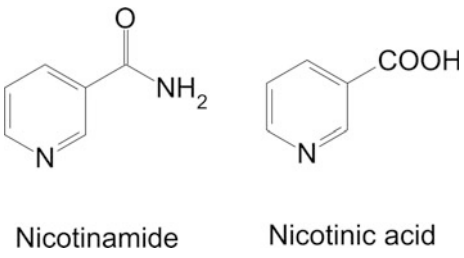

Fig. 4.3 Biochemical structure of nicotinamide and nicotinic acid. BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

and amino acid metabolism (NRC 2011). In addition, NAD and NADP also play an important role in the synthesis of fatty acids and cholesterol, respectively.

Nicotinic acid is contained in dried torula yeast, dried brewer's yeast and rice bran (600–300 mg/kg); wheat bran, dried fish, sunflower seed, rapeseed, liver and lung meal and dried distillers solubles (300–100 mg/kg); and fishmeal, corn gluten, meat and bone meal, meat meal, dried brewers grains, poultry by-product, alfalfa meal (100–40 mg/kg) and green vegetables.

4.2.1.4 Biotin

Biotin is a monocarboxylic acid that is slightly soluble in water and alcohol, and insoluble in organic solvents. The dry material and aqueous solutions are stable at 100 °C and light stable. The vitamin is destroyed by acids, alkali and oxidizing agents such as peroxides or permanganate. Avidin, a protein found in raw egg white, binds biotin and makes it unavailable to fish and other animals; treatment with heat causes the denaturation of avidin and the bound biotin becomes available again to fish.

Biotin acts as a coenzyme in some carboxylation reactions where the transfer of carbon dioxide from one compound to another occurs. Biotin plays a key role in carbohydrate and fat metabolism, since it is a component of the enzymes pyruvate carboxylase and acetyl coenzyme A carboxylase. Biotin is also involved in purine and protein synthesis, some deamination reactions and in the urea cycle.

Dietary sources rich of biotin include dried brewer's yeast, dried torula yeast, dried distillers solubles, rapeseed, sunflower seed (1–2 mg/kg); dried brewers grains, liver and lung meal, rice bran, and cottonseed (1–0.5 mg/kg); and soybean meal, dried skim milk, alfalfa, oats, dried blood meal, dried fish solubles, fishmeal, wheat bran (0.5–0.2 mg/kg), legumes and green vegetables.

4.2.1.5 Pantothenic Acid

Pantothenic acid may be considered as a dihydroxydimethylbutyric acid bonded to alanine. It is a component of 3-phospho-adenosine-5-diphospho-pantotheine (known as acetyl coenzyme A), which is a coenzyme with a key role in the acetylation reactions.

Pantothenic acid is important for carbohydrate, fat and protein metabolism and for producing energy from the major food nutrients. Carbohydrates, fats and proteins are first converted into acetyl coenzyme A before they are oxidized. Moreover, acetyl coenzyme A is involved in the synthesis of fatty acids, cholesterol, steroids, haemoglobin and in the acetylation of choline (NRC 2011). The compound generally used in fish nutrition is the calcium salt, under the form of a white crystalline powder readily soluble in water and insoluble in organic solvents.

Rich dietary sources of pantothenic acid are dried brewer's yeast and dried torula yeast (130–100 mg/kg); sunflower seed, wheat bran, dried skim milk and alfalfa (50–25 mg/kg); and rice bran, fishmeal, soybean meal, linseed, maize, cottonseed, poultry by-products (25–10 mg/kg), liver, kidney and green vegetables.

4.2.1.6 Choline

Choline is a very hydroscopic compound, soluble in water and stable to heat in acid, but not in alkaline solutions. Unlike the other water-soluble vitamins, choline has no coenzyme functions. Choline is an essential component of phospholipids and acetylcholine, which are involved in the cell membranes and the transmission of nerve impulses, respectively. It acts as a precursor of betaine, which is a source of methyl groups in trans-methylation reactions (i.e. synthesis of methionine), and in the form of the phospholipid lecithin plays an important role in the transport of lipid within the body.

Choline is contained in rapeseed, poultry by-products, shrimp, liver and dried fish solubles (7,000–6,000 mg/kg); dried brewer's yeast and sunflower seed (6,000–4,000 mg/kg); brown fishmeal, dried torula yeast, wheat germ, white fish, cottonseed, soybean, meat and bone (4,000–2,000 mg/kg); and heat bran, dried brewers grains, linseed meal, sesame, alfalfa and rice bran (2,000–1,000 mg/kg).

4.2.1.7 Myo-inositol

One of the optically active forms of hexahydroxycyclohexane, myo-inositol, is a crystalline powder soluble in water and insoluble in alcohol and ether, and is a highly stable compound. It is a constituent of inositol phospholipids and a structural component in biological membranes under the form of phosphatidylinositol, which is involved in signal transduction of several metabolic processes (NRC 2011). It is also an important structural component of skeletal, heart and brain tissues. It plays an important role in the growth of liver and bone marrow cells, liver lipid (cholesterol) transport, and in the synthesis of ribonucleic acid. It has no coenzyme functions.

Rich dietary sources of myo-inositol are animal tissues (skeletal, brain, heart and liver), dried brewer's yeast, fishmeal, cereal grains and legumes. In plant tissues, myo-inositol exists in a phosphorylated form as phytic acid which interferes with mineral absorption and therefore is considered as an anti-nutritional factor.

Concerning the beneficial effects of vitamin B-group in fish, Hardy and coworkers examined the effects of dietary pyridoxine on disease resistance in fingerlings of chinook salmon (*Oncorhynchus tshawytscha*) fed with 5, 10, 20 or 40 mg pyridoxine/kg diet at two different protein levels (30 and 65%) for 8 weeks at 16 °C; a group vaccinated with heat-killed *V. anguillarum* by intra-peritoneal injection and then exposed to this bacterial pathogen (Hardy et al. 1979). Significant differences in survival were observed in relation to diet, with approximately 80% mortality (vaccinated and non-vaccinated) in fish fed with 5 mg/kg, compared to <20% mortality in those fed with 20 or 40 mg of pyridoxine/kg diet. No effects were observed on antibody production; moreover, an increased protection of non-vaccinated fish was observed at the higher pyridoxine levels.

Jian carp fed with ≥ 5 mg vitamin B_6/kg diet showed higher white blood cell count, hemoagglutination titre, activities of lysozyme, acid phosphatase and total iron-binding capacity than fish fed with vitamin-B_6- unsupplemented diet (1.7 mg vitamin B_6/kg diet in basal diet) (Feng et al. 2010). The survival rate, leucocyte phagocytic activity, lectin potency, acid phosphatase activity, lysozyme activity and immunoglobulin M content of Jian carp (*Cyprinus carpio)* injected with *Aeromonas hydrophila* were all improved with an increase in dietary thiamine levels up to 0.8–1.1 mg vitamin B_1/kg diet (Feng et al. 2011). Again, Jiang and colleagues found in Jian carp an improvement of red and white blood cell counts, phagocytosis activity, hemoagglutination titre, lysozyme activity and anti-*Aeromonas hydrophila* antibody titre following the administration of 687.3 mg/kg diet of myo-inositol (Jiang et al. 2010). In the same fish species, Wen and coworkers indicated that immunoglobin M content, serum lysozyme activity and antibody titre after *A. hydrophila* challenge were positively affected by the dietary pantothenic acid levels (Wen et al. 2010).

In grouper, Lin and coworkers reported that a dietary folic acid level of 0.8 mg/kg diet is needed to maximize fish growth and non-specific immune responses. Akhtar and colleagues reported that in rohu (*Labeo rohita*), dietary B_6 supplementation at a level of 100 mg/kg diet enhanced immune responses and resistance to high-temperature stress (Akhtar et al. 2012; Lin et al. 2011).

4.2.1.8 Vitamin C

Vitamin C is an essential micronutrient and must therefore be supplied through feeding; fish are unable to synthesize this vitamin, known as ascorbic acid in its L-natural form (Fig. 4.4), due to the lack of an enzyme of the biosynthetic pathway. Unlike other water-soluble vitamins, vitamin C has no coenzyme functions, but acts as a cofactor in many reactions involving hydroxylating enzymes.

Vitamin C is involved in several physiological functions including growth, development, reproduction and possibly lipid metabolism through its action on carnitine synthesis. Vitamin C also plays a significant role in the immune response, response to stressors and resistance to infectious diseases of fish (Caruso and Genovese 1995; Navarre and Halver 1989).

