**UNIVERSITÀ DEGLI STUDI DI PADOVA**

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea magistrale in Biologia Marina


**TESI DI LAUREA**

# Effect of short-chain fatty acids inclusion in a plant feedstuffs-based diet on growth, immune response, and disease resistance of European seabass

**Relatore: Prof.ssa Angela Trocino**
**Dipartimento di Biomedicina Comparata e Alimentazione - Padova**

**Correlatore: Dott.ssa Helena Peres**
**Departamento de Biologia - Universidade do Porto**
**CIIMAR**

**Laureando: Filippo Bonin**

**ANNO ACCADEMICO 2021/2022**

# INDEX

**Abstract**

With the increase of the aquaculture sector and the growing demand for a new type of feed, novel aquafeeds start including a high level of plant feedstuffs (PF). However, most PF contains antinutritional factors that may negatively influence nutrient digestion, metabolic utilization and immune-related response.

The aim of this work was to assess the potential role of short-chain fatty acids (SCFAs) as functional ingredients in fish fed with a high content of PF diet on growth performance, immune status, and disease resistance of European seabass, *Dicentrarchus labrax*, one of the most harvested fish species in the Mediterranean area.

European seabass juveniles (initial body weight 15.2 g) were randomly allocated in groups of 25 fish in twenty-one tanks and fed for 56 days with seven isoproteic (44% crude protein) and isolipidic (18% crude lipids) diets supplemented with different SCFAs (SA: sodium acetate, SP: sodium propionate, SB: sodium butyrate) and different percentage supplementation; namely: Control (non-supplemented), SA 0.25% (sodium acetate at 0.25%), SA 0.50%, SP 0.25%, SP 0.50%, SB 0.25%, SB 0.50%. After the growth trial, three fish per tank were randomly sampled to assess the immune status. Six more fish per tank were randomly collected and infected with 100 µl of *Vibrio anguillarum* ($1.2x10^7$ CFU/$ml^{-1}$) and sampled 4- and 24-hours post-injection in order to evaluate blood parameters and innate immune parameters. Addittionally, fifteen fish per tank were infected with *Vibrio anguillarum* for the survival trial and the mortality was recorded for three weeks.

No differences were recorded in the growth performance and feed utilization efficiency. Haematology parameters decreased with time except for mean corpuscular haemoglobin concentration (MCHC), which tended to recover at 24h post-infection. Mean corpuscular volume (MCV) was higher in fish fed SB than SA. Regarding innate immune parameters, lysozyme and peroxidase activity decreased at 4- and 24-hours. Peroxidase activity showed a tendency to recover at 24h in diet SA 0.25% and 50% and SP 0.25%. Lysozyme activity was higher in fish fed SB.

Survival was highest in fish fed SB 0.50%.

In conclusion, SCFAs supplementation doesn't compromise growth performance, feed utilization efficiency, and whole-body composition of European seabass. Furthermore, SCFAs may not enhance the immune response of European seabass injected with *Vibrio anguillarum*. However, the cumulative survival of European seabass was improved in fish fed the sodium butyrate 0.50% supplemented diet.

**Riassunto**

Con l'aumento del settore delle acquacolture e la crescente domanda per un nuovo tipo di mangime, i nuovi mangimi hanno iniziato ad includere un elevato livello di componenti vegetali. Tuttavia, molte componenti vegetali contengono fattori antinutrizionali che possono influenzare negativamente la digestione, il metabolismo e le risposte immunitarie.

Lo scopo di questo studio è stato di valutare il ruolo potenziale degli acidi grassi a corta catena (SCFAs) come ingredienti funzionali in un mangime per pesci con un alto contenuto di componenti vegetali, su performance di crescita, stato immunitario e resistenza ai patogeni del branzino europeo, *Dicentrarchus labrax*, una delle specie maggiormente allevate nella zona mediterranea.

Giovanili di branzino (peso iniziale 15.2 g) sono stati distribuiti casualmente in 21 tank, in gruppi di 25 pesci per tank, e alimentati per 56 giorni con sette diete isoproteiche (44% proteina cruda) e isolipidiche (18% lipidi crudi) integrate con differenti SCFAs (SA: sodio acetato, SP: sodio propionato, SB: sodio butirato) a differenti concentrazioni; rispettivamente: Controllo (non-integrata), SA 0.25% (sodio acetato allo 0.25%), SA 0.50%, SP 0.25%, SP 0.50%, SB 0.25%, SB 0.50%. Alla fine del periodo di alimentazione, tre pesci per tank sono stati campionati casualmente per valutare lo stato immunitario. Altri sei pesci per tank sono stati selezionati casualmente e infettati con 100 µl di *Vibrio anguillarum* ($1.2x10^7$ CFU/$ml^{-1}$) e campionati 4- e 24- ore post-infezione in maniera da valutare i parametri del sangue e i parametri di immunità innata. In più, quindici pesci per tank sono stati infettati con *Vibrio anguillarum* per valutare la sopravvivenza e la mortalità è stata registrata per tre settimane.

Non è stata notata nessuna differenza nella performance di crescita e nell'efficienza di utilizzo del mangime. I parametri ematologici sono calati nel tempo, ad eccezione della concentrazione media di emoglobina corpuscolare (MCHC), la quale ha mostrato una tendenza a recuperare dopo 24 ore post-infezione. Il volume corpuscolare medio (MCV) è risultato maggiore nei pesci alimentati con un mangime integrato con SB piuttosto che in quelli alimentati con un mangime integrato con SA. Riguardo ai parametri di immunità innata, l'attività di lisozima e perossidasi è diminuita a 4 e 24 ore post-infezione. L'attività della perossidasi ha mostrato una tendenza a recuperare dopo 24 ore in pesci alimentati con SA 0.25% e 0.50% e SP 0.25%. L'attività del lisozima è risultata maggiore in pesci alimentati con SB.

La sopravvivenza maggiore è stata registrata nei pesci alimentati con SB 0.25%.

In conclusione, l'integrazione di SCFA non compromette le prestazioni di crescita, l'efficienza di utilizzo del mangime e la composizione corporea del branzino. Inoltre, gli SCFA potrebbero non migliorare la risposta immunitaria della spigola europea a cui è stato iniettato Vibrio anguillarum. Tuttavia, la sopravvivenza del branzino è stata migliorata nei pesci alimentati con la dieta integrata di sodio butirato 0,50%.

**List of abbreviations**

- AA – Acetic Acid/Acetate
- BA – Butyric Acid/Butyrate
- DGI – Daily Growth Index
- DHA – Docosahexaenoic Acid
- EAA – Essential Amino Acids
- EPA – Eicosapentaenoic acid
- EU – European Union
- FAO – Food and Agriculture Organization of the United Nations
- FBW – Fish Body Weight
- FCR – Feed Conversion Ratio
- FE – Feed Efficiency
- FI – Feed Intake
- FOS - Fructooligosaccharides
- LPS - Lipopolysaccharides
- LW – Live Weight
- MOS - Mannanoloigosaccharides
- NSPs – Non-Starch Polysaccharides
- OECD – Organization for Economic Co-operation and Development
- PA- Propionic Acid/Propionate
- SA – Sodium Acetate/Na-acetate
- SB – Sodium Butyrate/Na-butyrate
- SCFAs – Short-Chain Fatty Acids
- SDGs – Sustainable Development Goals
- SGR – Specific Growth Rate
- SP – Sodium Propionate/Na-propionate
- TGC – Thermal Growth Coefficient
- TOS – Transgalactooligosaccharides
- Tt – Thousand tons
- UN – United Nations
- USD – United States Dollar
- USFDA – United States Food and Drug Administration
- WG – Weight Gain

## 1. Introduction

The demand for animal protein is estimated to increase by 52% by 2050 with the human population increase. Globally, fish feed about 3,3 billion people with almost 20% of their average animal protein intake (FAO, 2020).
For centuries, the ocean has provided sustenance for millions of people. Indeed, it's estimated that more than 90% of fish stocks are now fully exploited (FAO, 2020). If this trend is not reversed, the total collapse of all world fisheries should hit around 2048 (Worm et al., 2006). The natural fish stocks are shrinking, and it has become necessary to develop more sustainable solutions in order to continue relying on the oceans as an important food source (National Geographic Society, 2019). Aquaculture stands as a sustainable way to explore marine bioresources (DeWeerdt, 2020).

### 1.1 Aquaculture

Aquaculture contributes to securing global food supplies. Fish aquaculture is the fastest growing animal food sector to support the growing human population (Xiong et al., 2018). Compared to land-farmed animals, aquaculture is one of the most sustainable ways to produce protein (source: UN SDGs), with a high feed conversion ratio and good protein retention (Fry et al., 2018).
In 2018, aquaculture covered 46% of total fish production (including crustaceans, molluscs and algae) (Figure 1), with 82.1 million tons produced, with an increase of 3.2% from 2017 and a total farm gate value of USD 263.6 billion (FAO, 2020).

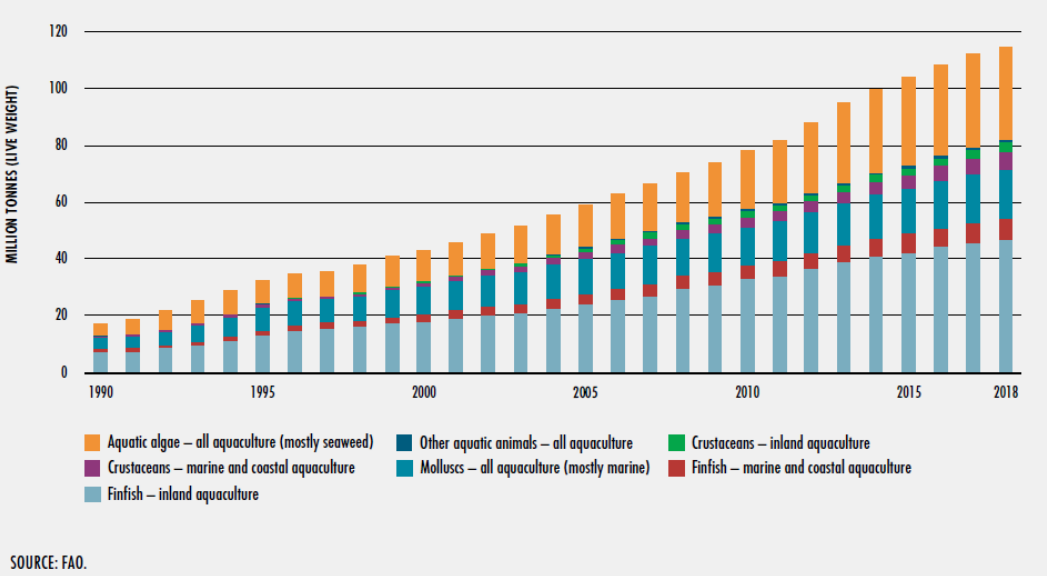


**Figure 1** World aquaculture production of aquatic animals and algae, 1990-2018. Source: FAO.

China is by far the major producer, with more farmed aquatic food than the rest of the world combined in 2018 (47559.1 Tt LW), followed by India (7066.0 Tt), Indonesia (5426.9 Tt), Vietnam (4134.0 Tt) and Bangladesh (2405.4 Tt). In Europe, Norway is the primary producer (1354.9 Tt) followed by Spain (347813.6 tons),

Turkey (311681 tons), France (187098.2 tons) and the United Kingdom (184932 tons) (FAO, 2020).
In 2018, Italy's aquaculture production accounted for 96 thousand kg of fish, crustaceans and molluscs, with a production decrease of 10%, while its value decreased by 15%, between 2008 and 2018. Portugal produced 28 thousand kg of fish, crustaceans and molluscs, with a production decrease by 18%, while its value decreased by 5% (OECD, 2021).
The most produced fish species worldwide are grass carp, *Ctenopharyngodon idellus* (10.5% of the total finfish production), silver carp, *Hypophthalmichthys molitrix* (8.8%), Nile tilapia, *Oreochromis niloticus* (8.3%), common carp, *Cyprinus carpio* (7.7%) and bighead carp, *Hypophthalmichthys nobilis* (5.8%).
European seabass, *Dicentrarchus labrax* was the 31$^{st}$ produced aquaculture species in 2019 (FAO, 2021), with Turkey as the major producer. Total production in 2019 was estimated to be 236.215 tons.

A significant proportion of world fisheries and aquaculture production is processed into fishmeal and fish oil. In 2017, out of a total world fisheries and aquaculture production of 174.0 Mt, 15.6 Mt were used to produce fishmeal and fish oil (Green, 2018). However, a decreasing trend was observed, with a past production for fishmeal and fish oil of 16.3 Mt in 2012, 17.9 Mt in 2009 up to 30 Mt in 1994 (FAO, 2017). Fishmeal is made from various small pelagic fishes with little demand for human consumption such as menhaden, herrings, anchovies and sardines and is obtained after milling and drying of fish and fish parts (Shepherd & Jackson, 2013; Boyd, 2015). Its production fluctuates according to changes in the catches, dominated mainly by the El Niño-Southern Oscillation, and in 2018, it rose by about 18 million tons (FAO, 2020). According to FAO (2020), 25-35% of fishmeal and fish oil is produced from the by-products of fish processing, such as heads, bones and gut.

Fishmeal and fish oil are still considered the most nutritious and digestible ingredients for farmed fish, but as the prices are growing rapidly (1428,6 USD/metric ton in January 2022; source: World Bank) and overfishing of these species it's being not environmentally sustainable, it is becoming necessary to find cheaper solutions that give equally satisfactory results (Oliva-Teles, 2012).

### 1.2 Replace fish meal with alternative feeds

Feeding habits of aquaculture fish produced worldwide in various habitats present substantial differences. Generally, most freshwater fish species are omnivorous or herbivorous and most diadromous and marine species are carnivorous. Carnivorous fish have high protein requirements, with an optimum dietary protein level ranging from 40% to 55% of the diet (NRC, 2011). The optimal level of lipids in the diet varies between 7% and 15%. Especially, essential fatty acids are required, with marine fish naturally needing long-chain polyunsaturated fatty acids (usually EPA and DHA), while freshwater species generally need $C_{18}$ PUFA (as linoleic and α-linoleic acid) (Tocher, 2010). Fish do not require carbohydrates because have a very efficient gluconeogenesis that pertmits them to produce *de novo* glucose from non-carbohydrates substrates. Depending on the fish species and type of carbohydrates, carbohydrates may be a good source of energy. Generally, more than 20% of carbohydrates is not recommended for carnivorous fish, but the threshold is strongly dependent on the spcies (Craig & Helfric, 2017). Commercial feeds produced for carnivorous fish are frequently based on a wide range of protein sources, particularly from two main sources, plants and terrestrial animals, used as fish meal replacements (Glencross et al., 2007).

Most plant-based protein sources have different drawbacks that may limit their incorporation into aquaculture diets. Most of them are deficient in one or more EAA amino acids, with usually lysine and methionine as the first limiting EAA, followed by tryptophan, threonine, arginine and histidine (Oliva-Teles et al., 2015). It follows that there is a need for supplementation of these amino acids to fit different fish species requirements (Jobling et al., 2001). Also, the presence of non-starch polysaccharides (NSPs), like cellulose and hemicellulose, reduce energy bioavailability due to the deficiency or the absence of NSPs digestive enzymes, such as β-glucanases and β-xylanases (Kuz'mina, 1996; Sinha et al., 2011). Moreover, plant feedstuffs present several other anti-nutritional factors, such as tannins, lectins and phytic acid, if not destroyed or inactivated (Kumar et al., 2011), that limit their use in aquaculture due to their potential deleterious effect on animals (Gatlin III et al, 2007; Gemede & Ratta, 2014; Oliva-Teles et al., 2015).