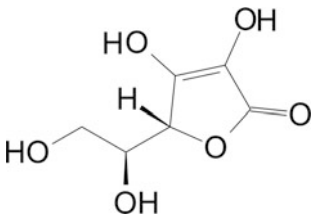

Fig. 4.4 Biochemical structure of *L*-ascorbic acid (reduced form). BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

Ascorbic acid acts as an antioxidant molecule; it is also required for numerous hydroxylation reactions such as those of tryptophan, tyrosine, lysine, phenylalanine and proline. Ascorbic acid plays a key role in maintaining the integrity of connective tissue, blood vessels and bone tissue, as it is involved in the formation of hydroxyproline from proline. Both amino acids are important constituents of collagen, mucopolysaccharides and chondroitin sulphate (i.e. intracellular substances that bind bone cells, blood capillary cells and connective tissue cells).

Ascorbic acid is also required for the conversion of folic acid into its metabolically active form of tetrahydrofolic acid, and for the conversion of tryptophan to serotonin. Another important function of vitamin C is catecholamine biosynthesis; stress response is controlled by the endocrine system via cortisol and catecholamines, whose synthesis depends upon ascorbic-acid-dependent hydroxylases. In stressful conditions, ascorbic acid requirement is increased, since this vitamin can compensate for the stress-induced down-regulation of the immune system.

Vitamin C plays a key role in fish immunity; ascorbic acid protects cells from oxidative damage, especially during the oxidative burst of macrophages, and is involved in the regeneration of vitamin E in its metabolically active form. In the presence of vitamin C, the toxicity of metals such as cadmium, nickel and lead is reduced, since they are converted into reduced forms, which are less absorbed and excreted more rapidly.

The tissue concentration of vitamin C is related to its dietary intake. It is stored in liver, head kidney and spleen; high levels in the head kidney are probably related to the presence of lymphopoietic tissues. The uptake of vitamin C in cells such as lymphocytes, neutrophils and leucocytes involves dehydroascorbic acid because ascorbic acid cannot cross their membrane. Once dehydroascorbic acid is taken up by the cells, it is rapidly reduced to ascorbic acid by an intracellular reductase. In some tissues such as brain, thymus and leukocytes, ascorbic acid accumulates and can be available when dietary vitamin C is depleted. There is a relationship between the ascorbic acid concentration (nmoles/10^8 cells) in leukocytes and the dietary intake of vitamin C. Major signs of ascorbate deficiency are reduced growth, anorexia, skeletal damages, internal and fin haemorrhage, distorted gill filaments, fin erosion, and increased mortality. Insufficient vitamin C levels in the body can damage tissues, since collagen synthesis (hydroxyproline) is catalysed by hydroxylases dependent upon ascorbic acid. Ascorbate deficiency also reduces complement activity in fish (the complement component C1q is rich in hydroxyproline and hydroxylysine).

Most fish require dietary ascorbic acid for normal growth and functions; vitamin C has also been shown to be effective in reducing the negative impacts of environmental (i.e. poor water quality such as high ammonia and low oxygen levels) and husbandry induced stress on health and disease resistance. Fish fed with diets containing high levels (>300 mg/kg) of ascorbic acid were found to survive better to toxic levels of copper (Hilton 1989) and nitrite (Wise et al. 1988). Ascorbic acid at high levels was able to protect fish from physiological stress (Hardie et al. 1991). In gilthead seabream, *Sparus aurata*, Henrique and colleagues found a lower concentration of plasma cortisol after hypoxia stress in fish fed with diets containing ascorbic acid compared to control fish fed without vitamin C (Henrique et al. 1998). In addition, Ji and colleagues reported a depression in lipogenesis and activation in lipolysis activity in black sea bream *Acanthopagrus schlegelii* fed with an ascorbate fortified diet (1,100 mg of *L*-ascorbyl 2-monophosphate-Mg/kg diet) (Ji et al. 2010).

Ascorbic acid deficiency signs vary with species, but the major signs of ascorbate deficiency include reduced growth, scoliosis and lordosis, caused by reduced synthesis of collagen. Other common deficiency signs include anorexia, lethargy, slow growth, dark skin colouration, internal and external haemorrhage, fin erosion, anaemia, reduced appetite, slow growth, and increased mortality (Li and Robinson 1999).

From currently available evidences, in fish, vitamin C acts as an immunomodulating agent, which affects immune function and disease resistance modulating non-specific rather than specific immunity (Blazer 1992; Landolt 1989; Roberts et al. 1995). Controversial results, however, are available on the role of dietary vitamin C levels in fish immunity (Mulero et al. 1998), perhaps due to different experimental protocols and fish species. Several studies have reported the beneficial effects of vitamin C on different immune parameters such as complement activity, leucocyte phagocytic activity, lysozyme content, antibody levels, cytokine production and lymphocyte proliferation (Caruso and Genovese 1995; Hardie et al. 1991; Li and Lovell 1985; Liu et al. 1989; Mulero et al. 1998; Navarre and Halver 1989; Thompson et al. 1993; Verlhac Trichet et al. 2015), and resistance to several bacterial and viral diseases (Durve and Lovell 1982; Li and Lovell 1985; Liu et al. 1989; Navarre and Halver 1989; Roberts et al. 1995). Ascorbic acid increases the natural cytotoxic activity of head kidney leucocytes of gilthead sea bream, *Sparus aurata*, against tumour cells (Cuesta et al. 2002). Ascorbic acid affected positively the immune response and disease resistance in large yellow croaker, *Pseudosciaena crocea*, and Indian major carp, *Labeo rohita*, respectively (Ai et al. 2006; Nayak et al. 2007). In Indian major carp fed with experimental diets supplemented with vitamin C at 500, 1,000 or 1,500 mg/kg diet for 60 days, Tewary and Patra showed higher specific growth rate in fish fed with a diet up to 1,000 mg vitamin C/kg compared with control fish fed with a diet depleted in vitamin C; phagocytic activity, respiratory burst activity and protection against challenge with *Aeromonas hydrophila* were also significantly enhanced by increasing vitamin C dietary supplementation (Tewary and Patra 2008). In Gilthead seabream (*Sparus aurata*) fed with diets containing 3,000 mg/kg ascorbic acid for 2, 4, 6, 8 or 10 weeks compared to 500 mg/kg (control group), Ortuño and colleagues found an

increase in the non-specific immune response parameters, although phagocytic activity of leukocytes peaked after 2 weeks of administration, natural haemolytic complement activity after 6 weeks and respiratory burst after 8 weeks (Ortuño et al. 1999). The increase in non-specific immune response parameters was only transient; for this reason, although there is evidence for a positive role of ascorbate in fish defence, there is no justification for prolonged therapy with vitamin C.

In contrast, in many fish species, no significant effects of dietary intake of vitamin C on respiratory burst by macrophages, phagocytosis and bactericidal activities were reported. In juvenile channel catfish fed for 20 weeks with six levels of vitamin C, no higher phagocytic index was found (Li and Lovell 1985). In Atlantic salmon fed with increasing ascorbic acid levels (50, 310 and 2,750 mg/kg for 26 weeks), there were no effects in the phagocytosis by head kidney macrophages or in the ability to produce superoxide anion, as well as in the antibody response to *A. salmonicida* (Hardie et al. 1991). In the same species, fed with high vitamin C (3.17 g/kg diet) for about 6 months, Thompson and coworkers found that leucocyte respiratory burst activity and bactericidal activity were not significantly different from fish fed with low vitamin C (0.082 g/kg diet) (Thompson et al. 1993).

In fish, vitamin C biosynthesis does not occur due to the lack of the last enzyme of the biosynthetic pathway, *L*-gulonolactone oxidase, and thus ascorbic acid must be provided in the diet. Rich dietary sources of ascorbic acid are green vegetables, lemon, fresh insects, liver and kidney. The minimum amount of ascorbic acid in the diet needed for normal growth and health generally ranges from 10 to 50 mg/kg of diet (NRC 1993); the required amount varies among fish species, but intraspecies differences such as fish strain, size and age also affect it (Li and Robinson 1999). The requirement of dietary ascorbate has been reported to be 50–100 mg/kg of the diet in channel catfish (Li et al. 1998) and rainbow trout (Sato et al. 1982).