One of the most available plant protein sources is soybean meal because of its reasonably balanced amino acid profile, stable composition, supply throughout the year and a reasonable price compared to fishmeal (Hernández et al., 2007). Despite this, soybean has a lower content of EAA than fishmeal, especially lysine, methionine, threonine and tyrosine (Gatlin III et al., 2007). Other plant-based ingredients that can be used as a possible replacement of fishmeal are rapeseed (Gómez-Requeni et al., 2004), peas (Allan et al., 2000; Allan & Booth, 2004), wheat (Guttieri et al., 2004; Tusche et al., 2012), corn gluten (Fournier et al., 2004; Lu et al., 2015) and carobs (Couto et al., 2016).

Algae are also being more and more considered as alternatives. Algae generally contain all the essential amino acids (Fleurence, 1999), and are rich in n-3 PUFA (Wahbeh, 1997) and antioxidants (Yuan & Walsh, 2006). Macroalgae have been widely investigated (Evans & Critchley, 2014), containing varying levels of nutrients, depending on various factors such as species, harvest season and

environmental conditions (Jensen, 1993). Moreover, marine macroalgae are rich in bioactive compounds that can be used to produce a variety of second metabolites (Gupta & Abu-Ghannam, 2011). Problems of using macroalgae in fish feed include the high content of non-digestible polysaccharide components such as alginates and carrageenans, which reduce their nutritionally available energy content. Microalgae are also rich in essential amino acids and PUFA, and are generally recognized as a good source of vitamins. Still, the cost of production is very high (Heimaiswarya et al., 2011).

After a long ban by the EU, in 2013, most of the terrestrial animal by-products were partially re-allowed to be used in animal feeds (EU Regulation n. 294/2013). These animal proteins, such as poultry meal, blood meal, and feather meal, can be used in relatively high levels in aquafeeds (Wang et al., 2006). These sources have high protein levels and relatively balanced amino acid profiles (El-Sayed, 1998).

In the end, insects are also considered as promising fishmeal alternatives (Gasco et al., 2020; Tran et al., 2022; Bordignon et al., 2022). Insects are a natural food source for some fish species, being rich in amino acids, lipids, vitamins and minerals (Henry et al., 2015). On the 1$^{st}$ of July 2017, the EU Regulation 2017/893 was released, authorizing the use of insect proteins as fish feed, but only the ones derived from a few species.

All these alternatives are very promising but often need specific functional ingredients to overcome the problem of the antinutritional factors.

### 1.3 Functional ingredients

Functional ingredients are feeds with dietary ingredients targeting specific functions or product characteristics that can be used in the manufacture of functional food products (Gabaudan, 2013). Certain additives aim for the feed quality, such as pellet binders, antioxidants and feed preservatives, others are enzymes used to improve the bioavailability of certain nutrients or to reduce the antinutrients effects (e.g., phytase) and others are used to improve the animal's performance and health (Encarnação, 2016).
Different options are available specifically to manage and regulate fish gut microbiota, such as probiotics, prebiotics and phytogenic substances (Montalban-Arques et al., 2015; Encarnação, 2016).
Some functional ingredients as prebiotics seems to be an available alternative to antibiotics to promote animal health and well-being (Ringø et al., 2010; Carbone & Faggio, 2016), usually associated with increased survival against bacterial infections and extreme environmental conditions (Safari et al., 2014; Selim & Reda, 2015; Azimirad et al., 2016).
Moreover, some functional ingredients positively modulate the gut microbiota (Nayak, 2010). Gut microbiota is known to be very important in the absorption and metabolism of feed (Turnbaugh & Gordon, 2009; Ridaura et al., 2013), regulating gut motility (Backhed et al., 2004) and intestinal barrier homeostasis (Wayman, 2016). The commensal bacteria contribute to the health of animals because of the role of bacterial metabolic end-products of short-chain fatty acids from the fermentation of dietary fiber, principally acetate, propionate and butyrate (Palmer & Rolls, 1983) that plays a crucial role in regulating energy homeostasis and other physiological drives (Hu et al., 2018) and influence the activity of digestive enzymes (Chen et al., 2022).

#### 1.3.1 Probiotics

Probiotics are live microbial feed supplements that beneficially affect the host animal by improving intestinal balance (Fuller, 1989).
Probiotics control gut microbiota and help restore the microbiota that has been disturbed to a beneficial composition. Numerous probiotic strains enhance growth performance by acting on rising digestive enzyme activity and feed utilization (Wang, 2006; Wang, 2007; Szer et al., 2008). Also, probiotics control the emergence of opportunistic pathogens by competing for nutrients, space, and attachment sites in the gut (Merrifield et al., 2010). There are different mechanisms with which probiotics act, such as the production of inhibitory compounds against pathogens as lactoferrin (Vega-Bautista et al., 2019) and lysozyme (Leśnierowski & Cegielska-Radziejewska, 2012), competition for essential nutrients and adhesion sites, modulation of interactions with the environment and the development of beneficial immune response (Merrifield et al., 2010).
It is improbable to find a probiotic bacteria that meet all of these characteristics (Ciorba, 2012), but with the combined application of multiple favourable probiotic

it may be possible to have more significant benefits than the individual application (Wang et al., 2008; Merrifield et al., 2010).
Probiotics must be viable at their site of action, surviving the stressful condition of feed processing. One of the major challenges is the high temperature of feeds are submitted during the extrusion process (Castex et al., 2014).

### 1.3.2 Prebiotics

Prebiotics are selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota, that confers benefits upon host wellbeing and health. (Gibson et al., 2004). Prebiotics are fermented by bacteria present in the host gut, such as *Bifidobacterium* and *Lactobacillus*, which produce short-chain fatty acids (SCFAs), which cause a decline in the gut pH, resulting in colonic and systemic health effects (Guerreiro et al., 2018). They must have against gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption and must be fermented by gut bacteria, which are associated with health and well-being (Gibson et al., 2004). Among prebiotics, there are several oligosaccharides, including inulin, TOS, lactulose, MOS, FOS, and more.
In fish, prebiotics leads to several benefits, such as improved growth performance, feed utilization, carcass composition, health status, disease resistance, gut morphology, oxidative stress, and microbiota modulation (Merrifield et al., 2010; Ringø et al., 2010; Dimitroglou et al., 2011; Hoseinifar et al., 2020; Juárez et al., 2021).
The effect of prebiotics may depend on several factors, such as prebiotic source, supplementation level, fish species, development stage, rearing conditions, and diet composition (Guerreiro et al., 2018). For this reason, despite most of the studies reporting positive effects on growth, others reported a lack of effects (Hoseinifar et al., 2014; Guerreiro et al., 2015; Guerreiro et al., 2017).
Diets enriched with prebiotics seem to have a beneficial effect on fish gut morphology, health, and physiology by increasing gut absorptive area, density, and height of microvilli, and villi structure complexity, increasing resistance to diseases by improving mucosal epithelium (Anguiano et al., 2013), and by incrementing digestive enzyme activities, like proteases, lipases, and amylases (Soleimani et al., 2012; Eshaghzadeh et al., 2015). Probiotics have also been reported to increase liver enzyme activities as catalase and glutathione peroxidase (Zhang et al., 2013).
Moreover, prebiotics activates the fish innate immune system by directly stimulating it or enhancing commercial microbiota growth (Song et al., 2014). Generally, prebiotics enhances fish's immune status, increasing immune parameters such as white blood cells, lysozyme activity, complement cells, and immunoglobulins (Soleimani et al., 2012; Akrami et al., 2013; Guerreiro et al., 2016).

### 1.3.3 Immune stimulants

Immune stimulants are chemical compounds that activate white blood cell which may increase animal resistance to infection (Raa, 2000). Immune stimulants could have different chemical nature, such as structural elements of bacteria, like nucleotides from the cell wall of baker's yeast (Raa, 1996).
Yeast β-glucans have been applied in aquaculture as immune-stimulants to modulate the innate immune system of fish and shrimp and improve their survival (Welker et al., 2012; Meena et al., 2013; Bai et al., 2014).
β-glucans and MOS can prevent intestinal cloning by removing bacteria such as *Salmonella dublin*, enhancing growth performance (Dimitroglou et al., 2010) and stimulating the immune system, i.e., by improving gut mucus and lysozyme activity (Torrecillas et al., 2011).
Bacterial LPS can either enhance or suppress immunity by affecting T cells, B cells, macrophages, and other immune components in different animal species (Swain et al., 2008).

### 1.3.4 Phytogenic substances

Phytogenic substances are plant-derived substances with benefits on animal performance and well-being (Menanteanu-Ledouble et al., 2015). These active plant ingredients can exercise multiple effects on the animal, such as stimulation of appetite, antimicrobial action, direct reduction of gut bacteria, stimulation of gastric juices, enhancement of the immune system, and anti-inflammatory and antioxidant properties (Windisch et al., 2008; Chakraborty & Hancz, 2011; Chakraborty et al., 2014; Reverter et al., 2014). The most commonly used phytogenic substances are essential oils and extracts of aromatic plants, containing plant's active compounds as secondary metabolites, including terpenoids, phenol-derived aromatic components, and aliphatic components (Chakraborty et al., 2014).
For example, oregano's essential oil has strong antibacterial and antioxidative properties (Burt, 2004; Baser, 2008), garlic has a good effect on bacterial disease control and immunostimulation (Nya et al., 2010) and rosemary has high antioxidant properties (Abutbul et al., 2004).

Among gut microbiota beneficial effects modulate by functional ingredients, the production of short-chain fatty acids is known having beneficial effects on fish (Ng & Koh, 2017).

## 1.4 Short-chain fatty acids

### 1.4.1 General characteristics

Short-chain fatty acids are the end products of microbial anaerobic metabolism in aquatic animals, as in other animals. Microbial metabolism converts indigestible dietary carbohydrates into SCFAs through carbohydrate fermentation at gut level (Piazzon et al., 2017). The most abundant SCFAs in the gut of fish are acetate, propionate and butyrate (Hao et al., 2017), followed by valerate, isobutyrate and isovalerate (those three accounting for only 2-9% of the total SCFAs production) (German, 2009; Hao et al., 2017). The concentration of SCFAs increases in the distal intestine of fish (Leenhouwers et al., 2007). The highest concentration of SCFAs was found in the posterior region of the gut (Clements et al., 1994), where the absorption happens. The concentrations of SCFAs in terms of molar ratio are different among fish species and gut regions within a species, usually presented by the ratio acetate:propionate:butyrate:valerate, in herring cale, *Odax cyanomelas*, the ratio is 83:8:9:1 (Clements et al., 2014). SCFAs are used in diverse biosynthetic processes by the host (den Besten et al., 2013). They are mainly used in the proximity of the gut, but a small proportion of acetate and propionate reaches the liver from the colon capillaries and through the portal vein, where is used as substrates for the Krebs cycle and gluconeogenesis (Tan et al., 2014; Ohira et al., 2017), with acetate also used as energy source for skeletal muscles and lipid synthesis in fish (Asaduzzaman et al., 2018)

In mammals, acetate is produced from pyruvate through acetyl-CoA, Wood-Ljungdahl path or arabinoxylan by *Bifidobacterium* spp. (Louis et al., 2014; Reichardt et al., 2018). Propionate is produced from lactate through the acrylate pathway, succinate through the succinate pathway, or deoxyhexose sugars through the propanediol pathway (Louis et al., 2014; Reichardt et al., 2018). Butyrate is synthesized from butyryl-CoA (Louis et al., 2014; Reichardt et al., 2018), or a combination of lactate and acetate (Flint et al., 2015). The synthesis of SCFAs depends on the substrate composition, gut microbial composition (i.e., *Lactobacillus* spp., *Clostridium* spp.) (Flint et al., 2015; Ríos-Covián et al., 2016), environmental conditions (Louis et al., 2014) and fish species, with herbivorous freshwater species having lower SCFAs concentration than herbivorous saltwater species (Mountfort et al., 2002; Hao et al., 2017). Carnivorous species have been reported to have a relatively higher concentration than herbivorous and omnivorous species (Clements et al., 2014; Hao et al., 2017). The main substrates to SCFAs production are indigestible polysaccharides divided into three categories: starch, such as amylose and amylopectin; starch-like, like glycogen, and non-starch polysaccharides, which include cellulose and pectins (Tan et al., 2014).

### 1.4.2 Short-chain fatty acids in aquaculture

SCFAs and their salts are “Generally Recognized as Safe” (source: USFDA) and are often used as growth promoters and antimicrobials in the livestock feed industry (Hoseinifar et al., 2017).

In aquaculture, SCFAs have demonstrated efficacy in growth performance and bioavailability of minerals, as well as improvement in host’s immune status, disease resistance, intestinal structure and function, and gut microbiota composition (Tran et al., 2020).

Solid acids and their salts can be directly added to the diet through specific pre-mixtures available on the market, while blended and liquid acids can be sprayed onto the diet (Mustafa & Al-Faragi, 2022).

#### Butyrate and Na-butyrate

Butyrate (BA) and sodium butyrate (SB) have received augmented attention because of their beneficial effect on the gut, growth promotion, immunostimulants effect and antioxidant properties (Abdel-Latif et al., 2020). Table 1 provides a general overview on the use of BA and SB supplemented in feed in aquaculture. Dietary BA studied on African sharptooth catfish (*Clarias gariepinus*) and Nile tilapia (*Oreochromis niloticus*) fingerlings (Omosowone et al., 2018) showed promising results in improving the growth, carcass protein content and nutrient utilization while only Nile tilapia fingerlings fed with a diet containing 1.5 g BA/kg showed a significant improvement on survivability. In a study conducted on barramundi (*Lates calcarifer*) (Aalamifar et al., 2020), fish fed with 5 and 10 g BA/kg showed an improvement in growth, total alkaline protease and lipase activity, hepatic superoxide dismutase activity and serum total protein concentration, 5 g BA/kg increased red blood analyser and serum haemolytic activity while 10 g BA/kg improved the serum lysozyme activity. A group fed with 2.5 g BA/kg enhanced hepatic catalase activity and serum haemolytic activity. All the groups feed with diets containing BA improved the fillet protein content, and red blood cells, and white blood cells count. Sustained-release microencapsulated sodium butyrate studied on common carp (*Cyprinus carpio*) pre-fed with oxidized soybean oil (Liu et al., 2014) highlighted improvement on weight gain and immune response, but reduced feed conversion rate and had no effects on organ indices, intestinal mucosal morphology and gut-adhesive bacterial population. Moreover, Owen et al. (2006) observed no impact on weight gain, specific growth rate and feed conversion ratio of African sharptooth catfish (*Clarias gariepinus*) fed with Na-butyrate supplemented diet. SB also seemed to alter the bacterial community

in the hindgut. Rimoldi et al. (2016) reported that European seabass (*Dicentrarchus labrax*) feed a high percentage of soybean meal supplemented with SB showed the up-regulation of TNF-α and normalize the inflammatory reaction in the distal intestine. Dietary SB in rainbow trout (*Oncorhynchus mykiss*) (Mirghaed et al., 2019) highlighted an increase in intestinal lysozyme, complement, and bactericidal activities. *da* Silva et al. (2016) stated that dietary SB supplementation improved the growth performance and feed efficiency of whiteleg shrimp (*Litopenaeus vannamei*), increased serum agglutination tire and positively altered the intestinal microbiota, and had no effect on phosphorous retention.

**Acetate and Na-acetate**

Table 2 provides a general overview on the use of acetate (AA) and Na-acetate (SA) integrated in feed in aquaculture. AA and SA have received less attention than the other SCFAs (Hoseinifar, 2017). In a study conducted on rainbow trout (*Oncorhynchus mykiss*) (Sugiura et al., 2006), dietary supplementation with 50 g AA/kg enhanced the phosphorous digestibility and decreased stomach pH, but without effects on endogenous acid secretion and intestinal pH. Dietary acetate studied on Siberian sturgeon (*Acipenser baerii*) (Zare et al., 2021) showed improvement in final body weight, weight gain and specific growth rate, high lysozyme activity and alternative complement. A suitable DHA/EPA ratio, which balances with fatty acids is important to improve immune function by increasing the related enzymes. Zhang et al. (2020) reported that dietary SA studied in zebrafish (*Danio rerio*) leads to increased body fat mass, energy gain, energy conversion efficiency, higher weight gain, and daily feeding rate. However, only fish fed with 0.15% SA showed an increased level of intestinal acetate. Yellowfin seabream (*Acanthopagrus latus*) did not have relevant growth-promoting effects and feed utilization whit a diet comprehending SA (Sangari et al., 2021).