The amount of ascorbic acid that must be added to the diet for normal function is also affected by the form of the vitamin added to the diet. Since ascorbic acid is extremely sensitive to dietary components (such as fats and trace minerals) that increase its oxidation, or to oxygen, high moisture, heat and pressure conditions such as those used during manufacture of most aquatic animal feeds, the most successful method to keep the stability of ascorbic acid during feed manufacture and storage is to chemically bind the C-2 carbon of its molecule to a chemical moiety such as a fatty acid, sulphate or phosphate. One of the most common methods used to stabilize ascorbic acid is to coat it with ethylcellulose or fat. Coated products are somewhat more stable than crystalline ascorbic acid; however, the protective coating may be damaged during feed manufacture, and losses of ascorbic acid can still be considerable. Concerning vitamin C supplementation, ethylcellulose-coated ascorbic acid has been the predominant form of the vitamin used in fish feeds for many years; however, it is relatively unstable during feed manufacture and storage, whereas the phosphorylated forms are the most stable and thus they provide a more precise amount of vitamin C.

4.2.2 *Fat-Soluble Vitamins (Vitamin A, D, E and K)*

4.2.2.1 Vitamin A

Vitamin A exists only in animal tissues, and it is present either in the form of retinol (alcohol, vitamin A_1, which is found in mammals and marine fish, Fig. 4.5) or 3, 4-dehydroretinol (vitamin A_2, which is found in freshwater fish together with vitamin A_1). A precursor of vitamin A (provitamin) is beta-carotene, a carotenoid pigment found in vegetable tissues. This can be converted into vitamin A after ingestion by fish; the conversion efficiency depends on the fish species and on the isomeric form of the carotenoid, since the isomer 'trans' is associated with the highest biological activity.

The main biological function of vitamin A is related to vision: in the retina, vitamin A is combined with a specific protein (opsin) to form a visual pigment, which in turn is involved in the reception and transmission of light from eye to brain. In addition, vitamin A is crucial in a number of physiological processes such as cell differentiation, embryo development, development of epithelial cells, including mucus-producing cells from stem cells to fully functional layers, and differentiation of immune cells in response to exposure to pathogens. This vitamin is involved in skin protection and maintenance of the mucous secreting epithelial tissues of the reproductive and gastrointestinal tract (NRC 2011). Fish liver oils and animal liver meals are rich in retinol, halibut and cod liver oil contain 9,000 µg/g and 180 µg/g retinol, respectively, animal liver 25–100 µg/g retinol. Also, carrots and spinach are a source of retinol.

In Atlantic salmon, the addition of 10,000 IU vitamin A/kg significantly improved the serum antibacterial activity, but no enhanced effect was observed when the added vitamin A was increased up to 25,000 IU vitamin A/kg (Thompson et al. 1994). Similar results have been reported for Atlantic salmon rainbow trout (Thompson et al. 1995) where supplementation with vitamin A caused increased classical complement activity, and leukocyte migration, but not the serum immunoglobulin level, lysozyme activity and phagocyte respiratory burst activity compared to control fish fed with a vitamin A-free diet. In juvenile Japanese flounder, Hernandez and colleagues observed significantly higher antibacterial activity in the serum of fish fed with diets containing 10,000 and 25,000 IU vitamin A kg^{-1} compared to fish fed with diets containing no vitamin A (Hernandez et al. 2007).

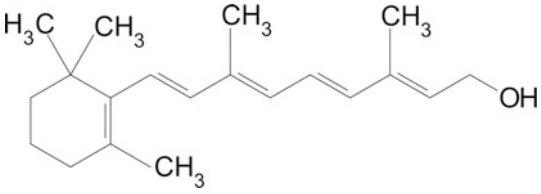

Fig. 4.5 Biochemical structure of retinol (alcohol, vitamin A_1, which is found in mammals and marine fish). BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

Concerning the effects of vitamin A on fish skeletogenesis, Fernández and Gisbert studied this aspect in flatfish, showing that bone and cartilage development and homeostasis at different life stages (embryonic, larval, juvenile or adult stages) are primarily regulated by retinoic acid, the active metabolite of vitamin A (Fernández and Gisbert 2011). The impact of dietary vitamin A on flatfish development and aquaculture depends on several factors: the chemical form and dose of this vitamin in the diet (i.e. retinal, used as the chromophore of rhodopsin in the eye, or retinoic acid, the main active metabolite of vitamin A, involved in morphogenesis and cell proliferation and differentiation and the development of nervous and skeletal systems), the duration of this supply, the fish developmental stage and the ontogeny of the species. Therefore, dietary vitamin A requirements need to be adapted for each flatfish species. During early larval development, retinyl palmitate and retinyl acetate are recommended, although retinyl palmitate is more stable and less toxic than retinol, retinal and retinoic acid, and a source of lipids (retinyl esters), while retinyl acetate has the advantage that is well accumulated and less metabolized by live preys; conversely, both retinoid compounds are recommended in diets for juveniles and broodstocks, since at these developmental stages fish have a fully developed liver with the necessary enzymes to convert those compounds into retinol, retinal and retinoic acid.

4.2.2.2 Vitamin D

Vitamin D occurs in nature under the forms of vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol), which are mostly found in plants and in animals, respectively. In the liver, both forms are hydroxylated to the 25-hydroxy forms; the 25-hydroxy-cholecalciferol is then converted into 1, 25-dihydroxycalciferol, which is the biologically active form of vitamin D and is responsible for the synthesis of the calcium-binding protein in the intestinal epithelial cells.

Vitamin D is important for the development, growth and maintenance of healthy bones during life. Its primary function is in the calcium metabolism, as vitamin D is required for intestinal absorption of calcium and for the calcification of growing bone tissue, and influences the action of parathyroid hormone on bone. Vitamin D functions through the vitamin D-receptor inducing the expression of various calcium-binding and transport proteins in the intestine to stimulate active calcium uptake; its function is important in maintaining bone mineralization, since vitamin D acts directly on osteoblasts, to inhibit proliferation, modulate differentiation and regulate mineralization of the extracellular matrix. Vitamin D is also involved in alkaline phosphatase activity, and in phosphorus metabolism. Additional functions of vitamin D regard its role in differentiation of bone, skin and blood cells, maintenance of blood calcium level, and the deposition and oxidation of citrate in bone; in insulin and prolactin secretion, muscle function, immune and stress responses, and melanin synthesis.

Cholecalciferol is contained in fish liver oils (cod liver oil 2–10 μg/g) and fishmeal, as well as in animal liver. Excessive intake of vitamin D may result in irreversible calcification of the heart, kidneys and other soft tissues (NRC 2011).

Dietary requirements of vitamin D in fish were recently reviewed (Lock et al. 2010). Addition of 2.4 IU vitamin D_3/kg diet for an optimal performance of fish is suggested (Darias et al. 2011; NRC 1993). However, vitamin needs are a function of several factors such as developmental stage, physiological, environmental and genetic conditions. Vitamin D_3 affects some innate immune parameters of the gilthead sea bream, *Sparus aurata* (Cerezuela et al. 2009). The immunostimulant effect was greater on the cellular (leucocyte peroxidase content, phagocytic, respiratory burst and natural cytotoxic activities) than humoral immune parameters (peroxidase and complement activity). Darias and colleagues provided a general overview about the requirements of vitamins D and C in fish and on the role of these vitamins in fish skeletogenesis and in the development of skeletal deformities (Darias et al. 2011). In addition, Darias and coworkers found an influence of high levels of dietary vitamin D_3 (42 and 120 IU/g diet) in the development of skeletal malformations in early juvenile of European sea bass (Darias et al. 2010), while no significant difference was observed in fry Atlantic salmon fed with three different vitamin D_3 levels (8,200 and 2,280 IU/g diet) for 14 weeks neither in growth parameters nor in skeletal malformations or histopathological changes among the groups (Graff et al. 2002). These results confirm the difficulty to establish an optimal dose of vitamin that could meet the requirements of the different fish species.

4.2.2.3 Vitamin E

Vitamin E (tocopherols) is a group of fat-soluble factors required for normal growth, reproduction, physiological functions and health of vertebrates. Of the eight naturally occurring isomers of tocopherol, the alpha isomer is the most widely distributed and has the greatest vitamin activity.

In general, the most widely documented function of vitamin E is its extracellular and intracellular antioxidant activity, protecting from oxidative damage the highly unsaturated fatty acids present in cell membranes and other reactive compounds (i.e. vitamins A and C). Vitamin E plays distinct metabolic functions including membrane integrity, cell signalling, gene expression and eicosanoids synthesis; many of these functions have a relevant impact on immune system and disease resistance.