**Propionate and Na-propionate**

Table 3 provides a general overview of the use of propionic acid (PA) and Na-propionate (SP) integrated in feed in aquaculture. Pourmozaffar et al. (2017) investigated propionate-supplemented diets at different rates in whiteleg shrimp (*Litopenaeus vannamei*), showing no enhancement in growth performance, but increased expression of immune-related prophenoloxydase, lysozyme, penaeidin-3α and crustin genes in the hepatopancreas. Furthermore, propionate studied in Nile tilapia (*Oreochromis niloticus*) (El-Adawy et al., 2018) enhanced the antibacterial effect of oxytetracycline and generally improved the immune system

and resistance against pathogens. In one of the first studies of SP (Ringø et al., 1991), arctic char (*Salvelinus alpinus*) fed with 10 g SP/kg led to the depressed growth of fish. Safari et al. (2016) highlighted increased expression TNF-α, IL-1β and lysozyme genes in the intestine and decreased expression of SOD, CAT and HSP70 genes in the liver of zebrafish (*Danio rerio*) fed with diets added whit SP.

**Table 1** Use of butyrate and sodium butyrate incorporated in feed in aquaculture. This table is adapted from Ng & Koh, 2017; Abdel-Latif et al., 2020; Tran et al., 2020.

| Organic acids/salts | Species | Initial live weight (g) | Dose (g/kg diets) | Effect | Reference |
|---|---|---|---|---|---|
| Butyrate | African sharptooth catfish, *Clarias gariepinus* | 42.39 ± 1.17 | 0.5, 1, 1.5 and 2 | • Improved growth of over 600%<br>• ↑ carcass protein and ↑ nutrient utilization<br>• No significant effects in survivability | Omosowone et al., 2018 |
| Butyrate | Nile tilapia, *Oreochromis niloticus* | 25.5 ± 0.5 | 0.5, 1, 1.5 and 2 | • Improved FBW, WG, SGR, FCR, FI of over 400%<br>• ↑ carcass protein and ↑ nutrient utilization | Omosowone et al., 2018 |
| Butyrate | Barramundi, *Lates calcarifer* | 12 ± 0.2 | 2.5, 5, 10 | • 5 and 10 g BA/ kg diet: ↑ growth, total alkaline protease, and lipase activities<br>• ↑ fillet protein content<br>• ↑ hepatic CAT activity at 2.5 g BA/kg diet<br>• ↑ hepatic SOD activity and serum TP concentration at 5 and 10 g BA/kg diet<br>• ↑ serum LYZ activity at 10 g BA/kg diet<br>• ↑ RBA in the fish group fed 5 g BA/kg feed | Aalamifar et al., 2020 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | • ↑ serum haemolytic activity in 2.5 or 5 g BA/kg diet<br>• ↑ RBCs and WBCs count in BA diets | |
| **Organic acids/salts** | **Species** | **In. weight (g)** | **Dose (g x kg$^{-1}$)** | **Effect** | **Reference** |
| Na-butyrate (microencapsulated) | Common carp, *Cyprinus carpio* | 6.22 ± 0 | 0 and 0.3 | • Improved weight gain<br>• Reduced FCR<br>• No effects on organ indices and intestinal mucosal morphology<br>• No effects on adhesive bacterial population<br>• Improved immune response | Liu et al., 2014 |
| Na-butyrate | Whiteleg shrimp, *Litopenaeus vannamei* | 2.53 ± 0.03 | 0, 5, 10 and 20 | • Improved growth performance and feed efficiency<br>• No effect on P retention<br>• Altered intestinal microbiota<br>• Increased serum agglutination titre | *da* Silva et al., 2016 |
| Na-butyrate | African sharptooth catfish, *Clairas gariepinus* | 64 ± 1.4 | 0, 2 and 20 | • No effects on weight gain, SGR and FCR<br>• Tended to alter the bacterial community in the hindgut of catfish | Owen et al., 2006 |
| Na-butyrate | European seabass, *Dicentrarchus labrax* | 514 ± 67.4 | 0.2% | • Up-regulation of TNF-α<br>• Normalize the inflammatory reaction in the distal intestine caused by the high SBM diet | Rimoldi et al., 2016 |

| Organic acids/salts | Species | In. weight (g) | Dose (g x $kg^{-1}$) | Effect | Reference |
|---|---|---|---|---|---|
| Na-butyrate | Rainbow trout, *Oncorhynchus mykiss* | 34.97 ± 1.87 | 1.5, 2.5 and 5 | • ↑ intestinal LYZ, complement, and bactericidal activities<br>• Overexpression of LYZ, IL-1b, IL-10, TNFa, TGFb, antioxidant enzymes (e.g., SOD, CAT, and GSH-Px) and Tj protein (e.g., claudin-4, occludin and ZO-1)<br>• After a challenge with *Streptococcus iniae*, fish fed 2.5 and 5 g SB/kg diet had significantly higher survivability<br>• 5 g SB/kg is recommended to reduce fish mortality | Mirghaed et al., 2019 |

**Table 2** Use of acetate and sodium acetate incorporated in feed in aquaculture. This table is adapted from Ng & Koh, 2017; Tran et al., 2020.

| Organic acids/salts | Species | In. weight (g) | Dose ($g \times kg^{-1}$) | Effect | Reference |
|---|---|---|---|---|---|
| Acetate | Rainbow trout, *Oncorhynchus mykiss* | 234.4 ± 24.2 | 0 and 50 | • Enhanced P digestibility<br>• No effects on endogenous acid secretion<br>• Tended to decrease stomach pH, but no effect on intestinal pH | Sugiura et al., 2006 |
| Acetate | Siberian sturgeon, *Acipenser baerii* | 54.85 ± 0.36 | 0 and 2% | • Improvement of FBW, WG and SGR<br>• High activity of lysozyme and alternative complement<br>• Significant modification of muscle n3/n6 ratios | Zare et al., 2021 |
| Na-acetate | Zebrafish, *Danio rerio* | 66 ± 0.7 mg | 0%, 0.05%, 0.1%, 0.15% and 0.2% | • Increased level of intestinal acetate in 0,15% Na-acetate fish fed<br>• Promotion of body fat mass, energy gain and energy conversion efficiency<br>• Higher weight gain and daily feeding rate | Zhang et al., 2020 |
| Na-acetate | Yellowfin seabream, *Acanthopagrus latus* | ~6.5 | 0, 5 and 10 | • Not relevant growth-promoting effect and feed utilization | Sangari et al., 2021 |

**Table 3** Use of propionate and sodium propionate incorporated in feed in aquaculture. This table is adapted from Ng & Koh, 2017; Tran et al., 2020.

| Organic acids/salts | Species | In. weight (g) | Dose ($g \times kg^{-1}$) | Effect | Reference |
|---|---|---|---|---|---|
| Propionate | Whiteleg shrimp, *Litopenaeus vannamei* | 10.2 ± 0.04 | 0, 0,5%, 1%, 2% and 4% | • Not enhance the growth performance<br>• Increased the expression of prophenoloxydase, lysozyme, penaeidin-3α and crustin genes in the hepatopancreas | Pourmozaffar et al., 2017 |
| Propionate | Nile tilapia, *Oreochromis niloticus* | 52 ± 3.75 | 0 and 0,2 | • Enhanced effect of antibacterial effect of OTC<br>• Improved immune system and resistance against pathogens | El-Adawy et al., 2018 |
| Na-propionate | Arctic char, *Salvelinus alpinus* | ~310 | 0 and 10 | • Depressed growth of fish fed | Ringø, 1991 |
| Na-propionate | Zebrafish, *Danio rerio* | 0.42 ± 0.6 | 0, 5, 10 and 20 | • Increased the expression of TNF-α, IL-1β and lysozyme genes in the intestine<br>• Decreased expression of SOD, CAT and HSP70 genes in the liver of the fish | Safari et al., 2016 |

### 1.5. European seabass (*Dicentrarchus labrax*, Linnaeus, 1758)

#### 1.5.1 Biology

European seabass (Figure 2) is a marine teleost belonging to the Moronidae family. It has an elongated body with 8 to 10 dorsal spines, 12 to 13 dorsal soft rays, 3 anal spines and 10 to 12 anal soft rays. The posterior edge of the operculum is finely serrated, and the lower part possesses strong denticles directed forward. It has 2 flat opercula spines, and the mouth is moderately protractile. Vomerine teeth are present anteriorly in a crescent band (Fisher et al., 1987). It is a coastal marine fish that lives in shallow waters (<100 m) (Lloris, 2002) from the north-east Atlantic to the Mediterranean and Black Sea (Kottelat & Freyhof, 2007). It is a euryhaline (0-40 ‰ salinity) and eurythermal (2-32° C) species that enter coastal waters and river mouths in summer, but migrates offshore in colder weather and occur in deep water during winter in the northern range. Young fish form school, but adults appear less gregarious (Frimodt, 1995).

**Figure 2** European seabass. Source: The Fish Society.

Gonochoric species, show group spawning with pelagic eggs. Breeding occurs only once per year, mostly in winter (December to March) for the Mediterranean population and up to summer (June) for the Atlantic population (Kousolaki et al., 2015). In the Mediterranean area, first sexual maturity generally occurs between 2 and 4 years of age, while in the Atlantic area, sexual maturity happens later (males between 4-7 years and females between 5-8 years) (source: Fishbase).

### 1.5.2 Seabass aquaculture

The traditional extensive method sets special barriers in proper lagoon sites to capture fish during autumn migration to the open sea. Those barriers stay open from February until May for the lagoon to be naturally stocked with fry. In this system, seabass is usually cultured in polyculture with seabream, mullets and eels. Seabass reaches a commercial size of 400-500 g in 37 months, with a total lagoon production of 50-150 kg/ha/yr (FAO, 2022). Figure 3 provides a glance at this method.

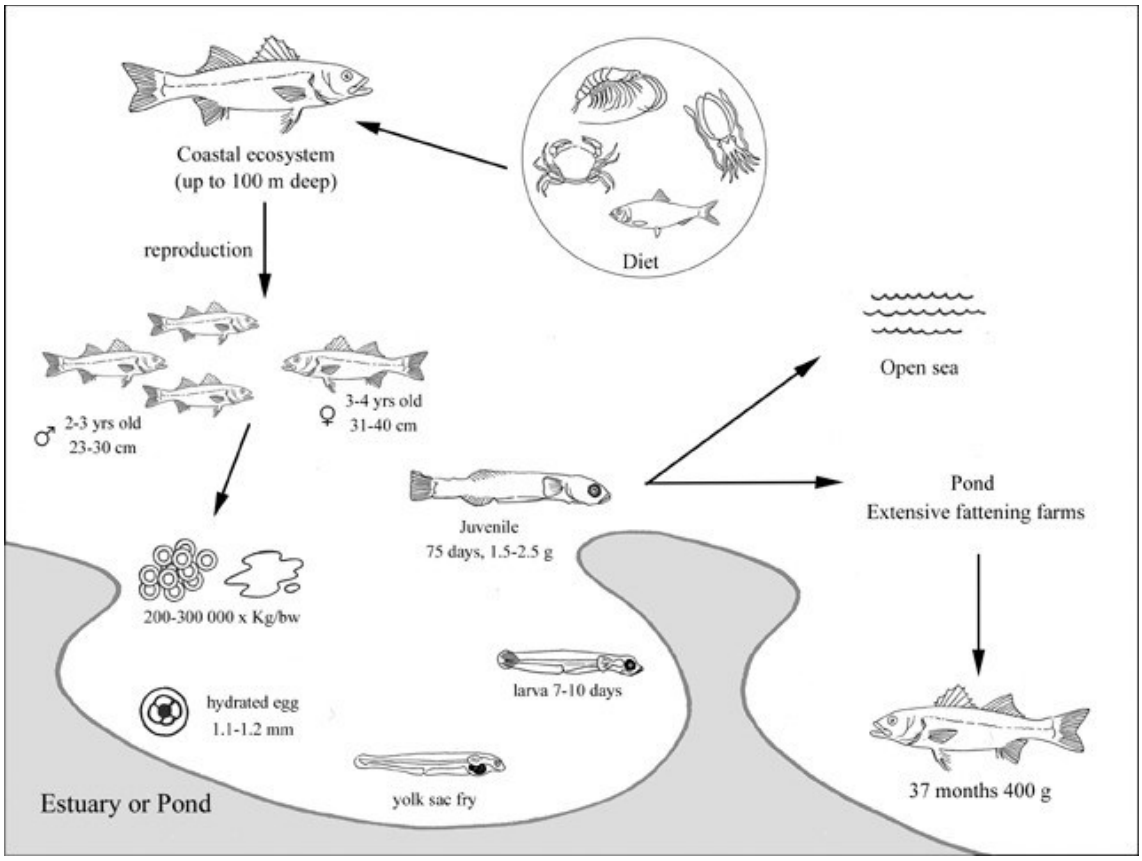


**Figure 3** Seabass extensive production cycle. Source: FAO, 2022.

Intensive seabass aquaculture is mostly located in the Mediterranean area and essentially consists in two phases: firstly, a hatchery-prefattening phase, with the fish reaching a weight from 1 to 20 g in 3-8 months, and secondly a fattening phase until the reaching of the market size which is typically from 240 to 450 g, in 12-20 months. Hatcheries are typically inland, in temperature-controlled systems, whereas the bulk of the ongrowing is in sea cages in natural waters (Vandeputte et al., 2019). Figure 4 provides a glance of this method.

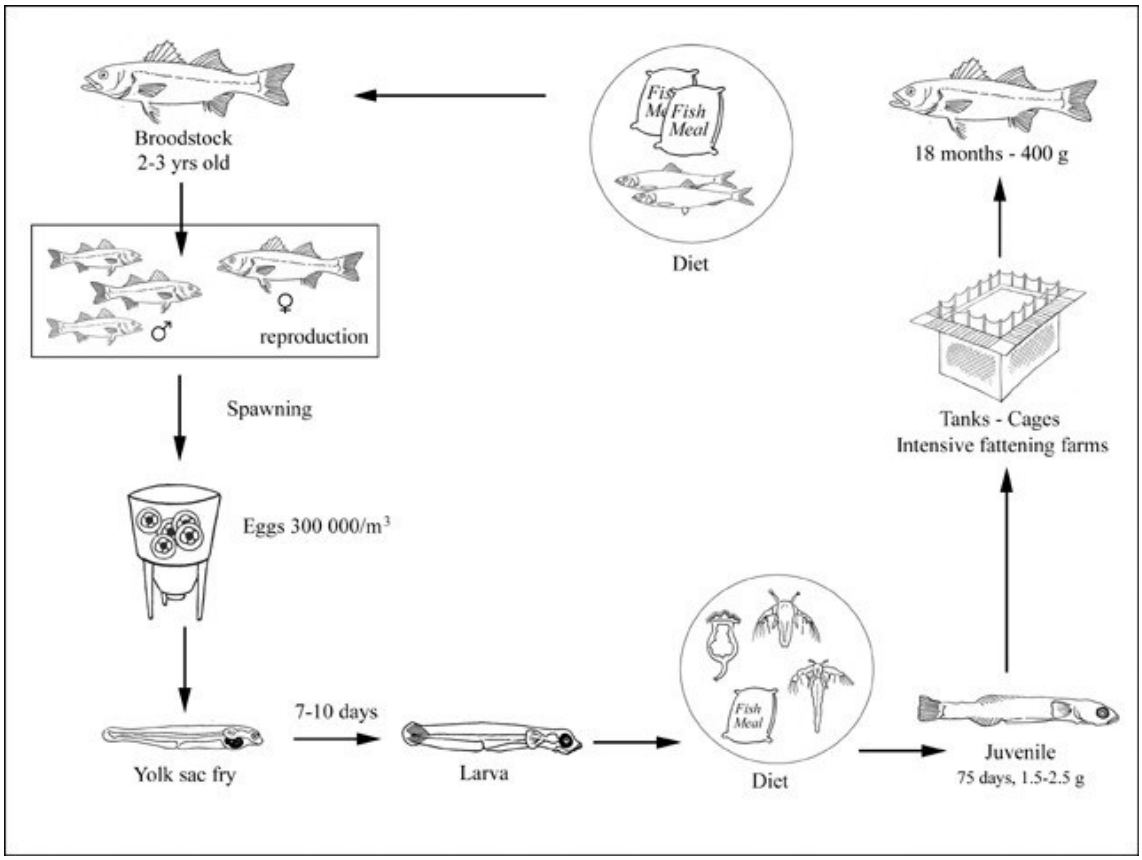


**Figure 4** Seabass intensive production cycle. Source: FAO, 2022.

Most hatcheries have settled their own broodstock units to secure a constant supply of good quality fish eggs. Broodstock ages varies between 5 and 8 years for females, and between 2 and 4 years for males. Sexual maturity could be reached naturally or through the induction of ovulation by photoperiod manipulation or hormonal treatments. During the spawning season, breeders were transferred into spawning tanks, in order to better monitor the performance. The male:female ratio is maintained at 2:1. Males are selected when they start releasing sperm spontaneously or by stripping, while female maturation is assessed when extracted oocytes from the ovary reach the late-vitellogenic stage (FAO, 2022).

Before the harvesting, fish are generally fasted for several days and then harvested with dipnets or vacuum pumps in very high densities (70-100 kg/$m^3$), before killing by asphyxiation in chilled water. Fish are therefore slaughtered as soon as possible (FAO, 2022).

## 2. Objectives of the study

European seabass is one of the most farmed fish Mediterranean area. With the increasing of acquaculture sector it's becoming necessary to find new types of feeds. Consequentially, alternative sources as plant-feedstuff are becoming more and more studied in novel aquafeeds. However, functional ingredients as short-chain fatty acids are necessary to counteract the effect of PF antinutricional factors.

The present study evaluated the effect of a plant-feedstuff supplemented with different short-chain fatty acids (sodium acetate, sodium propionate and sodium butyrate) in different concentrations (0.25% and 0.50%) on growth performance, immune status, and disease resistance on European seabass.

In a second step, fish were infected with the bacteria *Vibrio anguillarum*, in order to evaluate blood parameters and immune innate parameters after 4- and 24-hours post-infection.

This study was carried out during the Erasmus+ Traineeship in Porto, Portugal. Growth trial was conducted at CIIMAR- Centro Interdisciplinar de Investigação Marinha e Ambiental in Matosinhos, Portugal. Analysis was carried out at CIIMAR and at FCUP - Faculdade de Ciências da Universidade do Porto in Porto, Portugal.

This study was approved by the ORBEA Animal Welfare Committee of CIIMAR (ORBEA; reference ORBEA_CIIMAR_30_2019) in compliance with the European Union directive 2010/63/EU and the Portuguese Law (DL 113/2013) and conducted by certified scientists (Functions a, b, c & d defined in article 23 of European Union Directive 2010/63).

## 3. Materials and methods

### 3.1 Experimental diets

Seven experimental diets were formulated to be isoproteic (43% crude protein) and isolipidic (18% crude lipid), containing a mixture of plant feedstuff and fish meal as the protein sources (corresponding to 75% and 25% of total dietary protein content, respectively). A mixture of fish oil and rapeseed oil (50:50) was used as the main lipid source. All the diets had the same composition, differing only in the type and concentration of SCFAs; namely: Control (non-supplemented), SA 0.25% (sodium acetate at 0.25%), SA 0.50%, SP 0.25%, SP 0.50%, SB 0.25%, SB 0.50%. SCFAs were included in the diets at a percentage concentration of 0,25% and 0,50%. All the ingredients were mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA), through a 3 mm diet. Pellets were then dried in an oven at 50°C for 48 hours, and stored in the refrigerator until used. The ingredients and proximate composition of the experimental diets are presented in Table 4.

**Table 4** Ingredient and proximate composition (% dry matter) of the experimental diets

| | CONTROL | SA 0.25 | SA 0.50 | SP 0.25 | SP 0.50 | SB 0.25 | SB 0.50 |
|---|---|---|---|---|---|---|---|
| Fish meal[1] | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| CPSP[2] | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Soybean meal[3] | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Corn gluten[4] | 17.5 | 17.6 | 17.6 | 17.6 | 17.6 | 17.6 | 17.6 |
| Wheat gluten[5] | 5.98 | 5.98 | 5.98 | 5.98 | 5.98 | 5.98 | 5.98 |
| Rapeseed[6] | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 | 5.00 |
| Wheat meal[7] | 17.6 | 17.3 | 17.0 | 17.3 | 17.0 | 17.3 | 17.0 |
| Fish oil | 7.13 | 7.13 | 7.13 | 7.13 | 7.13 | 7.13 | 7.13 |
| Rapeseed oil | 7.13 | 7.13 | 7.13 | 7.13 | 7.13 | 7.13 | 7.13 |
| Vitamin premix[8] | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Choline chloride (50%)[9] | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Mineral premix[10] | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| Binder[11] | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Dicalcium phosphate | 0.67 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 | 0.68 |
| Methionine[12] | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |
| Taurine[13] | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 | 0.30 |
| SA 0,25% | - | 0.25 | - | - | - | - | - |
| SA 0,50% | - | - | 0.50 | - | - | - | - |
| SP 0,25% | - | - | - | 0.25 | - | - | - |
| SP 0,50% | - | - | - | - | 0.50 | - | - |
| SB 0,25% | - | - | - | - | - | 0.25 | - |
| SB 0,50% | - | - | - | - | - | - | 0.50 |
| | | | | | | | |
| Proximate composition (% of dry matter) | | | | | | | |
| Dry Matter (DM, %) | 95.8 | 95.1 | 95.3 | 93.9 | 95.6 | 94.6 | 95.6 |
| Protein | 43.9 | 43.2 | 44.0 | 42.9 | 43.3 | 43.3 | 43.1 |
| Lipids | 17.5 | 17.4 | 17.7 | 18.2 | 18.0 | 17.7 | 17.6 |
| Ash | 4.35 | 3.91 | 8.29 | 3.04 | 15.2 | 4.13 | 4.86 |
| Energy (kJ $g^{-1}$ DM) | 22.7 | 23.1 | 22.6 | 22.8 | 22.9 | 22.8 | 22.7 |

CP: Crude protein; CL: Crude lipids.

[1] Sorgal, S.A., Ovar, Portugal (CP: 70.3; CL: 12.0).

[2] Soluble fish protein concentrate, Sorgal, S.A., Ovar, Portugal (CP: 73.3; CL: 8.3).

[3] Sorgal, S.A., Ovar, Portugal (CP: 49.7; CL: 1.3).

[4] Sorgal, S.A., Ovar, Portugal (CP: 61.3; CL: 1.5).

[5] Sorgal, S.A., Ovar, Portugal (CP: 81.2; CL: 0.6).

[6] Sorgal, S.A., Ovar, Portugal (CP: 32.4; CL: 1.3).

[7] Sorgal, S.A., Ovar, Portugal (CP: 14.5; CL: 1.6).

[8] Vitamins (mg*kg$^{-1}$ diet): retinol, 18000 (IU kg$^{-1}$ diet); calciferol, 2000 (IU kg$^{-1}$ diet); alpha tocopherol, 35; menadion sodium bis., 10; thiamin, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.

[9] Choline chloride (50%), Sorgal, S.A., Ovar, Portugal.

[10] Minerals (mg*kg$^{-1}$ diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dicalcium phosphate, 8.02 (g kg$^{-1}$ diet); potassium chloride, 1.15 (g kg$^{-1}$ diet); sodium chloride, 0.4 (g kg$^{-1}$ diet).

[11] Binder, AQUACUBE, Agil, UK.

[12] Feed-grade methionine, Sorgal, S.A., Ovar, Portugal.

[13] Feed-grade taurine, Sorgal, S.A., Ovar, Portugal.

### 3.2 Growth trial

The growth trial was carried out at CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental in Matosinhos, Portugal.

The trial was performed in a thermoregulated recirculating aquatic system equipped with 21 cylindrical fiberglass tanks of 300 L water capacity, supplied with a continuous flow of mechanically and biologically filtered seawater and aerification provided through air stones. During the trial, the water parameters were checked daily, room and water temperature were maintained at ± 22°C, salinity at ± 26‰, $NO_2$ below 0.5 mg/L, dissolved oxygen was maintained above 80%, and photoperiod for 12 hours light and 12 hours dark.

European seabass (*Dicentrarchus labrax*) juveniles were provided by Atlantik Fish, Lda., Castro Marim, Portugal. After transportation to the experimental facilities fish were maintained in quarantine for 15 days. Fish were then transferred to the experimental room and acclimatized to the experimental facilities and water parameters for 2 weeks. At the end of the acclimatization period, 21 homogenous groups of 25 European seabass (IBW = 15.22±0.03 g) were randomly distributed to the experimental tanks. Each diet was randomly assigned to triplicate groups. During the trial, fish were fed by hand, twice a day, 6 days per week, until apparent satiety. The trial lasted 10 weeks, and at the end of the trial fish were weighed following one day of feed deprivation.

For whole-body composition, 6 fish from the initial stock and 3 fish from each tank at the end of the trial were randomly collected and stored at -20°C until analysis. Wet weight, liver, and viscera weights of these fish were recorded to

determination of hepatosomatic and visceral index. The remaining fish were fed three more days, to minimize the stress.

At the sampling day, 4 hours after the morning meal, three fish from each tank (9 per treatment) were randomly sampled for blood analysis.

Blood samples were collected according to Peres et al. (2015). Briefly, the blood was divided into two aliquots and placed in two Eppendorf tubes. One aliquot was immediately centrifuged, and the plasma was frozen at -80°C until analysis. The other aliquot was used for haematological analysis (WBC and RBC count, haematocrit and haemoglobin)

### 3.3 Challenge and fish survival

#### 3.3.1 Bacterial growth

*Vibrio anguillarum* DSMZ 21597 was cultivated in brain heart infusion broth (BHI) for 24 hours at 25°C. The cultures were then centrifugated at 4000xg for 15 minutes at room temperature. Bacterial cells were resuspended in sterile BHI to reach a final concentration of $1x10^7$ CFU/ml. To confirm the injection concentration for the final challenge, serial dilution of bacterial suspension was plated.

#### 3.3.2 Pre-Challenge

Two weeks before the end of the growth trial, a pre-challenge was performed. A total of 30 fish were injected with different concentrations ($5x10^7$, $1x10^7$ and $5x10^6$ CFU/ml) of *Vibrio anguillarum* in order to assess the LD50 (the dose that kills 50% of the fish). After the injection, the fish were randomly put in three tanks. The water temperature was increased from 22 to 24°C. Mortality was recorded daily.

### 3.3.3 Challenge trial

At the end of the growth trial, the remaining fish were relocated to the challenge room in 14 tanks, 2 tanks for each dietary treatment, of 100L of water capacity. The water temperature was increased up to 24°C, salinity 35 ‰ and the photoperiod was 12 hours light and 12 hours dark.

Six fish per tank (18 per diet) were intraperitoneally injected with 100 ul of *Vibrio anguillarum* ($1.2x10^7$ CFU/$mL^{-1}$) according to the pre-challenge results.

Fish were sampled for the haematological profile and immune parameters, 4- and 24- hours after the injection .

### 3.3.4 Survival trial

In order to assess the survival, 15 fish per tank (30 per diet) were injected with the previously evaluated concentration of *Vibrio anguillarum* and put in 14 tanks, 2 tanks for each dietary treatment, of 100 L water capacity, 30 fish were injected with phosphate buffered saline solution (PBS) for control. The dead fish were weighed, and examined for clinical signs and the approximate timing of death was recorded. Mortality was recorded daily for 3 weeks.

## 3.4. Analysis

### 3.4.1 Chemical analysis

Feed proximate composition and whole-body composition analysis were performed according to standard methods (AOAC, 1980). Protein content was assessed by the Kjeldahl method following acid digestion (Tecator® Digestion System 40, Höganäs, Sweden; model 1016 Digester) at 450°C, using Kjeltec distillation (Tecator® Kjeltec system, Höganäs, Sweden; model 1026 Distilling Unit) and titration (Titronic® universal, Radnor, PA). Lipid content was assessed by extraction with petroleum ether using a Soxtec system (FOSS®, Hilleroed, Denmark; model ST243). Dry matter was measured by drying the samples at 100°C until constant weight. Energy was measured using a calorimetric bomb (Parr®, 6200 calorimeter). Ashes were assessed by incineration at 450°C for 16 hours in a muffle furnace.

### 3.4.2 Haematological analysis

Total erythrocytes (RBC) and leucocytes (WBC) counts were performed with fresh blood diluted in HBSS using a hemacytometer. Haematocrit was assessed after microcentrifugation at 10000xg for 10 minutes at room temperature. Haemoglobin concentration was determined with Drabkin's reagent using an analytical kit (Spinreact, Spain).

Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated:

- MCV ($\mu m^3$) = (Ht/RBC) x 10
- MCH (pg $cell^{-1}$) = (HH/RBC) x 10
- MCHC (g 100 $ml^{-1}$) x 100

### 3.4.3 Plasmatic immune parameters analysis

#### Lysozyme activity

To analyse plasma lysozyme activity, 15 µl of plasma were added in duplicate in a 96-well plate. To each well, 250 µl of a standard bacterial suspension (*Micrococcus lysodeikticus*) was added. Then, 265 µl of sodium hydrogen propionate tampon solution and 265 µl of the standard bacterial solution were added in duplicate as control samples. The absorbance was read at 450 nm, 0,5 and 4,5 minutes after addition of the bacterial suspension, using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA). The amount of lysozyme in the sample was calculated using the formula of the standard curve.

#### Anti-protease activity

Plasma anti-protease activity was assessed by the ability of plasma to inhibit activity according to Machado et al. (2021). Briefly, 10 µl of plasma were mixed with 10 µl of a trypsin solution and incubated for 10 min. at 22°C. After that, 100 µl of phosphate-buffered saline and 125 µl of azocasein solution were added and incubated for one hour at 22°C. After that, 250 µl of trichloroacetic acid was added and incubated for a further 30 min at 22°C.

The samples were centrifuged at 10000xg for 5 min and 100 µl of supernatant was added in duplicate wells to a 96-well plate containing 100 µl of sodium hydroxide.

A positive control with 120 µl of PBS was added and 10 µl of trypsin with 110 µl of PBS was added as a reference. The absorbance was measured at 450 nm, using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA). The percentage of non-inhibited trypsin was calculated by multiplying the sample absorbance x 100 and dividing it for the reference absorbance. Anti-protease activity was expressed in the percentage of inhibited trypsin, calculated from the difference between total trypsin to non-inhibited trypsin.

**Protease activity**

To determine plasma protease activity, 10 µl of plasma were mixed with 100 µl of sodium bicarbonate and 125 µl of azocasein, and incubated for 24 hours at room temperature. After, 250 µl of trichloroacetic acid 10% were added and centrifuged at 6000xg for 5 min at 25°C. After that, 100 µl of supernatant were added in duplicate wells of a 96-well plate containing 100 µl of sodium hydroxide.

As positive control was used 100 µl of sodium bicarbonate with 10 µl of trypsin, and as negative control, 110 µl of sodium bicarbonate was used. The absorbance was read at 450 nm, using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA).

**Bactericidal activity**

Plasma bactericidal activity was determined according to Machado et al. (2018). Briefly, 20 µl of plasma was added to duplicate wells of a U-shaped 96-well plate. Hank's balanced salt solution (HBSS) was added instead of plasma for positive control. To each well, 20 µl of *Vibrio anguillarum* 1.2 x $10^7$ cfu*$ml^{-1}$ were added and incubated for 2.5 h at room temperature. After the incubation 25 µl of iodonitrotetrazolium was added and incubated for 10 min at 25°C to allow the formation of formazan. Plates were then centrifuged at 2000xg for 10 min and the precipitate was dissolved in 200 µl of dimethyl sulfoxide. The absorbance was measured at 490 nm, using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA). Bactericidal activity is expressed as a percentage, calculated from the difference between bacteria surviving compared to the number of bacteria from positive control (100%).