Vitamin E is particularly required under situations of chronic and acute stress. Inclusion of adequate amounts of α-tocopherol in fish diets markedly enhances fish health and promotes resistance to stress and, potentially, to infectious diseases (Blazer 1992; Izquierdo and Betancor 2015). Indeed, vitamin E has a synergistic effect with n-3 highly unsaturated fatty acids (HUFA) and vitamin C, maintaining the integrity of immune responses, the normal resistance of erythrocytes to

hemolysis and the permeability of capillaries and enhancing the non-specific immune responses and disease resistance.

The predominant role of vitamin E in antioxidant defence mechanisms is to increase the stability of tissue lipids to oxidation in many fish species. The antioxidant activity of tocopherols and tocotrienols is related to their abilities to donate their phenolic hydrogen to lipid-free radicals, thereby retarding the auto-catalytic lipid peroxidation processes. Lipid-free radicals have an unpaired electron that makes them highly reactive towards unsaturated lipids, on which they capture a hydrogen atom of a double bond, create a new free radical and initiate the chain of reactions that rapidly lead to lipid autoxidation.

The specific location as a structural component of cell membranes allows vitamin E to play a role in the control of peroxidation of polyunsaturated fatty acids (PUFA). Tocopherols can also act as quenchers of free radicals like the singlet oxygen; this prevents the propagation of free radical reactions in membranes and lipoproteins, and maintains homeostasis of labile metabolites in the cells.

Moreover, α-tocopherol is responsible for maintaining membrane stability of fish fillet throughout its shelf life, delaying colour deterioration and rancid flavour. Fillet quality can be markedly improved by supplementing vitamin E in diets for rainbow trout, Atlantic salmon or European sea bass. In addition to its antioxidant function, vitamin E influences membrane fluidity and maintenance, and is necessary in capillaries and heart muscle; it plays also an important role in cellular respiration and in the biosynthesis of deoxyribonucleic acid and coenzyme Q.

Deficiency of this vitamin causes a wide variety of pathological conditions, such as reduced growth and survival, lower stress resistance, epithelial pigmentation loss, haematological alterations and exophthalmia (Hamre et al. 1997). Moreover, a marked immunodepression was detected under vitamin E depletion (Blazer and Wolke 1984; Hardie et al. 1990), confirming that vitamin E interacts with the immune system; diets low or depleted in vitamin E resulted in high mortality rates in European sea bass infected with *Aeromonas salmonicida* (Obach et al. 1993). Vitamin-E-depleted diets also impaired antibody response in rainbow trout (Verlhac et al. 1993). In gilthead sea bream, vitamin-E-depleted diets reduced the serum alternative complement pathway activity in comparison to fish fed with 150 mg/kg of α-tocopherol supplementation (Montero et al. 1998).

Gilthead sea bream fed with a vitamin-E-deficient diet showed a faster elevation of plasma cortisol levels, reduced growth and lower survival under repeated chasing stress (Montero et al. 2001). Conversely, dietary supplementation with vitamin E was shown to improve immune response and disease resistance in several fish species (Blazer and Wolke 1984; Cuesta et al. 2001; Hardie et al. 1990; Lin and Shiau 2005; Montero et al. 1998, 1999, 2001; Oliva-Teles 2012; Ortuño et al. 2000). Blazer and Wolke studied the effects of alpha-tocopherol on the specific and non-specific immunity in rainbow trout (*Salmo gairdneri*) maintained on a control diet (140 mg vitamin E/kg feed), and a tocopherol-deficient diet (0.6 mg/kg) for 12–17 weeks and found that fish fed with the tocopherol-deficient diet had significantly reduced humoral immunity (hemoagglutination, hemolysin and bacterial agglutination) compared to the others (Blazer and Wolke 1984). Hardie and colleagues

studied the effects of vitamin E on disease resistance in Atlantic salmon by feeding levels of 7, 86, 326 or 800 mg vitamin E/kg diet for 20 weeks (Hardie et al. 1990). When fish from the 7 and 326 mg/kg groups were bath-exposed to *A. salmonicida*, the rate of mortality was significantly increased in the low vitamin E group. There is no effect of vitamin E level on macrophage function (superoxide production), lymphokine production or humoral immune response to *A. salmonicida*, whereas an effect on non-specific serum factors was found. Fish fed with the vitamin-E-depleted diet showed an impaired complement function, a reduction in total hemolytic activity and a reduced ability to opsonize bacteria. Wise and coworkers reported an increase in macrophages phagocytic activity in channel catfish *Ictalurus punctatus* fed with diets supplemented with 60 and 2,500 mg/kg diet of vitamin E (Wise et al. 1993).

The effects of vitamin E on fish immune response, however, are sometimes not consistent in the same fish species. For example, in gilthead seabream (*Sparus aurata*) fed with vitamin E-supplemented diets (600, 1,200 or 1,800 mg tocopherol acetate/kg diet) for 15, 30 or 45 days compared to a commercial diet (100 mg/kg) as control, a dietary intake of 1,200 mg vitamin E for 30 days of treatment was effective to stimulate the non-specific immune system of sea bream (serum haemolytic activity and the phagocytosis of head kidney leucocytes), although leucocyte migration and respiratory burst activity remained unaffected (Ortuño et al. 2000).

In Nile tilapia *Oreochromis niloticus*, some immune parameters (serum protein, lysozyme or alternative complement activity) were affected by dietary lipid and vitamin E levels, although these nutrients had no effect on fish resistance to *Streptococcus iniae* infection or on antibody titre against that bacterial infection (Lim et al. 2009).

In Japanese flounder, *Paralichthys olivaceus*, Wang and colleagues observed that although high dietary intake of vitamin E improved non-specific immune responses and disease resistance, dietary vitamin E and n-3 HUFA had a synergistic effect on that response (Wang et al. 2006). In sub-adult turbot fed with graded levels of vitamin E (0, 120, 240, 480 and 960 mg/kg diet) for 15 weeks, Niu and coworkers reported significant increases in the final weight, specific growth rate, serum lysozyme activity, phagocytic index and superoxide dismutase activity with increasing vitamin E levels, with the best results in the diet with 480 mg/kg vitamin (Niu et al. 2014).

Vitamin E needs to be replenished through the diet. Most commercial feeds contain 150–400 mg of vitamin E per kg of diet, which seem adequate to maintain the immune function of salmonids, marine fish and catfish (Lall 2000). Vitamin E requirements vary depending on fish species, size, age, water conditions, stressing situations, etc. In addition, α-tocopherol biological activity might be, at least partially, damaged after steam pelleting and storage at room temperature, and the requirement of vitamin E increases as the levels of polyunsaturated fatty acids increase. Generally, the dietary vitamin level required for promoting health benefits is about 10–100 times higher than that required for normal growth; however, the molecular mechanisms implied in the enhancement of immune function are not well

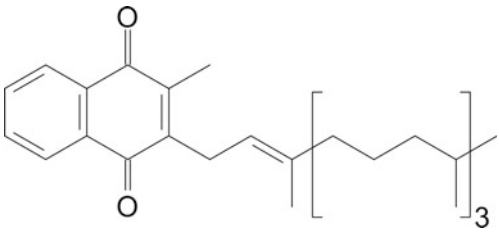

Fig. 4.6 Biochemical structure of phylloquinone (vitamin K_1). BKchem version 0.13.0, 2009 (http://bkchem.zirael.org/index.html) has been used for drawing this structure

understood in fish and more research is needed to define the specific dietary requirements for this vitamin.

Rich dietary sources of tocopherol are alfalfa meal, wheat germ meal (125–100 mg/kg); whole chicken egg (100–75 mg/kg); rice bran (75–50 mg/kg); dried brewers grains, dried distillers solubles, barley grain, soybean meal and maize grain (50–25 mg/kg); and corn gluten, wheat bran, fishmeal, sunflower seed, cottonseed (25–10 mg/kg), vegetable oils and green vegetables.

4.2.2.4 Vitamin K

The term 'vitamin K' is used to identify all the derivatives of naphthoquinone. There are three major forms of vitamin K, two of natural origin (vitamin K_1 or phylloquinone, Fig. 4.6), and vitamin K_2 or multiprenylmenaquinones, which are synthesized by green plants and bacteria, respectively; the third compound is the vitamin K_3 or menadione, which is a synthetic product.