**Peroxidase activity**

Total peroxidase activity was measured according to Machado et al. (2018). 15 µl of plasma was diluted in a 96-well plate with 135 µl of HBSS $Ca^{2+}$ and $Mg^{2+}$ free. 150 µl of HBSS were added in duplicate as blank. Then, to each well, 50 µl of 10 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB) and 50 µl of $H_2O_2$ 5mM were added. After 2 minutes, 50 µl of sulfuric acid 2M was added to each well to stop the reaction. The absorbance was read at 450 nm, using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA). Peroxidase activity (unity*plasma $mL^{-1}$) was determined following the assumption that one unity of peroxidase produces an absorbance mutation of one unity of optic absorbance.

**3.5 Statistical analysis**

Data are presented as means and pooled standard error of the mean (S.E.M.). The probability level for rejection of the null hypotheses was 0.05. Statistical analysis of growth parameters and whole-body composition data were done by two-way analysis of variance (ANOVA) with type of fatty acid and fatty acid supplementation as fixed factors. Haematological parameters and innate immune parameters were examined by three-way ANOVA with time (0 h, 4- and 24-hours post-infection), type of fatty acid and fatty acid supplementation as the main factors. Non-orthogonal contrasts between control and other treatments were performed.

Significative differences among the interaction fatty acid*level*time were analysed by two-way ANOVA, while significative differences between the interactions fatty acid*level, level*time, and fatty acid*time were analysed by one-way ANOVA or t-test. Significative differences between means were calculated by Tukey's multiple range test. Non-orthogonal contrasts between control and other treatments were performed.

All statistical analysis were done using SPSS 28.0 software package for Windows.

## 4. Results

Fish promptly accepted all the experimental diets. Growth performance and feed utilization efficiency were unaffected by the dietary inclusions of the different SCFAs, as shown in Table 5. Statistical analysis indicated a significant ($P<0.05$) effect of fatty acid in protein efficiency ratio (PER), regardless level of supplementation. Non-orthogonal contrast indicated a difference in initial body weight (IBW) between the control diet and diet SP 0.50%.

As shown in table 6, the protein and ash content of fish collected before the growth trial was higher than dietary treatments, while initial lipid content was far lower. Comparing dietary treatments, protein lipid, and ash, HIS and VSI did not differ significantly. Significative interactions were found the level of supplementation for protein and lipid content and for the interaction between fatty acids and the lipid and ash content level. Non-orthogonal contrast indicated significative differences between the control diet and diet SP 0.25% for protein and lipid content and between the control diet and diets SA 0.25%, SP 0.50% and SB 0.25%.

Regarding haematological parameters, as referred in table 7, all decreased with time, without significative differences among fatty acids or their level of supplementation compared to time 0h (pre-infection). Only the mean corpuscular haemoglobin concentration showed an increase in SA 0.25%, SP 0.50 and SB 0.50% diets. Mean corpuscular volume was markedly higher in fish fed with sodium butyrate than sodium acetate, especially al 4- and 24-hours post-infection. All the parameters showed a general tendency to recover at 24h.

As shown in table 8, innate immune parameters presented a decrease in lysozyme activity and peroxidase activity at 4- and 24-hours post- infection in all the fish, regardless the diets. In general, lysozyme activity was higher in fish fed with sodium butyrate when compared with sodium acetate, regardless the inclusion level of SB. Peroxidase activity recovered at 24h in fish fed sodium acetate 0.25% and 0.50%, and at sodium propionate at 0.25%. Anti-proteases activity and bactericidal activity showed non-significative variations among all the diets, while proteases activity was higher in fish fed all dietary treatments compared with the control diet.

The survival trial showed a difference in survival rate only for fish fed diet SB 0.50% and diet SP 0.25%, as shown in figure 5. However, the cumulative survival was above 60% for all the fish. Fish fed diet SB 0.50% stop dying after 2.5 days, while, as a whole, fish stop dying after 6 days.

**Table 5** Growth performance and feed utilization efficiency of European seabass fed the experimental diets.

| | CONTROL | SA 0.25 | SA 0.50 | SP 0.25 | SP 0.50 | SB 0.25 | SB 0.50 | SEM |
|---|---|---|---|---|---|---|---|---|
| Initial body weight (g) | 15.2* | 15.2 | 15.2 | 15.2 | 15.2* | 15.2 | 15.2 | 0.01 |
| Final body weight (g) | 43.9 | 44.3 | 43.4 | 43.0 | 43.7 | 44.8 | 44.2 | 0.26 |
| Weight gain (g*kg *ABW$^{-1}$*day$^{-1}$) | 17.4 | 17.4 | 17.2 | 17.1 | 17.3 | 17.6 | 17.4 | 1.71 |
| Daily Growth Index[1] | 1.88 | 1.89 | 1.85 | 1.83 | 1.86 | 1.92 | 1.88 | 0.01 |
| Feed Intake (g*kg*ABW$^{-1}$*day$^{-1}$)[2] | 21.9 | 22.5 | 21.2 | 21.0 | 21.2 | 21.5 | 21.2 | 0.15 |
| Feed Efficiency[3] | 0.79 | 0.77 | 0.81 | 0.81 | 0.81 | 0.82 | 0.82 | 0.01 |
| Protein Efficiency Ratio[4] | 1.80 | 1.79 | 1.84 | 1.90 | 1.88 | 1.89 | 1.90 | 0.01 |
| Survival rate (%) | 98.7 | 100.0 | 100.0 | 100.0 | 98.7 | 100.0 | 98.7 | 0.31 |

| Parameters | Fatty acid | Level | Fatty acid*Level | Fatty acid | | |
|---|---|---|---|---|---|---|
| | | | | SA | SP | SB |
| IBW | Ns | Ns | Ns | - | - | - |
| FBW | Ns | Ns | Ns | - | - | - |
| WG | Ns | Ns | Ns | - | - | - |
| DGI | Ns | Ns | Ns | - | - | - |
| FI | Ns | Ns | Ns | - | - | - |
| FE | Ns | Ns | Ns | - | - | - |
| PER | 0.035 | Ns | Ns | A | AB | B |

*Data are presented as means (n=9 samples). P-values from two-way ANOVA (p≤0.05). If the interaction was significant, Tukey post-hoc test was used to identify differences in experimental treatments. Different capital letters indicate differences among the type of fatty acid regardless of supplementation level. Means in the same row with the superscript symbol * indicate differences between the control diet and the other diets at 0h, # at 4h, and $ at 24h.*

Average Body Weight (ABW) = [initial body weight (IBW) + final body weight (FBW)]/2.

[1] Daily Growth Index = [(Final weight$^{1/3}$ – Initial weight$^{1/3}$)/time in days] *100.

[2] Feed Intake = dry feed intake/ABW/days.

[3] Feed Efficiency = wet weight gain/dry feed intake

[4] Protein efficiency ratio = weight gain/protein intake

**Table 6** Whole-body composition analysis (% dry matter basis).

| | INITIAL | CONTROL | SA 0.25 | SA 0.50 | SP 0.25 | SP 0.50 | SB 0.25 | SB 0.50 | SEM |
|---|---|---|---|---|---|---|---|---|---|
| Protein | 61.3 | 52.4* | 53.0 | 53.4 | 55.0* | 53.5 | 54.3 | 51.5 | 0.35 |
| Lipid | 7.97 | 32.5* | 30.3 | 31.0 | 28.6* | 34.0 | 32.8 | 31.9 | 0.81 |
| Dry matter | 95.5 | 96.6 | 97.3 | 97.0 | 97.0 | 96.9 | 13.8 | 97.1 | 0.11 |
| Ash | 32.7 | 17.4* | 14.9* | 15.2 | 16.4 | 13.3* | 13.8* | 14.4* | 0.65 |
| HSI[1] | - | 1.44 | 1.21 | 1.25 | 1.34 | 1.33 | 1.26 | 1.36 | 0.03 |
| VSI[2] | - | 9.33 | 8.72 | 9.13 | 8.39 | 9.34 | 9.87 | 10.20 | 0.36 |

| Two-way ANOVA | | | |
|---|---|---|---|
| **Parameters** | **Fatty acid** | **Level** | **Fatty acid*Level** |
| Protein | Ns | 0.043 | Ns |
| Lipid | Ns | 0.004 | 0.026 |
| Dry matter | Ns | Ns | Ns |
| Ash | Ns | Ns | 0.025 |
| HSI[1] | Ns | Ns | Ns |
| VSI[2] | Ns | Ns | Ns |

| Parameters | Fatty acid*Level | | | | | |
|---|---|---|---|---|---|---|
| | 0.25 | | | 0.50 | | |
| | SA | SP | SB | SA | SP | SB |
| Protein | - | - | - | - | - | - |
| Lipid | - | - | - | A | B | AB |
| Ash | - | - | - | B | A | AB |

*Data are presented as means (n=9 samples). P-values from two-way ANOVA (p≤0.05). Means in the same row with the superscript symbol * indicate differences between the control diet and the other diets at 0h, # at 4h, and $ at 24h.*

[1] Hepatosomatic index = (liver weight/whole body weight) x 100.

[2] Visceral index = (visceral weight/whole body weight) x 100.

**Table 7** Haematological parameters of European seabass fed the experimental diets.

| Parameters | CONTROL | | | SA 0.25 | | | SA 0.50 | | | SP 0.25 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h |
| Haematocrit (%) | 31.9 | 22.2 | 27.0$ | 32.6 | 18.8 | 22.7 | 28.7 | 22.2 | 22.3 | 30.2 | 21.3 | 21.3 |
| Haemoglobin (g/dL) | 1.83 | 1.73 | 1.11 | 1.82 | 1.15 | 1.24 | 1.76 | 1.38 | 1.26 | 1.69 | 1.13 | 1.16 |
| MCV ($m^{3)}$ | 188.8 | 106.5# | 107.3 | 109.0 | 79.4# | 102.0 | 112.8 | 82.2# | 105.4 | 122.6 | 91.5# | 97.6 |
| MCH (pg $cell^{-1}$) | 6.80 | 5.78 | 5.75 | 6.06 | 4.75 | 5.69 | 6.86 | 5.12 | 5.90 | 6.77 | 4.86 | 5.29 |
| MCHC (g 100 $ml^{-1}$) | 5.72 | 5.62 | 5.39$ | 5.65 | 6.15 | 5.51 | 6.27 | 6.20 | 5.66 | 5.63 | 5.24 | 5.45 |
| RBC ($x10^6$ $\mu l^{-1}$) | 2.74 | 2.15# | 1.94$ | 3.02 | 2.26 | 2.24$ | 2.54 | 2.73# | 2.13 | 2.50 | 2.37# | 2.20 |
| WBC ($x10^4$ $\mu l^{-1}$) | 8.71* | 4.03 | 5.68 | 7.31 | 4.15 | 7.33 | 6.72* | 3.65 | 5.77 | 8.89 | 3.70 | 7.13 |

| Parameters | SP 0.50 | | | SB 0.25 | | | SB 0.50 | | | SEM | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h |
| Haematocrit (%) | 35.8 | 22.5 | 26.0$ | 30.5 | 21.5 | 24.2 | 29.2 | 20.2 | 25.3$ | 0.75 | 0.46 | 0.51 |
| Haemoglobin (g/dL) | 1.76 | 1.17 | 1.35 | 1.66 | 1.18 | 1.38 | 1.51 | 1.33 | 1.01 | 0.05 | 0.10 | 0.04 |
| MCV ($m^{3)}$ | 137.5 | 88.3# | 119.7 | 107.5 | 87.7# | 109.9 | 111.6 | 105.8 | 113.9 | 3.11 | 2.48 | 2.08 |
| MCH (pg $cell^{-1}$) | 6.66 | 4.60 | 6.18 | 6.43 | 4.81 | 6.22 | 5.74 | 6.44 | 4.52 | 0.18 | 0.33 | 0.19 |
| MCHC (g 100 $ml^{-1}$) | 5.13 | 5.21 | 5.15 | 5.40 | 5.44 | 5.85 | 5.28 | 6.48 | 3.98$ | 0.17 | 0.32 | 0.15 |
| RBC ($x10^6$ $\mu l^{-1}$) | 2.68 | 2.55# | 2.18 | 2.55 | 2.48# | 2.22$ | 2.63 | 1.94 | 2.23$ | 0.06 | 0.06 | 0.04 |
| WBC ($x10^4$ $\mu l^{-1}$) | 6.83* | 3.37 | 6.13 | 7.26 | 3.48 | 5.77 | 8.54 | 4.33 | 5.57 | 0.24 | 0.11 | 0.23 |

| | Three-way ANOVA | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameters | Time | Fatty acid | Level | Fatty acid*Level | Fatty acid*Time | Time*Level | Fatty acid*Time*Level | Time | | | Fatty acids | | |
| | | | | | | | | 0h | 4h | 24h | SA | SP | SB |
| Haematocrit (%) | <0.001 | Ns | Ns | Ns | Ns | Ns | Ns | C | A | B | - | - | - |
| Haemoglobin (g/dL) | <0.001 | Ns | Ns | Ns | Ns | Ns | Ns | B | A | A | - | - | - |
| MCV ($m^{3)}$ | <0.001 | 0.02 | Ns | Ns | Ns | Ns | Ns | C | A | B | a | ab | b |
| MCH (pg $cell^{-1}$) | <0.001 | Ns | Ns | Ns | Ns | Ns | Ns | B | A | A | - | - | - |
| MCHC (g 100 $ml^{-1}$) | Ns | Ns | Ns | Ns | Ns | Ns | Ns | - | - | - | - | - | - |
| RBC ($x10^6$ $\mu l^{-1}$) | <0.001 | Ns | Ns | Ns | Ns | Ns | <0.001 | B | A | A | - | - | - |
| WBC ($x10^4$ $\mu l^{-1}$) | <0.001 | Ns | Ns | 0.004 | Ns | Ns | Ns | C | A | B | - | - | - |

*Data are presented as means (n=9 samples for data 0h, n=6 samples for data 4h and 24h). P-values from three-way ANOVA (p≤0.05). If the interaction was significant, Tukey post-hoc test was used to identify differences in experimental treatments. In the three-way ANOVA, different capital letters indicate differences among time regardless of type of fatty acid and supplementation level.*

*Different small letters indicate difference among type of fatty acid regardless of time and supplementation level. Means in the same row with different superscript symbols indicate differences between contrasts. * indicate differences between 0h, # between 4h, and $ between 24h.*