The major role of vitamin K is related to blood coagulation, since it facilitates the production and/or release of various plasma proteins including prothrombin and thromboplastin as well as of blood-clotting factors VII, IX and X. The metabolic role of vitamin K involves enzyme vitamin-K-dependent carboxylase, which converts the specific glutamyl residues in the plasma proteins into γ-carboxyglutamyl residues, which are required for the normal interaction of the vitamin-K-dependent clotting factors with phospholipid surfaces. Vitamin K also has an important role in calcium transport; the action of osteocalcin, the major bone matrix protein, is vitamin-K-dependent (NRC 2011). Vitamin K is contained in fishmeal (2 mg/kg), liver meal and green vegetables such as spinach.

4.3 Minerals

Minerals are inorganic elements considered to be essential nutrients for fish life. There are about 20 elements (Table 4.2) that should be introduced with diet in levels sufficient to sustain health and maintain the ability of fish to cope with stress and to resist diseases. Some minerals may increase immunocompetence of fish, making them less susceptible to diseases. According to their concentration, the

Table 4.2 Essential mineral elements for fish life. There are about 20 elements that should be introduced with diet in levels sufficient to sustain health and maintain the ability of fish to cope with stress and to resist diseases

Macroelements		Trace or microelements	
Principal cations	Principal anions		
Calcium (Ca)	Phosphorus (P)	Iron (Fe)	Fluorine (F)
Magnesium (Mg)	Chlorine (Cl)	Zinc (Zn)	Vanadium (V)
Sodium (Na)	Sulphur (S)	Manganese (Mn)	Chromium (Cr)
Potassium (K)		Copper (Cu)	Molybdenum (Mo)
		Iodine (I)	Selenium (Se)
		Cobalt (Co)	Tin (Sn)
		Nickel (Ni)	Silicon (Si)

mineral elements are usually classified into macroelements and microelements; macrominerals are required in large amounts, ranging from tenths of a gramme to some grammes per day; conversely, microminerals are required in very small amounts, ranging from microgrammes to milligrammes per day (Webster and Lim 2015).

The general functions of minerals are as follows:

(1) Minerals are essential constituents of skeletal structures and soft tissues.
(2) Minerals are essential for the transmission of nerve impulses and muscle contraction.
(3) They are involved in the maintenance of acid–base and osmotic balances and regulate the exchange of water and solutes within fish body, the pH of the blood and other body fluids.
(4) Minerals are essential components of many enzymes, vitamins, hormones, proteins and lipids and enzyme activators, or cofactors in metabolism.
(5) Many minerals have biological functions in the defence mechanism and immunocompetence of the fish.

To satisfy their metabolic requirements, fish can absorb dissolved minerals in the aquatic environment through the skin, gill membrane and the digestive tract.

4.3.1 Macrominerals

4.3.1.1 Calcium

Among the macrominerals, calcium (Ca) is an essential component of bones and cartilage; it is also necessary for blood clotting, through stimulation of the release of thromboplastin from the platelets.

(1) It is involved in the transmission of nerve impulses through its control over acetylcholine production and in muscle contraction.
(2) Together with phospholipids, calcium plays a key role in the regulation of the cell membrane permeability and in the uptake of nutrients.
(3) Calcium is believed to be essential for the absorption of vitamin B_{12} from the gastrointestinal tract.
(4) Calcium is an activator for several enzymes, such as pancreatic lipase, acid phosphatase, cholinesterase and ATPases.

Calcium is readily absorbed in the proximal end of the intestine (through vitamin D), gills, skin and fins of fish and crustacea. In general, dietary calcium absorption is made easier by dietary lactose. Conversely, excess of lipids or fibres in the diet and the presence of phytic acid, which is present in cereal grains and oilseed meals, prevent the absorption of calcium. Calcium is contained in high amounts in the shell of oysters, rock phosphate (40–30%); shrimp and crab meal (20–10%); white fish, meat meal (10–5%); and brown fish, skim milk, poultry by-product and alfalfa meal (1–5%).

4.3.1.2 Magnesium

Magnesium (Mg) has different biological functions, including the formation of bones and cartilage, the activation function for several biological catalysing molecules, muscular and nervous functions (Webster and Lim 2015), and some important role with relation to the synthesis of proteins. Interestingly, magnesium may be found in notable amounts as Mg salt of phytic acid (also named phytin).

In Atlantic salmon, both unvaccinated and vaccinated against *Vibrio anguillarum* and fed with diets containing various levels of Mg (0, 300 and 500 mg kg^{-1} of diet), El-Mowafi and colleagues did not observed differences in serum antibodies, serum lysozyme and hemolytic activities, between vaccinated and unvaccinated fish fed, as well as among the three Mg levels (El-Mowafi et al. 1997).

In channel catfish *Ictalurus punctatus* fed with diets containing various Mg levels (0, 200–1,000 mg kg^{-1} of diet), no significant differences in total cell count, red blood cell count, hematocrit and haemoglobin were found (Lim and Klesius 2003). A similar result was observed in the same species challenged with *Edwardsiella ictaluri*.

In grass carp *Ctenopharyngodon idella* fed with diets containing various levels (76.9–2,481.2 mg kg^{-1} of diet) of Mg, Wang and coworkers observed higher superoxide dismutase, glutathione peroxidase and lysozyme activities in fish fed with a diet containing 692.0 mg Mg kg^{-1} with respect to the control diet (Wang et al. 2011).

4.3.1.3 Phosphorus

Phosphorus (P) is an essential mineral, which is a component of fish bones and cartilage, in the forms of calcium phosphate and hydroxyapatite. It is also important in energy metabolism as a component of adenosine mono-, di- and triphosphate, respectively, and as a component of nucleic acids, phospholipids and enzymes, and is important in antibody formation. Inorganic phosphates serve as buffers in the regulation of the normal acid–base balance of animal body fluids. Fish absorb phosphorus from the water through their skin, fins and gills; however, the concentration of phosphorus in fresh and seawater is low, and consequently a dietary source of phosphorus is needed to satisfy phosphorus requirements.

In channel catfish, Eya and Lovell suggested that a dietary P level of 0.4% is sufficient to meet the growth requirement and to improve the resistance of fish against *Edwardsiella ictaluri* infection (Eya and Lovell 1998). In European white fish (*Coregonus lavaretus*), Jokinen and colleagues found that fish fed with the low-P diet were significantly smaller than fish fed with diets containing adequate P, and had lower plasma immunoglobulin M (IgM) levels than those of fish fed with the high-P diet, although there were no differences in serum lysozyme and antibody production between the two groups (Jokinen et al. 2003).

A greatest fraction (50–80%) of the phosphorus contained in plant foods (e.g. soybean or oilseeds) occurs in the form of calcium or magnesium phytate. This organic form of P is poorly utilized and must first be hydrolysed within the gastrointestinal tract by the enzyme phytase. Phosphorus absorption can be inhibited if the diet contains an excess of calcium over phosphorus; calcium may bind P forming insoluble salts (tricalcium phosphates). Therefore, highly digestible sources of phosphorus (i.e. monocalcium and dicalcium phosphate) are often added to fish diets to meet the amount of phosphorus required for growth, bone mineralization and physiological functions. Conversely, P-deficient diets can result in reduced growth and insufficient bone mineralization with severe malformations.

Phosphorus is contained in bone (20–10% P); meat and bone meal, white fish, shrimp and poultry by-product (5–2%); and rice and wheat bran, dried brewer's yeast, sunflower and sesame seeds (2–1%).

4.3.1.4 Sodium, Potassium and Chloride Ions

Sodium, potassium and chloride ions are all central components of body fluids and cells, and are needed to maintain osmotic pressure and acid–base equilibrium. They also play important roles in water metabolism. Potassium, sodium and chloride are readily absorbed from the gastrointestinal tract, skin, fins and gills of fish.

Sodium is the main cation of the extracellular fluids; it regulates osmotic pressure and maintains acid–base balance. Sodium is also important for muscle contraction and plays a role in nerve function and carbohydrate absorption.

Potassium is a major cation of the intracellular fluids and regulates intracellular osmotic pressure and acid–base balance, together with sodium. Potassium has a

stimulating effect on muscle irritability; it is also required for glycogen and protein synthesis and the metabolic breakdown of glucose. If it present in excess, it may interfere with magnesium absorption.

Chloride is the main anion of extracellular fluids (i.e. gastric juice) and is essential for the regulation of osmotic pressure and acid–base balance. It also plays a specific role in the transport of oxygen and carbon dioxide in the blood.