**Table 8** Innate immune parameters of European seabass fed the experimental diets.

| Parameters | CONTROL | | | SA 0.25 | | | SA 0.50 | | | SP 0.25 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h |
| Lysozyme (μg ml$^{-1}$) | 21.0$^{*}$ | 14.9 | 8.3$^{\$}$ | 16.9 | 16.1 | 4.4$^{\$}$ | 16.4 | 14.1 | 7.2 | 28.1$^{*}$ | 14.6 | 9.7 |
| Peroxidase (unity ml$^{-1}$) | 181.4 | 35.3$^{\#}$ | 143.1$^{\$}$ | 165.8 | 42.2 | 120.7 | 191.1 | 64.6 | 139.5 | 127.2 | 82.2$^{\#}$ | 132.2 |
| Proteases activity (%) | 15.8$^{*}$ | 8.64$^{\#}$ | 10.8 | 20.3 | 9.82 | 10.3 | 23.7$^{*}$ | 9.39 | 11.3 | 18.6 | 10.8$^{\#}$ | 10.8 |
| Anti-proteases activity (%) | 87.5$^{*}$ | 85.7 | 90.3 | 87.4 | 86.7 | 89.0 | 87.2 | 88.0 | 87.1 | 86.9 | 84.2 | 90.3 |
| Bactericidal activity (%) | 50.7$^{*}$ | 43.1$^{\#}$ | 45.5 | 44.7$^{*}$ | 52.3$^{\#}$ | 44.3 | 49.0 | 53.0$^{\#}$ | 44.0 | 48.7 | 47.2 | 48.8 |

| Parameters | SP 0.50 | | | SB 0.25 | | | SB 0.50 | | | SEM | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h |
| Lysozyme (μg ml$^{-1}$) | 19.0 | 16.0 | 8.4 | 24.2 | 12.1 | 8.4 | 18.9 | 14.1 | 11.5 | 1.11 | 0.53 | 0.47 |
| Peroxidase (unity ml$^{-1}$) | 126.6 | 62.0 | 47.5$^{\$}$ | 179.0 | 67.1 | 54.2$^{\$}$ | 220.6 | 62.0 | 35.3$^{\$}$ | 12.3 | 4.8 | 10.1 |
| Proteases activity (%) | 16.7 | 9.94 | 10.5 | 18.4 | 11.3$^{\#}$ | 10.4 | 23.3* | 10.4$^{\#}$ | 10.9 | 0.91 | 0.25 | 0.15 |
| Anti-proteases activity (%) | 84.2$^{*}$ | 83.6 | 89.8 | 87.9 | 78.7 | 89.0 | 86.2 | 85.4 | 89.7 | 0.5 | 1.0 | 0.2 |
| Bactericidal activity (%) | 48.5 | 47.7 | 42.4 | 46.1 | 47.0 | 48.0 | 46.1 | 49.2 | 55.1 | 0.7 | 1.0 | 1.5 |

| | Three-way ANOVA | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameters | Time | Fatty acid | Level | Fatty acid*Level | Fatty acid*Time | Time*Level | Fatty acid*Level*Time | Time | | | Fatty acid | | |
| | | | | | | | | 0h | 4h | 24h | SA | SP | SB |
| Lysozyme (μg ml$^{-1}$) | <0.001 | 0.015 | Ns | Ns | Ns | 0.01 | Ns | C | B | A | a | ab | b |
| Peroxidase (unity ml$^{-1}$) | <0.001 | Ns | Ns | Ns | 0.002 | Ns | Ns | B | A | A | - | - | - |
| Proteases activity (%) | Ns | <0.001 | <0.001 | <0.001 | <0.001 | 0.034 | <0.001 | - | - | - | b | a | a |
| Anti-proteases activity (%) | <0.001 | Ns | Ns | Ns | Ns | Ns | Ns | B | A | C | - | - | - |
| Bactericidal activity (%) | Ns | Ns | Ns | Ns | 0.011 | Ns | Ns | - | - | - | - | - | - |

| Parameters | Time*Level | | | | | | Fatty acid*Time | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.25 | | | 0.50 | | | SA | | | SB | | |
| | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h | 0h | 4h | 24h |
| Lysozyme (µg ml$^{-1}$) | C | B | A | C | B | A | - | - | - | - | - | - |
| Peroxidase (unity ml$^{-1}$) | - | - | - | - | - | - | A | B | B | B | A | A |
| Proteases activity (%) | - | - | - | - | - | - | - | - | - | B | A | A |
| Bactericidal activity (%) | - | - | - | - | - | - | AB | B | A | A | A | B |

| Parameters | Fatty acid*Level | | | | | |
|---|---|---|---|---|---|---|
| | 0.25 | | | 0.50 | | |
| | SA | SP | SB | SA | SP | SB |
| Lysozyme (µg ml$^{-1}$) | - | - | - | - | - | - |
| Peroxidase (unity ml$^{-1}$) | - | - | - | - | - | - |
| Proteases activity (%) | B | AB | A | - | - | - |
| Bactericidal activity (%) | - | - | - | - | - | - |

*Data are presented as means (n=9 for data 0h, n=6 for data 4h and 24h). P-values from three-way ANOVA (p≤0.05). If the interaction was significant, Tukey post-hoc test was used to identify differences in experimental treatments. In the three-way ANOVA, different capital letters indicate differences among time regardless of the type of fatty acid and supplementation level. Different small letters indicate differences among the type of fatty acids regardless of time and supplementation level. Means in the same row with different superscript symbols indicate differences between contrasts. * indicate differences between 0h, # between 4h, and $ between 24h.*

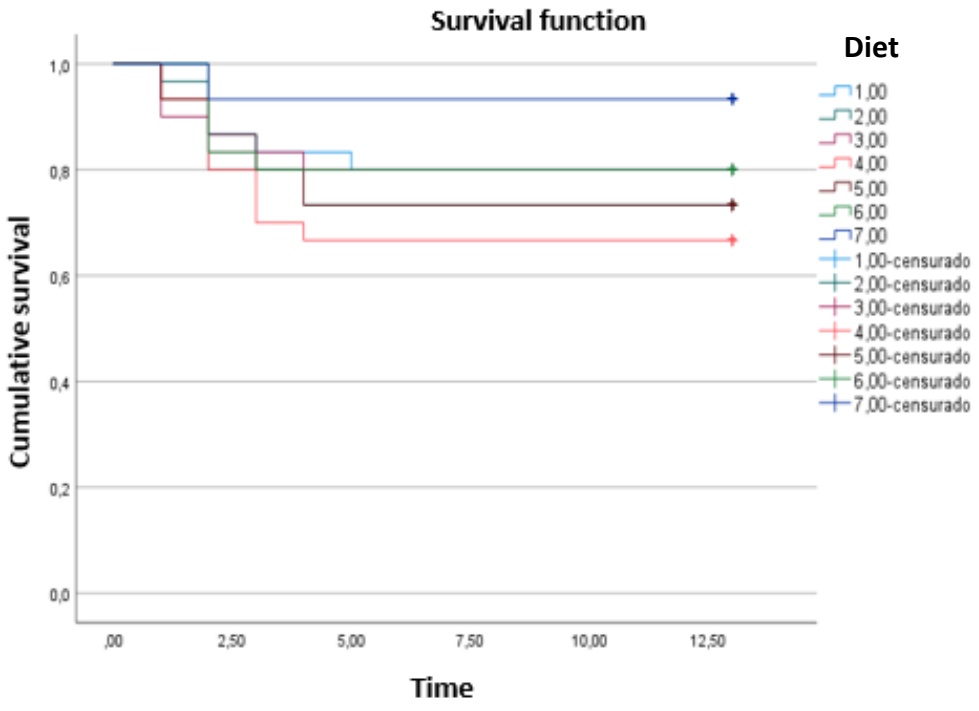


**Figure 5** Cumulative survival during the survival trial

## 5. Discussion

The main objective of this work was to assess the potential role of SCFAs as functional ingredients in a plant-based feedstuff on growth, immune response and disease resistance in European seabass, *Dicentrarchus labrax*, one of the most important farmed species in European aquaculture.

Kaushik et al. (2004) highlighted that European seabass could be fed a diet almost devoid of fish meal without significant differences in growth performance.

Results of the growth trial confirm that European seabass had a high growth rate even when fed a high plant protein diet. Moreover, this trial also indicates that supplementation of sodium acetate, sodium propionate and sodium butyrate up to 0.50% in a PF diet in European seabass does not affect growth performance compared with the control diet. Similar results have been reported for other fish species (Owen et al., 2006; Sangari et al., 2021). However, several studies have reported an increase (Liu et al., 2014; Omosowone et al., 2018; Aalamifar et al., 2020) or a decrease in growth performance (Ringø, 1991).

Fish promptly accepted all the diets, and no differences in voluntary feed intake were observed, indicating that SCFAs didn't negatively affect the palatability of the feed. Nevertheless, fishmeal and soluble fish protein concentrate (CPSP) had to be added to increase palatability.

Whole-body composition analysis identified a relatively high percentage of lipids in fish carcasses, nonetheless with the values reported for seabass of the same size (Peres & Oliva-Teles, 2002; Martins et al., 2018). This result may be related, at least in part, to the high dietary PF inclusion level. In fact, it has been reported that plant-based diets influence lipid metabolism and fat deposition, leading to a slight increase in lipid content in whole-body composition than fish fed commercial diets (Kaushik et al., 2004; Messina, 2007).

Fish fed diet SA 0.25% showed the lowest protein efficiency ratio (PER), per Sangari et al. (2020) who reported a reduction of PER in yellowfin seabream, *Acanthopagrus latus*, fed sodium acetate and sodium propionate. The lower PER value followed the same trend as feed efficiency compared to the other dietary treatments, suggesting a lower nutrients absorption. However, several studies did not report a significant effect of dietary supplementation with SCFAs in diet on the growth performance and feed utilization of aquaculture species, such as rainbow trout, *Oncorhynchus mykiss* (Pandey & Satoh, 2007), hybrid tilapia (Ebrahimi et al., 2017) and catfish, *Clarias gariepinus* (Owen et al., 2006).

The whole-body protein content of the fish-fed diet SP 0.25% was significantly higher than that of the other groups. This increase may be related to the protein-sparing effect of SP that may have promoted the utilization of lipid rather than

protein for energy production leading to higher protein accretion in whole-body composition (Kaushik et al., 1995). Protein sparing effect by lipids helps preserve protein for growth purposes, using lipids as an energy source (Thirunavukkarasar et al., 2022). An additional point might be the high value of PER for diet SP 0.25%.

Significative differences in ash content for diet SA 0.25%, SP 0.50%, SB 0.25% and 0.50% compared to the control diet might be related to the positive effect of SCFAs on mineral bioavailability. SCFAs alter the acidity in fish gut, modifying the mineral transport mechanism (Hoseinifar et al., 2016). Furthermore, SCFAs affect the ability of elements to chelate and form complexes by chelation with sodium ions and other elements' ions, reducing the precipitation between ions and phosphates (Cross et al., 1989). Moreover, SCFAs enhance the proliferation of epithelial cells in the gastrointestinal mucosa, increasing the absorption area for minerals (Baruah et al., 2007). Even if not statistically significant, also ash content of fish fed diet SA 0.50% and SP 0.25% was lower than control diet, supporting the previous statements.

According to studies conducted on humans and other animals, SCFAs play a significant role in controlling immunological and metabolic problems by inhibiting HDAC (histone deacetylase) and activating GPCRs (G protein-coupled receptor) (Morrison & Preston, 2016; Sun et al., 2017). Rapid gut lumen absorption allows for the rapid action of acetate, propionate, and butyrate in a variety of inflammatory processes, including systemic autoimmune responses (Sun et al., 2017).

Haematological parameters, such as red blood cell count (RBC) and white blood cell count (WBC), are considered valid indices to assess fish health (Hoseinifar et al., 2011). Haematological analysis is particularly crucial for illness diagnosis since it can deliver a trustworthy evaluation by non-lethal ways (Satheeshkumar et al., 2012). The physiological status and health of a fish cultivation can be determined by its hematological profile, so hematology in conjunction with other common diagnostic techniques could be used to identify and evaluate stress-causing conditions and/or diseases that have an impact on production performance (Tavares-Dias & Moraes, 2007., Pavlidis et al., 2007). As routine, stock health monitoring in fish farms, it is particularly recommended to monitor the values of RBC, Hb, and WBC (Fazio, 2019).

In the present study, after the injection, fish presented a significant decrease in all the haematological parameters over time, except for MCHC. A decrease in haematocrit, haemoglobin and RBC, and normal MCHC, might indicate anaemia. This result agrees with the study of Bandeira Junior et al. (2018) on silver catfish, *Rhamdia quelen*, infected with *Citrobacter freundii*. The lower value of RBC after infection might suggest that the anaemia is non-regenerative. According to Tiwari & Pandey (2014), this type of anaemia can be found in animals with a chronic severe bacterial infection, such as *Vibrio* spp. In fish, this kind of infections are

characterized by lethargie, exophthalmus, ulcers and skin lesions, haemorrhagies in the internal organs, ascites, paleness of kidney and liver (Korun, 2008).

Sepulcre et al. (2007) suggested that *Vibrio anguillarum* may inhibit the respiratory burst of European seabass WBC as an important virulence mechanism. This mechanism has been demonstrated for rainbow trout (Boesen et al., 2001), while gilthead seabream's WBC seems able to kill the bacterium *in vitro* (Chaves-Pozo et al., 2005; Sepulcre et al., 2007). This mechanism could explain the significative low level of WBC at 4h investigated in this experiment. The subsequent recovery showed at 24h might be explained according to Paiva et al. (2004), stating that the increase in WBC represents a nonspecific immune response to the bacterial infection, following the releasing of immature neutrophils. However, further analysis on the characterization of WBC must be conducted to confirm this hypothesis.

The innate immune system is a fundamental defence mechanism of fish against non-self and danger signals (Magnadóttir, 2006). Through humoral and cell-mediated pathways, the immune system of teleost fish has defense mechanisms against bacteria that operate in a multifactorial way to stop bacterial colonization (Biller-Takahashi et al., 2013). For instance, according to Demers & Bayne (1997), fish lysozyme, an enzyme with antibiotic characteristics that is generated by leucocytes, has a wider activity than mammalian lysozyme and has frequently been utilized as an indicator of non-specific immune functions, which are crucial for preventing infections in fish. Moreover, peroxidase is used as an indicator of oxidative stress, due to its role as antioxidant enzyme (Mylonas & Kouretas, 1999).

The significant decrease in lysozyme activity and peroxidase activity during the challenge might be explained by the lower WBC values recorded at 4h. Fish fed diet of SP 0.50% showed a significative higher value compared with the control diet at 0h, agreeing with the results obtained by Safari et al. (2021) in a study conducted on African cichlid, *Labidochromis lividus*, fed a combination of dietary symbiotic and SP. The recovery of peroxidase activity shown in fish fed diet SA 0.25% and 0.50%, and SP 0.25% suggest an improvement in activation of phagocytic cells and a better development of an inflammatory response (Faurschou et al., 2003).

As far as our knowledge, there are no studies that reported a general decrease in innate immune parameters. However, several studies reported an improvement in fish species fed with SCFAs. Aalamifar et al. (2020) reported a significant increase in serum lysozyme activity in barramundi, *Later calcarifer*, and fed dietary butyrate. Common carp, *Cyprinus carpio*, fed microencapsulated SB improved the immune response (Liu et al., 2014), while rainbow trout, *Oncorhynchus mykiss*, showed an improvement in lysozyme and bactericidal activity (Mirghaed et al., 2019). Zare et al. (2021) demonstrated that Siberian sturgeon, *Acipenser baerii*,

fed dietary acetate improved lysozyme activity, and zebrafish, *Danio rerio*, fed SP increased the expression of lysozyme genes (Safari et al., 2016).

Our study showed that dietary SCFAs supplementation increase the immune of European seabass against *Vibrio anguillarum*, especially dietary supplementation of sodium butyrate at 0.50%. This result agrees with a previous study by Li et al. (2022) on zebrafish, *Danio rerio*.

## 6. Conclusion

European seabass is one of the most important farmed fish in the Mediterranean area, which farming is expected to grow in the future due to the constantly growth of protein demand.

This study aimed to assess the potential role of SCFAs as functional ingredients in a plant-based feedstuff on growth, immune response and disease resistance in European seabass.

Growth performance, feed utilization efficiency, and whole-body composition indicated that SCFAs do not compromise European seabass performance.

After a bacterial challenge with *Vibrio* anguillarum, general decrease of haematological parameters and innate immune parameters was observed, may indicate that SCFAs did not enhance the immune response of European seabass. However, the cumulative survival of European seabass was improved in fish fed the sodium butyrate 0.50% supplemented diet.

Further works have to be conducted to further study the mecanism behind the increased survival rate of fish fed SCFAs supplemented diets and assess economical values for a commercial application.

## 7. References


- Aalamifar, H., Soltanian, S., Vazirzadeh, A., Akhlaghi, M., Morshedi, V., Gholamhosseini, A., Mozanzadehm M.T. (2020). Dietary butyric acid improved growth, digestive enzyme activities and humoral immune parameters in Barramundi (*Lates calcarifer*). *Aquaculture nutrition*, 26(1): 156-164.
- Abdel-Latif, H.M.R., Abdel-Tawwab, M., Dawood, M.A.O., Menanteau-Ledouble, S., El-Matbouli, M. (2020). Benefits of Dietary Butyric Acid, Sodium Butyrate, and Their Protected Forms in Aquafeeds: A Review. *Reviews in Fisheries Science & Aquaculture*, 28(4): 421-448.
- Abutbul, S., Golan-Goldhirsh, A., Barazani, O., Zilberg, D. (2004). Use of *Rosamarinus officinalis* as a treatment against *Streptococcus iniae* in tilapia (*Oreochromis* sp.). *Aquaculture*, 238: 97–10.
- Akrami, R., Iri, Y., Rostami, H.K., Mansour, M.R. (2013). Effect of dietary supplementation of fructooligosaccharide (FOS) on growth performance, survival, lactobacillus bacterial population and hemato-immunological parameters of stellate sturgeon (*Acipenser stellatus*) juvenile. *Fish & Shellfish Immunology*, 35: 1235–1239.
- Allan, G.L., Booth, M.A. (2004). Effects of cooking extrusion processing on digestibility of peas, lupins, canola meal and soybean meal in silver perch *Bidyanus bidyanus* (Mitchell) diets. *Aquaculture Research*, 35: 981-991.
- Allan, G.L., Parkinson, S., Booth, M.A., Stone, D.A.J., Rowland, S.J., Frances, J., Warner-Smith, R. (2000). Replacement of fish meal in diets for Australian silver perch, *Bidyanus bidyanus* IV. Effects of dehulling and protein concentration on digestibility of grain legumes. *Aquaculture*, *186*(3–4), 293–310.
- Anguiano, M., Pohlenz, C., Buentello, A., Gatlin III, D.M. (2013). The effects of prebiotics on the digestive enzymes and gut histomorphology of red drum (*Sciaenops ocellatus*) and hybrid striped bass (*Morone chrysops × M. saxatilis*). *Br J Nutr*., 109: 623-629.
- Asaduzzaman, M., Iehata, S., Akter, S., Kader, M.A., Ghosh, S.K., Khan, M.N.A., Abol-Munafi, A.B. (2018). Effects of host gut-derived probiotic bacteria on gut morphology, microbiota composition and volatile short chain fatty acids production of Malaysian Mahseer Tor tambroides. Aquaculture Reports 9: 53–61.
- Azimirad, M., Meshkini, S., Ahmadifard, N., Hoseinifar, S.H. (2016). The effects of feeding with symbiotic (*Pediococcus acidilactici* and fructooligosaccharide) enriched adult Artemia on skin mucus immune responses, stress resistance, intestinal microbiota and performance of angelfish (*Pterophyllum scalare*). *Fish & Shellfish Immunology*, 54: 516–522.

- Backhed, F., Ding, H., Wang, T., Hooper, L.V., Koh, G.Y., Nagy, A.; Semenkovich, C.F., Gordon, J.I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci.*, 101: 15718–15723.
- Bai, N., Gu, N., Zhang, W., Xu, W., Mai, K. (2014). Effects of β-glucan derivatives on the immunity of white shrimp *Litopenaeus vannamei* and its resistance against white spot syndrome virus infection. *Aquaculture*, 426–427: 66–73.
- Bandeira Junior, G., Casale dos Santos, A., de Freitas Souza, C., Dellaméa Baldissera, K., dos Santos Moreira, K.L., Leite da Veiga, M., de Ugualde Marques da Rocha, M.I., Castagna de Vargas, A.P., Alves da Cunha, M., Baldisserotto, B. (2018). *Citrobacter freundii* infection in silver catfish (*Rhamdia quelen*): Hematological and histological alterations. *Microbial Pathogenesis*, 125: 276-280.
- Baruah K., Sahu N.P., Pal A.K., Jain K.K., Debnath D., Mukherjee S.C. (2007). Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. *Aquaculture Research*, 38: 109–120.
- Baser, K.H.C. (2008). Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. *Curr. Pharm. Des.* 14: 3106–3119.
- Boesen, J.T., Larsen, M.H., Larsen, J.L., Ellis, A.E. (2001). In vitro interactions between rainbow trout (*Oncorhynchus mykiss*) macrophages and Vibrio anguillarum serogroup O2a. *Fish Shellfish Immunol.*, 11: 415-431.
- Bordignon, F., Gasco, L., Birolo, M., Trocino, A., Caimi, C., Ballarin, C., Bortoletti, M., Nicoletto, C., Maucieri, C., Xiccato, G. (2022). Performance and fillet traits of rainbow trout (*Oncorhynchus mykiss*) fed different levels of *Hermetia illucens* meal in a low-tech aquaponic system. *Aquaculture*, 546
- Boyd, C. E. (2015). Overview of aquaculture feeds: global impacts of ingredient use. In D. A. Davis (Ed.), *Feed and Feeding Practices in Aquaculture* (p. 403). Woodhead Publishing.
- Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in food – a review. *Int. J. Food Microbiol.*, 94: 223–253.
- Carbone, D., Faggio, C. (2016). Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of *Sparus aurata* and *Dicentrarchus labrax*. *Fish & Shellfish Immunology*, 54: 172–178.
- Castex, M., Durand, H., Okeke, B. (2014). Issues with industrial probiotic scale-up. *Aquaculture Nutrition, Gut Health, Probiotics and Prebiotics* John Wiley & Sons, Chichester, West Sussex, pp. 347–359.
- Chakraborty, S.B., Hancz, C. (2011). Application of phytochemicals as immunostimulant, antipathogenic and antistress agents in finfish culture. *Aquaculture*, 3: 103–119.

- Chakraborty, S.B., Horn, P., Hancz, C. (2014). Application of phytochemicals as growth-promoters and endocrine modulators in fish culture. *Aquaculture*, 6: 1–19.
- Chaves-Pozo, E., Muñoz, P., López-Muñoz, A., Pelegrín, P., García Ayala, A., Mulero, V., Meseguer, J. (2005). Early innate immune response and redistribution of inflammatory cells in the bony fish gilthead seabream experimentally infected with *Vibrio anguillarum*. *Cell Tissue Res.*, 320: 61-68.
- Chen, S., Luo, S., Yan, C. (2022). Gut Microbiota Implications for Health and Welfare in Farm Animals: A Review. *Animals*, 12(1): 93.
- Ciorba, M.A. (2012). A Gastroenterologist's Guide to Probiotics. *Clin. Gastroenterol. Hepatol.*, 10(9): 960-968.
- Clements, K.D., Gleeson, V.P., Slaytor, M. (1994). Short-chain fatty acid metabolism in temperate marine herbivorous fish. *Journal of Comparative Physiology B*, 164: 372-377.
- Couto, A., Barroso, C., Guerreiro, I., Pousão-Ferreira, P., Matos, E., Peres, H., Oliva-Teles, A., Enes, P. (2016). Carob seed germ meal in diets for meagre (*Argyrosomus regius*) juveniles: Growth, digestive enzymes, intermediary metabolism, liver and gut histology. *Aquaculture*, *451*, 396–404.
- Couto, A., Serra, C.R., Guerreiro, I., Coutinho, F., Castro, C., Rangel, F., Lavrador, A.S., Monteiro, M., Santos, R., Peres, H., Pousão-Ferreira, P., Gasco, L., Gai, F., Oliva-Teles, A., Enes, P. (2022). Black soldier fly meal effects on meagre health condition: gut morphology, gut microbiota and humoral immune response. *Journal of Insects as Food and Feed*, 0(0): 1-16.
- Craig, S., Kuhn, D.D. (2017). Understanding Fish Nutrition, Feeds, and Feeding. *Virginia Cooperative Extension*, 420-256.
- Cross, H., Debiec, H., Peterlik, M. (1989). Mechanism and regulation of intestinal phosphate absorption. *Mineral and Electrolyte Metabolism*, 16: 115–124.
- da Silva, B.C., do Nascimento Vieira, F., Pedreira Mouriño, J.L., Bolivar, N., Quadros Seiffert, W. (2016). Butyrate and propionate improve the growth performance of *Litopenaeus vannamei*. *Aquaculture Research*, 47(2): 612-623.
- Demers, N.E., Bayne, C.J. (1997). The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. *Developmental and Comparative Immunology*, 21: 363-373.
- den Besten, G., Van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D-J., Bakker, B.M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research*, 54: 2325–2340.

- DeWeerdt, S. (2020). Cultivating a sea change – Can aquaculture overcome its sustainability challenges to feed a growing global population? *Nature*, 588.
- Dimitroglou, A., Merrifield, D.L., Spring, P., Sweetman, J., Moate, R., Davies, S.J. (2010). Effects of dietary mannan oligosaccharides (MOS) and soybean meal on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). *Aquaculture*, 300: 182–188.
- El-Adawy, M., Abd El-Aziz, M., El-Shazly, K., Ali, N.G., Abu El-Magd, M. (2018). Dietary propionic acid enhances antibacterial and immunomodulatory effects of oxytetracycline on Nile tilapia, *Oreochromis niloticus*. *Environmental Sciences and Pollution Research*, 25: 34200-34211.
- El-Sayed, A. F. M. (1998). Total replacement of fish meal with animal protein sources in Nile tilapia, *Oreochromis niloticus* (L.), feeds. *Aquaculture Research*, *29*, 275–280.
- Encarnação, P. (2016). Functional feed additives in aquaculture feeds. In *Aquafeed formulation*, 217-237
- Eshaghzadeh, H., Hoseinifar, S.H., Vahabzadeh, H., Ringø, E. (2015). The effects of dietary inulin on growth performances, survival, and digestive enzyme activities of common carp (*Cyprinus carpio*) fry. *Aquacult Nutr.*, 21: 242–413.
- Evans, F.D., Critchley, A.T. (2014). Seaweeds for Animal Production Use. *Journal of Applied Phycology*, 26: 891-899.
- FAO (2017). The state of world fisheries and aquaculture.
- FAO (2020). The state of world fisheries and aquaculture.
- FAO (2021). Fishery and Aquaculture Statistics, 1976-2019.
- FAO (2022).
- Fazio, F. (2019). Fish hematology analysis as an important tool of aquaculture: A review. *Aquaculture*, 500: 237-242.
- Fleurence, J. (1999). Seaweed Proteins: Biochemical, Nutritional Aspects and Potential Uses. *Trends in Food Science and Technology*, 10: 25-28.
- Flint, H.J., Duncan, S.H., Scott, K.P., Louis, P. (2015). Links between diet, gut microbiota composition and gut metabolism. *Proceedings of the Nutrition Society*, 74: 13–22.
- Fournier, V., Huelvan, C., Desbruyeres, E. (2004). Incorporation of a mixture of plant feedstuffs as substitute for fish meal in diets of juvenile turbot (*Psetta maxima*). *Aquaculture*, *236*(1–4), 451–465.
- Frimodt, C. (1995). *Multilingual illustrated guide to the world's commercial warmwater fish*. Fishing News Books, Osney Mead, Oxford, England.
- Fry, J.P., Mailloux, N.A., Love, D.C., Milli, M.C., Cao, L. (2018). Feed conversion efficiency in aquaculture: do we measure it correctly? *Environ. Res. Lett*, 13: 024017.

- Fuller, R. (1989). Probiotics in man and animals. *J. Appl. Bacteriol.*, 66(5): 365-78.
- Gabaudan, J. (2013). Development of Functional Feed. *High Value Aquaculture Fin Fish Symposium*, Kagoshima, Japan 15-18 October 2013.
- Gasco, L., Acuti, G., Bani, P., Zotte, A. D., Danieli, P. P., De Angelis, A., Fortina, R., Marino, R., Parisi, G., Piccolo, G., Pinotti, L., Prandini, A., Schiavone, A., Terova, G., Tulli, F., Roncarati, A. (2020). Insect and fish by-products as sustainable alternatives to conventional animal proteins in animal nutrition. *Italian Journal of Animal Science*, 19(1), 360-372
- Gatlin III, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E.J., Stone, D., Wilson, R., Wurtele, E. (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture research*, 38: 551-579.
- Gatlin, D. M., Barrows, F. T., Brown, P., Dabrowski, K., Gaylord, T. G., Hardy, R. W., Herman, E., Hu, G., Krogdahl, A., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E. J., Stone, D., Wilson, R., Wurtele, E. (2007). Expanding the utilization of sustainable plant products in aquafeeds: A review. *Aquaculture Research*, *38*(6), 551–579.
- Gemede, H.F., Ratta, N. (2014). Antinutritional factors in plant food: Potential health benefits and adverse effects. *International Journal of Nutrition and Food Sciences*, 3: 284-289.
- Gibson, G.R., Probert, H.M., Van Loo, J.A.E., Rastall, R.A., Roberfroid, M.B. (2004). Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics, *Nutr. Res. Rev.*, 17: 259–275.
- Glencross, B. D., Booth, M., Allan, G. L. (2007). A feed is only as good as its ingredients - A review of ingredient evaluation strategies for aquaculture feeds. *Aquaculture Nutrition*, *13*(1): 17–34.
- Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J. A., Médale, F., Martin, S. A. M., Houlihan, D. F., Kaushik, S., Pérez-Sánchez, J. (2004). Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). *Aquaculture*, *232*(1–4), 493–510.
- Green, K. (2018). Fishmeal and fish oil facts and figures. https://www.seafish.org/document/?id=1B08B6D5-75D9-4179-9094-840195CEEE4B.
- Guerreiro, I., Couto, A., Machado, M., Castro, C., Pousão-Ferreira, P., Oliva-Teles, A., Enes, P. (2016). Prebiotics effect on immune and hepatic oxidative status and gut morphology of white sea bream (*Diplodus sargus*). *Fish & Shellfish Immunology*, 50: 168–174.

- Guerreiro, I., Oliva-Teles, A., Enes, P. (2015). Improved glucose and lipid metabolism in European sea bass (*Dicentrarchus labrax*) fed short-chain fructooligosaccharides and xylooligosaccharides. *Aquaculture*, 441: 57–63.
- Guerreiro, I., Oliva-Teles, A., Enes, P. (2018). Prebiotics as functional ingredients: focus on Mediterranean fish aquaculture. *Aquaculture*, 10: 800-832.
- Guerreiro, I., Serra, C.R., Pousão-Ferreira, P., Oliva-Teles, A., Enes, P. (2017). Prebiotics effect on growth performance, hepatic intermediary metabolism, gut microbiota and digestive enzymes of white sea bream (*Diplodus sargus*). *Aquaculture Nutrition*, 24(1): 153-163.
- Gupta, S., Abu-Ghannam, N. (2011). Bioactive potential and possible health effects of edible brown seaweeds. *Trends in Food Science & Technology*, 22(6): 315-326.
- Guttieri M.J., Bowen D., Dorsch J.A., Souza E. & Raboy V. (2004). Identification and characterization of a low phytic acid wheat. *Crop Science*, 44: 418-424.
- Hao, Y.T., Wu, S.G., Jakovlić, I., Zou, H., Li, W.X., Wang, G.T. (2017). Impacts of diet on hindgut microbiota and short-chain fatty acids in grass carp (*Ctenopharyngodon idellus*). *Aquaculture Research* 48: 5595–5605.
- Hao, Y.T., Wu, S.G., Jakovlić, I., Zou, H., Li, W.X., Wang, G.T. (2017). Impacts of diet on hindgut microbiota and short-chain fatty acids in grass carp (*Ctenopharyngodon idellus*). *Aquaculture Research*, 48: 5595–5605.
- Hemaiswarya, S., Raja, R., Ravi Kumar, R., Ganesan, V., Anbazhagan, C. (2011). Microalgae: a sustainable feed source for aquaculture. *World Journal of Microbiology and Biotechnology*, 27: 1737-1746.
- Henry, M., Gasco, L., Piccolo, G., Fountoulaki, E. (2015). Review on the use of insects in the diet of farmed fish: Past and future. *Animal Feed Science and Technology*, *203*(1), 1–22.
- Hernández, M. D., Martínez, F. J., Jover, M., García García, B. (2007). Effects of partial replacement of fish meal by soybean meal in sharpsnout seabream (*Diplodus puntazzo*) diet. *Aquaculture*, *263*(1–4), 159–167.
- Hoseinifar, S.H., Mirvaghefi, A., Merrifield, D.L., Amiri, B., Yelghi, S., Bastami, K. (2011). The study of some haematological and serum biochemical parameters of juvenile beluga (Huso huso) fed oligofructose. *Fish Physiology and Biochemistry*, 37: 91–96.
- Hoseinifar, S.H., Sharifian, M., Vesaghi, M.J., Khalili, M., Esteban, M.A. (2014). The effects of dietary xylooligosaccharide on mucosal parameters, intestinal microbiota and morphology and growth performance of Caspian white fish (*Rutilus frisii kutum*) fry. *Fish & Shellfish Immunology*, 39: 231–236.