Dietary sources rich in sodium, potassium and chlorine are shrimp meal, white fish, meat and bone meal (1–4% of sodium); soybean and rice bran (2–4% of potassium); wheat bran, cottonseed, rapeseed and sunflower (1–2% of potassium); and salt (60% as chloride ion).

4.3.1.5 Sulphur

Sulphur is an essential component of several key amino acids (methionine and cystine), vitamins (thiamine and biotin), the hormone insulin and the crustacean exoskeleton. Several key enzymes, such as coenzyme A and glutathione, contain sulphydryl groups. Sulphur is an essential component of heparin, chondroitin, fibrinogen and taurine under the form of sulphate. It is believed to be involved in the detoxification of aromatic compounds within the animal body.

Sulphur-containing amino acids and inorganic sulphates are readily absorbed from the gastrointestinal tract of fish. Dietary sources rich in sulphur amino acids are fishmeal and chicken eggs.

4.3.2 Microminerals

This category includes manganese, zinc, selenium, chromium, iodine and copper. Many of these minerals have biological functions that affect the host immune system and defence mechanisms. However, except for iron (Fe), little is known about the effects of dietary trace elements in fish immunity and disease resistance.

4.3.2.1 Chromium

Chromium (Cr) is an important micronutrient, which has two ionic forms: hexavalent Cr (Cr-6) and trivalent Cr (Cr-3). This latter is an essential nutrient and is required in energy metabolism. Chromium acts with other substances to control metabolism. Trivalent chromium is a component of the glucose tolerance factor (GTF), a low molecular weight compound which is released into the blood when there is a dramatic increase in glucose and/or insulin levels, and acts as a cofactor for the hormone insulin. Trivalent chromium is also believed to play an important role in cholesterol and amino acid metabolism.

Juveniles of rainbow trout, *Oncorhynchus mykiss*, fed for 6 weeks on diets supplemented with Cr at increasing concentrations showed significantly lower red blood cell count and hematocrit together with significantly higher serum lysozyme activity and higher percentages of phagocytic activity of head kidney macrophages in fish fed on 2,340 and 4,040 ppb Cr compared to control fish (Gatta et al. 2001).

Trivalent chromium is readily absorbed by fish from the gastrointestinal tract and the surrounding water. Rich dietary sources of trivalent chromium include shrimp, *Artemia salina*, shellfish, liver, poultry by-product and fishmeal (5–1 mg/kg dry weight).

4.3.2.2 Copper

Copper (Cu) is an essential component of several oxidation–reduction enzyme systems. It is a cofactor for enzymes such as superoxide dismutase, cytochrome oxidase and for enzymes used in the electron transport chain; it is also required for normal functioning of the brain, skeleton and spinal cord. Copper is involved in iron metabolism, and therefore haemoglobin synthesis and red blood cell production and maintenance, being a component of the enzyme caeruloplasmin (ferroxidase).

Copper is also necessary for the formation of pigment melanin and consequently skin pigmentation, for the formation of bone and connective tissue, and for maintaining the integrity of myelin sheath of nerve fibres.

Copper is readily absorbed from the gastrointestinal tract, gills, fins and skin of fish and crustacea. Dietary copper availability and absorption is reduced in the presence of phytates, and high dietary intakes of cadmium, iron, lead, zinc, inorganic sulphates and calcium carbonate. Moreover, high levels of dietary Cu can reduce growth, increase mortality, increase oxidative stress and reduce immune response.

Rich dietary sources of copper include corn gluten, linseed and soybean meal, dried brewers grains and cottonseed (50–20 mg/kg).

4.3.2.3 Iron

Iron is an essential mineral in the formation of red blood cells, since it combines with the respiratory pigments haemoglobin and myoglobin and so it transports oxygen within the body. It is also an essential component of various enzymes such as cytochromes, catalases, peroxidases and enzymes involved in energy metabolism. A deficiency of iron may cause nutritional anaemia, conversely if in high concentration iron can bind with phosphorus to form an insoluble iron–phosphate complex that results in phosphorus deficiency. Iron is an essential mineral with important effects on immune system function and resistance to infectious diseases.

In channel catfish fed with Fe-deficient diet for 13 weeks, Lim and Klesius found significantly lower final weight, serum iron, hematocrit and total blood cell count compared to fish fed with a control diet with optimal Fe content (Lim and

Klesius 1997). After challenge with *E. ictaluri*, fish fed with an iron-deficient diet died on day-1 post-challenge, while mortality was recorded on day-5 post-challenge in fish fed on control diet.

In Atlantic salmon, diet supplementation with Fe had no effect on haematology, antioxidant status or non-specific immune parameters (Andersen et al. 1998) except for a small but significant increase in catalase activity in head kidney.

In channel catfish, Lim and colleagues also showed that macrophage migration in either the absence or presence of *E. ictaluri* was higher in fish fed with Fe-supplemented diets, while dietary levels of either Fe or vitamin C or their interaction affected survival after challenge with *E. ictaluri* (Lim et al. 2000).

Iron is readily absorbed through the gastrointestinal tract, gills, fins and skin of fish. Dietary iron availability and absorption is usually depressed by high dietary intakes of phosphate, calcium, phytates, copper and zinc. In general, the ferrous iron (Fe^{2+}) is more available for absorption than ferric iron (Fe^{3+}). Reducing substances such as vitamin C enhance the absorption of iron.

Rich dietary sources of iron are blood and bone meal (0.3–0.2% Fe); coconut, sunflower, linseed, alfalfa, fishmeal, poultry by-product, dried brewer's yeast and rice bran (500–200 mg/kg).

4.3.2.4 Iodine

The importance of iodine concerns two thyroid hormones, thyroxine and tri-iodo-thyronine, and correlated metabolic functions. On the other side, iodine availability and biological absorption may be diminished when cobalt is highly available. All seafood and fish products contain remarkable iodine concentration, with peculiar reference to seaweed, marine fish and crustaceans.

4.3.2.5 Manganese

Manganese (Mn) is generally linked with bone formation, different metabolic functions and animal reproductions. In addition, it can act as activator for different enzymatic molecules; moreover, it may produce metal complexes with important biological consequences (Webster and Lim 2015). In particular, Mn deficiency has been reported to reduce natural defences against biological agents in rainbow trout (Inoue et al. 1998). On the other hand, Mn and zinc concomitant supplementation may be ineffective in sockeye salmon *Oncorhynchus nerka* when speaking of bacterial kidney disease resistance (Bell et al. 1984).

4.3.2.6 Selenium

The role of selenium (Se), in conjunction with vitamin E, is correlated with the defence of cells against oxidation (Webster and Lim 2015) through the production

of selenium–metal protein and selenide–metal complexes (the involved enzyme is glutathione peroxidase). The effect of Se- and vitamin E concomitant supplementation in channel catfish was questioned, while one or the other nutrient can show ameliorated answers (Wise et al. 1993). Anyway, the concentration of selenium has important effects on growth and immune answers in the same fish species (Wang et al. 1997).

4.4 Enzymes

Fish gut microbiota has been suggested to have a beneficial role in fish nutrition, due to the secretion of a wide range of enzymes (Ray et al. 2012), such as chitinase, cellulase and collagenase, helping animals in their digestion. Fish harbour proteolytic, amylolytic and cellulolytic bacteria in their digestive tracts (Bairagi et al. 2002; Ghosh et al. 2002; Roy et al. 2009; Saha et al. 2006). Endogenous enzymes produced by gut bacteria allow the utilization of carbohydrates commonly found in plant feedstuffs, such as mannose, xylose, raffinose, cellobiose and cellulose (Kar et al. 2008).

Other enzymes (named exogenous enzymes) can be provided through dietary supplementation (Castillo and Gatlin 2015) for carbohydrases to improve fish utilization of carbohydrates. Exogenous cellulases were provided to enhance growth and digestive enzyme activities of grass carp fed on plant protein sources (Zhou et al. 2013).

Lemos and Tacon have reviewed the benefits related to dietary supplementation with phytases in improving digestibility of dietary plant phytates, phosphorus and other minerals as well as organic substrates (protein, amino acids and lipids) (Lemos and Tacon 2016).

Aquaculture industry is considered as an emerging market for exogenous feed enzymes (Ravindran and Son 2011), which can increase digestibility of alternative protein sources for fishmeal. Recent research proposes the use of fish viscera extracts as a potential source of enzymes to be included as additives in fish feeds (González-Riopedre et al. 2013), although there are still many questions regarding real cost of production of enzyme extracts at industrial scale.

4.5 Probiotics

The use of probiotics as alternative feed additives to promote better growth, health maintenance and disease prevention is gaining increasing scientific and commercial interest (Dimitroglou et al. 2011; Kim and Austin 2006; Magnadottir 2010; Martinez-Cruz et al. 2012; Mohapatra et al. 2013); it is now widely accepted as the new eco-friendly alternative measure for sustainable aquaculture (Ram and Parvati 2012). Probiotics are originally defined as the organisms and substances which

contribute to the intestinal microbial balance (Parker 1974). The term 'probiotic' means promoter of life. Indeed, they help, in a natural way, to improve the overall health status of the host organism (Gismondo et al. 1999). According to Food and Agricultural Organization/World Health Organization, probiotics are 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO 2001). Such approach has a potential advantage as it introduces specific microbes in the intestine for restoring the intestinal microbial balance and exerting numerous beneficial effects in the host (Oliva-Teles 2012). Most probiotics proposed as biological control agents in aquaculture belong to the genera *Lactobacillus, Lactococcus, Leuconostoc, Carnobacterium, Enterococcus, Enterobacter, Shewanella, Bacillus, Aeromonas, Vibrio, Pseudomonas, Clostridium* and *Saccharomyces*.

Among the numerous health benefits attributed to probiotics, modulation of immune system is one of the most common, and their ability to stimulate the systemic and local immunity under in vitro and in vivo conditions is noteworthy. Supplementation with different probiotics (either monospecies or multispecies) can increase phagocytic, lysozyme, complement and respiratory burst activity, as well as the expression of various cytokines in fish; however, the exact mode of action of probiotics is yet to be established because they exert host specific and strain-specific differences in their activities.

Some possible benefits linked to probiotics' use are competitive exclusion of pathogenic bacteria (Balcázar et al. 2004), source of nutrients and enzymatic contribution to digestion (Garriques and Arevalo 1995), direct uptake of dissolved organic material mediated by the bacteria (Moriarty 1997), enhancement of the immune response against pathogenic microorganisms (Balcázar et al. 2004) and antiviral effects (Kamei et al. 1988; Girones et al. 1989).

The effectiveness of probiotics in terms of protection against infectious pathogens is often correlated with an increase of immunocompetence. Protection against Edwardsiellosis (Nayak 2010), lactococcosis (Vendrell et al. 2008) and several other diseases (Brunt and Austin 2005; Brunt et al. 2007; Sharifuzzaman and Austin 2009) has been successfully accomplished through probiotics feeding. It is well known that the microbiota in the gastrointestinal tract of aquatic animals can be modified, for example, by ingestion of other microorganisms; therefore, microbial manipulation constitutes a viable tool to reduce or eliminate the incidence of opportunist pathogens (Balcázar 2002).

The first report on the existence in seawater of bacteria with an inhibitory effect against *Vibrio sp*. has been ascribed to Gaixa (1889). Subsequently, Rosenfeld and Zobell studied antibiotic-producing marine microorganisms, and since then research aiming at developing biological control agents has started (Rosenfeld and Zobell 1947). Olsson and colleagues showed that *Thalassobacter utilis* played inhibitory effects against *Vibrio anguillarum* in aquaculture (Olsson et al. 1992).

Austin and coworkers used a probiotic *V. alginolyticus* strain (10^8 cells/ ml) applied through a bath treatment to Atlantic salmon; it was able to reduce mortality after exposures to *A. salmonicida*, and to a lesser extent after exposures to *V. anguillarum* and *V. ordalii* (Austin et al. 1995). In faecal extracts from turbot

juveniles, Olsson and colleagues found that the growth of *V. anguillarum* was inhibited by *Carnobacterium* cells; from this observation, they concluded that the turbot intestinal tract and faeces could serve as an enrichment site for *V. anguillarum*, and that intestinal bacteria with antagonistic activity against vibrios could be used to reduce the load of fish pathogenic vibrios in turbot hatcheries (Olsson et al. 1998). Robertson and colleagues reported that a strain of *Carnobacterium sp*., isolated from the intestine of Atlantic salmon and used at a concentration of 5×10^7 cells/g of feed, was able to control infections caused by *A. salmonicida*, *Vibrio ordalii* and *Yersinia ruckeri* in fry and fingerling salmonids (Robertson et al. 2000). Strains that are generally used as human probiotics (e.g. lactobacilli and enterococci) have been considered for studies in fish as a novel and safe treatment in aquaculture. Administration of *Lactobacillus rhamnosus* to rainbow trout for 51 days reduced the fish mortality caused by *A. salmonicida* from 52.6% (control group) to 18.9 and 46.3% in groups fed with a diet containing 10^9 and 10^{12} cells/g, respectively (Nikoskelainen et al. 2001). Moreover, after challenge with *Edwardsiella tarda*, survival rates of European eels (*Anguilla anguilla* L.) fed with *Enterococcus faecium* were significantly higher than control groups (Chang and Liu 2002).

Irianto and Austin found that *Aeromonas hydrophila, Vibrio fluvialis, Carnobacterium sp*. and an unidentified gram-positive coccus were beneficial for rainbow trout when administered as food additives, since their application reduced significantly the impact of furunculosis in farmed fish by competitive exclusion and enhanced cellular immunity (Irianto and Austin 2002). Sugita and colleagues, studying the antibacterial abilities of intestinal bacteria isolated from juveniles and larvae of Japanese flounder (*Paralichthys olivaceus*), reported that 53.3% of *Vibrio spp*. inhibited the growth of *Pasteurella piscicida* (Sugita et al. 2002). From the stomach and intestine of common clownfish (*Amphiprion percula*), 106 bacterial isolates were recovered (Vine et al. 2004); five of these isolates had inhibitory effects against a wide variety of pathogens such as *A. hydrophila, A. salmonicida, V. harveyi, V. anguillarum, V. damsela* and *V. alginolyticus*.

Studies reported also the presence of antifungal effects of different probiotics. In eels specimens (*Anguilla australis*), a strain of *Aeromonas* isolated from freshwater showed antagonistic activity against *Saprolegnia sp*., suppressing the growth of this opportunistic pathogen (Lategan and Gibson 2003).

Therapy based on probiotics offers a suitable alternative for controlling pathogens thereby overcoming the adverse consequences of antibiotics and chemotherapeutic agents. In aquaculture, probiotics, either in diet or bio-encapsulated, help in achieving natural resistance and high survival of larvae and post-larvae of fishes (Abraham et al. 2007).

Probiotics can be used as immunostimulants by means of specific dietary manipulation. An immunostimulant has been defined as ‘a naturally occurring compound that modulates the immune system by increasing the host’s resistance against diseases that in most circumstances are caused by pathogens’ (Kesarcodi-Watson et al. 2008). Treatment with immunostimulants is primarily aimed at enhancing the innate immune system and therefore as a preventive

measure in aquaculture. Immunostimulants and probiotics can, also, be effective during periods of stress in aquaculture, i.e. during sexual maturation and spawning or transfer to sea cages (Bagni et al. 2005). The non-specific immune system can be stimulated by probiotics. It has been demonstrated that oral administration of *Clostridium butyricum* bacteria to rainbow trout enhanced the resistance of fish to vibriosis, by increasing the phagocytic activity of leucocytes (Sakai et al. 1995). In addition, the administration of a lactic acid bacterium *Lactobacillus rhamnosus* (strain ATCC 53103) at a level of 10^5 colony-forming units (CFU) per gram of feed stimulated the respiratory burst in rainbow trout (*Oncorhynchus mykiss*) (Nikoskelainen et al. 2003).

A lot of immunological studies have been performed in several fish using different probiotics, and their effectiveness in stimulating the teleost immunity both under in vivo and in vitro conditions is noteworthy (Picchietti et al. 2009; Salinas et al. 2008).

Probiotics interact with the immune cells such as mononuclear phagocytic cells (monocytes and macrophages), polymorphonuclear leucocytes (neutrophils), etc., to enhance innate immune responses. Probiotics, in both in vitro and in vivo conditions, actively stimulate the proliferation of B lymphocytes in fish and elevation of immunoglobulin level by probiotics supplementation is reported in many animals including fish (Al-Dohail et al. 2009).

Different lactic acid bacteria (LAB), either in viable or non-viable form, can elevate immunoglobulin level in fish (Panigrahi et al. 2005).

Balcázar and colleagues found a rise in immunoglobulin level in *Salmo trutta* (but not at significant level) following the administration of *Lactococcus lactis* ssp. *lactis*, *Lactobacillus sakei* and *Leuconostoc mesenteroides* (10^6 CFU/g feed) for a period of 2 weeks. Moreover, probiotics are able to stimulate phagocytic activity, to induce the activation of the inflammatory response and to trigger antibody production (Balcázar et al. 2007).

Respiratory burst activity is an important innate defence mechanism of fish. Heat-inactivated *Lactobacillus delbrueckii* subsp. *lactis* and *B. subtilis* (5×10^7 CFU/ml) under in vitro conditions are able to enhance the activity of head kidney leucocytes in gilthead sea bream (*Sparus aurata*).

Lysozyme, one of the important bactericidal enzymes of innate immunity, is an indispensable tool of fish to fight against infectious agents. Probiotics can also enhance the lysozyme level in skin mucosa of fish (Taoka et al. 2006).

In teleosts, complement system plays a key role in adaptive immune responses. Probiotics can enhance natural complement activity of fish (Panigrahi and Azad 2007; Salinas et al. 2008), and dietary treatment with many probiotics is reported to stimulate the piscine complement components (Wang et al. 2008). It is also worth noting that non-viable probiotics can stimulate complement components in fish. Choi and Yoon recorded an increased complement activity in *O. mykiss* from at 4th week of feeding with the heat-inactivated probiotics (Choi and Yoon 2008).

Cytokines are protein mediators produced by immune cells and contribute to cell growth, differentiation and defence mechanisms of the host. Different strains of LAB can induce regulatory and pro-inflammatory cytokines while other probiotics

can increase intestinal inflammatory responses (Picchietti et al. 2009). In gut, probiotics exert a particular role since they are involved in many functions including immunostimulatory activity. Recent studies indicate that probiotics can stimulate the piscine gut immune system with marked increase in the number of Ig+ cells and acidophilic granulocytes (Picchietti et al. 2009; Salinas et al. 2008).

Picchietti and colleagues recorded increased T lymphocytes in gut without any change in CD_4 and $CD_{8\alpha}$ transcript in sea bass (*D. labrax*) after administration of *L. delbrueckii* ssp. *delbruecki*. (Picchietti et al. 2009).

Some bacteria used as candidate probiotics have, also, antiviral effects. Although the exact mechanism is not known, it has been reported that strains of *Pseudomonas sp*., *Vibrio* sp., *Aeromonas* sp. and groups of coryneforms isolated from salmonid hatcheries showed antiviral activity against infectious hematopoietic necrosis virus (IHNV) (Kamei et al. 1988).

The methods to select probiotic bacteria for use in aquaculture include collection of background information, acquisition/selection of potential probiotics, evaluation of the ability of potential probiotics to compete with pathogenic strains, assessment of the pathogenicity of the potential probiotics, evaluation of the effect of the potential probiotics in the host and economic cost/benefit analysis (Gomez-Gil et al. 2000).

Probiotics can be provided to the host or added to its aquatic environment via live food, via baths, via addition to water or via addition to artificial diet. Optimum dose of probiotics depends on the host and it is defined as the concentration required for colonization and subsequent proliferation in the gut and to exert beneficial effects including immunostimulatory activity. In fact, different in vitro and in vivo studies indicate that immune response of fish varies with the concentration of probiotics. The dose of probiotics is usually selected based on their ability to enhance the growth and to protect host. In aquaculture, the dose of probiotics usually varies from 10^6–10^{10} CFU/g feed.

Current regulations regarding the use of additives used in feed products in the European Union have addressed the need to protect human health, animal health and welfare, together with environmental sustainability. Initially, antibiotics were used as growth promoters in animals; subsequently, the ‘White Paper’ on Food Safety and the Regulation (EC) No. 178/2002 of the European Parliament was published, in order to establish a policy of food safety for the European Union, and the European Food Safety Authority (EFSA) was created. EFSA works on all stages of food production and supply (including primary production, animal feed and food for consumers). The authorization, marketing and use of feed additives are currently regulated under Council Directive 70/524/EEC. Before a feed additive may be marketed or used, it must be authorised in accordance with the provisions of the Directive. To obtain authorization, a manufacturer is required to submit a dossier containing data and studies demonstrating the efficacy and safety of the product for animals, consumers and the environment.

4.6 Antioxidants

Other than vitamin E, compounds with antioxidant properties against reactive oxygen species—such as carotenoids—are frequently included as bioactive components in fish diets, especially for the species appreciated for their pigmentation. Dietary supplementation with natural or synthetic carotenoid sources results in the typical colour of fish tissues (Ranjan 2016). Carotenoids (also referred to as tetraterpenoids) are hydrophobic molecules commonly located within cell membranes. Structurally, carotenoids are classified into carotene and xanthophyll. Carotenes (α, β, Y carotene and lycopene) are oxygen-free hydrocarbons, whereas xanthophylls (astaxanthin, canthaxanthin and zeaxanthin) are oxygen-containing hydrocarbons. Animals are unable to synthesize carotenoids, and astaxanthin is commonly used as feed ingredient to improve pigmentation of salmon. Astaxanthin and canthaxanthin are of significant importance for aquaculture feed. Salmonids preferentially absorb astaxanthin rather than canthaxanthin, zeaxanthin or carotenes.

Crustacean by-products have successfully been used as a source of carotenoids, although their high ash and chitin content—which reduce their digestibility—can severely limit the rate of inclusion in dietary formulations for fish. Other feed ingredients such as yellow corn, corn gluten meal and alfalfa are also used as sources of carotenoids in aquafeeds formulation.

Except for xanthophylls esters, carotenoids are absorbed without prior metabolic conversion. Compared to other nutrients, absorption of carotenoids is rather slow; for example, in salmonids, about 35% of dietary astaxanthin is absorbed along the proximal intestine. The intestinal absorption of carotenoids—by enterocyte brush border—involves their preliminary dispersion in lipid emulsions and solubilisation into mixed bile salt micelles. Since carotenoids are hydrophobic compounds, their digestion, absorption and transport processes depend on the presence of lipids; therefore, dietary lipids are considered important cofactors for carotenoid bioavailability. In salmonids, this latter varies with age and physiological status of fish. Carotenoid metabolism takes place in the liver or in the intestine. Only about 5–15% of the dietary carotenoids are utilized for muscle pigmentation: this low percentage is due to a low absorption rate in the gastrointestinal tract, deposition in other organs and metabolic transformation into colourless compounds that may eventually be excreted.

Carotenoids are precursors of vitamin A and play a role in protecting the immune system and increasing fish immunity and resistance to microbial diseases (Amar et al. 2000, 2004; Christiansen et al. 1995). Carotenoids have also been reported to increase the cytotoxic activity of killer cells, reduce tumour growth and promote wound healing (Shahidi and Brown 1998). In the presence of vitamins A, C and E, beta-carotene and astaxanthin supplementation allowed to enhance the defence mechanisms of rainbow trout (Amar et al. 2001).

In juvenile olive flounder (*Paralichthys olivaceus*), Pham and colleagues studied the effects of dietary carotenoids on growth, skin pigmentation, antioxidant

responses and fatty acid composition; dietary inclusion of paprika and the extract of Chlorophycea *Haematococcus pluvialis* at a concentration of approximately 100 mg/kg carotenoid was found to increase antioxidant activities and skin pigmentation of fish as efficiently as commercial synthetic astaxanthin (Pham et al. 2014).

In the European Union, several synthetic antioxidants such as ethoxyquin and butylated hydroxytoluene have been authorised for use as feed additives. Recently, the use of synthetic antioxidants in fish feed ingredients and the problem of maximum residual limits in fresh fillets from a food safety perspective have been studied (Lundebye et al. 2010). In farmed coho salmon, Ortiz and coworkers compared the effects of synthetic antioxidants butylated hydroxytoluene and ethoxyquin to those caused by two diets including natural antioxidants (a tocopherol isomer-rich mixture *versus* a tocopherol isomer–rosemary extract mixture) (Ortiz et al. 2012).

4.7 Conclusions

The aim of the present chapter was to underline how fish health is related with animals' nutrition. However, the formulation of cost-effective and nutritionally adequate aquaculture feeds requires further research, because of the great diversity of cultured fish species together with incomplete understanding of fish physiological responses. Therefore, it is still important to continue research in this field to modulate and adapt the choice of various additives, feeding regimes and supplementation times, to reach optimal production yield.

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