- Hoseinifar, S.H., Sun, Y-Z., Caipang, C.M. (2017). Short-chain fatty acids as feed supplements for sustainable aquaculture: an updated view. *Aquaculture Research*, 48: 1380-1391.
- Hoseinifar, S.H., Yousefi, S., Van Doan, H., Ashouri, G., Gioacchini, G., Maradonna, F., Carnevali, O. (2020). Oxidative Stress and Antioxidant Defense in Fish: The Implications of Probiotic, Prebiotic, and Synbiotics. *Reviews in Fisheries Science & Aquaculture*, 29(2): 198-217.
- Hu, J., Lin, S., Zheng, B., Cheung, P.C.K. (2018). Short-chain fatty acids in control of energy metabolism. *Crit. Rev. Food Sci. Nutr.*, 58: 1243–1249.
- Jensen, A. (1993). Present and future needs for algae and algal products. *Hydrobiologia*, 260: 15-23.
- Jobling, M. (2001). Feed Composition and Analysis. In D. Houlihan, T. Boujard, & M. Jobling (Eds.), *Food Intake in Fish* (p. 442). Blackwell Science Ltd.
- Juárez, O.E., Lafarga-De la Cruz, F., Lazo, J.P., Delgado-Vega, R., Chávez-García, D., López-Landavery, E., Tovar-Ramírez, D., Galindo-Sánchez, C.E. (2021). Transcriptomic assessment of dietary fishmeal partial replacement by soybean meal and prebiotics inclusion in the liver of juvenile Pacific yellowtail (*Seriola lalandi*). *Molecular Biology Reports*, 48: 7127-7140.
- Kaushik, S.J., Covès, D., Dutto, G., Blanc, D. (2004). Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. *Aquaculture*, 230: 391-404.
- Kaushik, S.J., Doudet, T., Médale, F., Aguirre, P., Blanc, D. (1995). Protein and energy needs for maintenance and growth of Nile tilapia (*Oreochromis niloticus*). *J. Appl. Ichthyol.*, 11(3–4): 290–296.
- Kottelat, M., Freyhof, J. (2007). *Handbook of European freshwater fishes*. Publications Kottelat, Cornol and Freyhof, Berlin.
- Kousoulaki, K., Saether, B.S., Albrektsen, S., Noble, C. (2015). Review on European sea bass (*Dicentrarchus labrax*, Linnaeus 1758) nutrition and feed management: a practical guide for optimizing feed formulation and farming protocols. *Aquaculture Nutrition*, 21: 125-151.
- Kumar, V., Sinha, A.K., Makkar, H.P.S., De Boeck, G., Becker, K. (2011). Phytate and phytase in fish nutrition. *Journal of Animal Physiology and Animal Nutrition*, 96(3): 335-364.
- Kuz'mina, V.V. (1996). Influence of age on digestive enzyme activity in some freshwater teleosts. *Aquaculture*, 148: 25–37.
- Leenhouwers, J.I., Ortega, R.C., Verreth, J.A.J., Schrama, J.W. (2007). Digesta characteristics in relation to nutrient digestibility and mineral absorption in Nile tilapia (*Oreochromis niloticus* L.) fed cereal grains of increasing viscosity. *Aquaculture*, 273: 556–565.

- Leśnierowski G., Cegielska-Radziejewska R. (2012). Potential possibilities of production, modification and practical application of lysozyme. *Acta Sci.Pol. Technol. Aliment*., 11(3): 223-230.
- Liu, W., Yang, Y., Zhang, J., Gatlin, D.M., Ringø, E., Zhou, Z. (2014). Effects of dietary microencapsulated sodium butyrate on growth, intestinal mucosal morphology, immune response and adhesive bacteria in juvenile common carp (*Cyprinus carpio*) pre-fed with or without oxidized oil. *British Journal of Nutrition*, 112(1): 15-29.
- Lloris, D. (2002). *A world overview of species of interest to fisheries. Chapter: Dicentrarchus labrax*.
- Louis, P., Hold, G.L., Flint, H.J. (2014). The gut microbiota, bacterial metabolites and colorectal cancer. *Nature Reviews Microbiology*, 12: 661–672.
- Lu, F., Haga, Y., Satoh, S. (2015). Effects of replacing fish meal with rendered animal protein and plant protein sources on growth response, biological indices, and amino acid availability for rainbow trout *Oncorhynchus mykiss*. *Fisheries Science*, *81*(1), 95–105.
- Machado, M., Azeredo, R., Fontinha, F., Fernández-Boo, S., Coinceição, L.E.C., Dias, J., Costas, B. (2018). Dietary Methionine Improves the European Seabass (*Dicentrarchus labrax*) Immune Status, Inflammatory Response, and Disease Resistance. *Front. Immunol.*,
- Machado, M., Moura, J., Peixoto, D., Castro-Cunha, M., Coinceição, L.E.C., Dias, J., Costas, B. (2021). Dietary methionine as a strategy to improve innate immunity in rainbow trout (*Oncorhynchus mykiss*) juveniles. *General and Comparative Endocrinology*, 302.
- Martins, N., Estevão-Rodrigues, T., Diógenes, A.F., Diaz-Rosales, P., Oliva-Teles, A., Peres, H. (2018). Taurine requirement for growth and nitrogen accretion of European seabass (*Dicentrarchus labrax*, L.) juveniles. *Aquaculture*, 494: 19-25.
- Meena, D.K., Das, P., Kumar, S., Mandal, S.C., Prusty, A.K., Singh, S.K., et al. (2013). Betaglucan: an ideal immunostimulant in aquaculture (a review). *Fish Physiol. Biochem.*, 39 (3): 431–457.
- Menanteanu-Ledouble, S., Krauss, I., Santos, G., Fibi, S., Weber, B., El-Matbouli, M. (2015). Effect of a phytogenic feed additive on the susceptibility of *Onchorhynchus mykiss* to *Aeromonas salmonicida*. *Diseases of Aquatic Organisms*, 115: 57-66.
- Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., Baker, R.T.M., Bøgwald, J., et al. (2010). The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture*, 302: 1–18.
- Messina, M.T. (2007). Varying plant protein source in the diet of sea bass *Dicentrarchus labrax* differently affects lipid metabolism and fat deposition. *Italian Journal of Animal Science*, 6: 806-808.

- Mirghaed, A.T., Yarahmadi, P., Soltani, M., Paknejad, H., Hoseini, S.M. (2019). Dietary sodium butyrate (Butirex® C4) supplementation modulates intestinal transcriptomic responses and augments disease resistance of rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.*, 92: 621–628.
- Montalban-Arques, A., De Schryver, P., Bossier, P., Gorkiewicz, G., Mulero, V., Gatlin III, D.M., Galindo-Villegas, J. (2015). Selective manipulation of the gut microbiota improves immune status in vertebrates. *Front. Immunol.*, 6: 512.
- Morrison, D.J., Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes*, 7: 189–200.
- Mustafa, S.A., Al-Faragi, J.K. (2022). Supplementation of Feed Additives on Aquaculture Feeds: A Review. *International Journal of Pharmaceutical Research*, 13(1): 561-567.
- Mylonas, C., Kouretas, D. (1999). Lipid peroxidation and tissue damage. *In Vivo*, 13: 295-309
- National Geographic Society (2019). Sustainable fishing. https://www.nationalgeographic.org/encyclopedia/sustainable-fishing/
- Nayak, S.K. (2010). Role of gastrointestinal microbiota in fish. *Aquaculture research*, 41: 1553-1573.
- Ng, W-K., Koh, C-B. (2017). The utilization and mode of action of organic acids in the feeds of cultured aquatic animals. *Reviews in Aquaculture*, 9: 342-368.
- NRC (2011). Nutrient Requirements of Fish and Shrimp. *The National Academy Press*, Washington, DC.
- Nya, E.J., Dawood, Z., Austin, B. (2010). The garlic component, allicin, prevents disease caused by *Aeromonas hydrophila* in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.*, 33: 293–300.
- OECD (2021). "OECD Review of Fisheries Country Notes".
- Ohira, H., Tsutsui, W., Fujioka, Y. (2017). Are short chain fatty acids in gut microbiota defensive players for inflammation and atherosclerosis? *Journal of Atherosclerosis and Thrombosis*, 24: 660–672.
- Oliva-Teles, A. (2012). Nutrition and health of aquaculture fish. *Journal of fish disease*, 35: 83-108.
- Oliva-Teles, A., Enes, P., Peres, H. (2015). Replacing fish meal and fish oil in industrial aquafeeds for carnivorous fish. *Feed and Feeding Practices in Aquaculture*, 203-233.
- Omosowone, O., Adeparusi, E.O., Dada, A. (2018). Comparison of dietary butyric acid supplementation effect on growth performance and body composition of *Clarias gariepinus* and *Oreochromis niloticus* fingerlings. *Iranian Journal of Fisheries Sciences*, 17(2): 403-412.

- Owen, M., Waines, P., Bradley, G., Davies, S. (2006). The Effect of Dietary Supplementation of Sodium Butyrate on the Growth and Microflora of *Clarias Gariepinus* (Burchell). *Paper Presented at the Proceedings of the XII International Symposium Fish Nutrition and Feeding*.
- Palmer, M.F., Rolls, B.A. (1983). The Activities of Some Metabolic Enzymes in the Intestines of Germ-Free and Conventional Chicks. Br. J. *Nutr.*, 50: 783–90.
- Pandey, A., Satoh, S. (2007). Effects of organic acids on growth and phosphorus utilization in rainbow trout *Oncorhynchus mykiss*. *Fish Sci*, 74: 867–874.
- Pavia, M.J.T.R., Ishikawa, C.M., Cocuzza das Eiras, A., Risaffi da Silveira, V. (2004). Effects of an experimental challenge with Mycobacterium marinum on the blood parameters of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1757). *Biological and Applied Sciences*, 47(6).
- Pavlidis, M., Futter, W.C., Kathario, P., Divanach, P. (2007). Blood cells of six Mediterranean mariculture fish species. *J. Appl. Ichtyol.*, 23: 70-73.
- Peres, H., Costas, B., Perez-Jimenez, A., Guerreiro, I., Oliva-Teles, A. (2015). Reference values for selected hematological and serum biochemical parameters of Senegalese sole (*Solea senegalensis* Kaup, 1858) juveniles under intensive aquaculture conditions. *J. Appl. Ichthyol.*, 31: 65-71.
- Peres, H., Oliva-Teles, A. (2002). Utilization of raw and gelatinized starch by European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture*, 205(3-4): 287-299.
- Piazzon, M.C., Calduch-Giner, J.A., Fouz, B., Estensoro, I., Simó-Mirabet, P., Puyalto, M., Karalazos, V., Palenzuela, O., Sitjà-Bobadilla, A., Pérez-Sánchez, J. (2017). Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiome*, 5: 164.
- Pourmozaffar, S., Hajimoradloo, A., Miandare, H.K. (2017). Dietary effect of apple cider vinegar and propionic acid on immune related transcriptional responses and growth performance in white shrimp, *Litopenaeus vannamei*. *Fish & Shellfish Immunology*, 60: 65-71.
- Raa, J. (1996). The Use of Immunostimulatory Substances in Fish and Shellfish Farming. *Fisheries Science*, 4(3): 229-288.
- Raa, J. (2000). The use of immune-stimulants in fish and shellfish feed. *Avances en Nutrición Acuícola V*. Memorias del V Simposium Internacional de Nutrición Acuícola. 19-22 Noviembre, 2000. Mérida, Yucatán, Mexico.
- Reichardt, N., Vollmer, M., Holtrop, G., Farquharson, F.M., Wefers, D., Bunzel, M., Duncan, S.H., Drew, J.E., Williams, L.M., Milligan, G., Preston, T., Morrison, D., Flint, H.J., Louis, P. (2018). Specific substrate-driven changes in human faecal microbiota composition contrast with functional

redundancy in short-chain fatty acid production. *The ISME Journal*, 12: 610–622.

- Reverter, M., Bontemps, N., Lecchini, D., Banaigs, B., Sasal, P. (2014). Use of plant extracts in fish aquaculture as an alternative to chemotherapy: current status and future perspectives. *Aquaculture*, 433: 50–61.
- Ridaura, V.K., Faith, J.J., Rey, F.E., Cheng, J., Duncan, A.E., Kau, A.L., Griffin, N.W., Lombard, V., Henrissat, B., Bain, J.R., et al. (2013). Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science*, 341: 1241214
- Rimoldi, S., Finzi, G., Ceccotti, C., Girardello, R., Grimaldi, A., Ascione, C., Terova, G. (2016). Butyrate and taurine exert a mitigating effect on the inflamed distal intestine of European sea bass fed with a high percentage of soybean meal. *Fisheries and Aquatic Sciences*, 19: 40.
- Ringø, E. (1991). Effects of dietary lactate and propionate on growth and digesta in Arctic char, *Salvelinus alpinus* (L.). *Aquaculture*, 96(3-4): 321-333.
- Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C.G., Salazar, N. (2016). Intestinal short chain fatty acids and their link with diet and human health. *Frontiers in Microbiology*, 7: 185.
- Safari, O., Shahsavani, D., Paolucci, M., Atash, M.M.S. (2014). Single or combined effects of fructo- and mannan oligosaccharide supplements on the growth performance, nutrient digestibility, immune responses and stress resistance of juvenile narrow clawed crayfish, *Astacus leptodactylus leptodactylus* Eschscholtz, 1823. *Aquaculture*, 432: 192–203.
- Safari, R., Hoseinifar, S.H., Kavandi, M. (2016). Modulation of antioxidant defense and immune response in zebra fish (*Danio rerio*) using dietary sodium propionate. *Fish Physiology and Biochemistry*, 42: 1733-1739.
- Sangari, M., Sotoudeh, E., Bagheri, D., Morammazi, S., Torfi Mozanzadeh, M. (2021). Growth, body composition, and hematology of yellowfin seabream (*Acanthopagrus latus*) given feeds supplemented with organic acid salts (sodium acetate and sodium propionate). *Aquaculture International*, 29: 261-273.
- Satheeshkumar, P., Ananthan, G., Senthilkumar, D., Khan, A.B., Jeevanantham, K. (2012). Comparative investigation on haematological and biochemical studies on wild marine teleost fihes from Vellar estuary, southeast coast of India. *Comp. Clin. Path.*, 21: 275-281.
- Selim, K.M., Reda, R.M. (2015). Beta-glucans and mannan oligosaccharides enhance growth and immunity in Nile tilapia. *North American Journal of Aquaculture*, 77: 22–30.
- Sepulcre, M.P., López-Castejón, G., Meseguer, J., Mulero, V. (2007). The activation of gilthead seabream professional phagocytes by different PAMPs underlines the behavioural diversity of the main innate immune cells of bony fish. *Mol. Immunol.*, 44: 2019-2026.

- Sepulcre, M.P., Sarropoulou, E., Kotoulas, G., Meseguer, J., Mulero, V. (2007). *Vibrio anguillarum* evades the immune response of the bony fish sea bass (*Dicentrarchus labrax* L.) through the inhibition of leukocyte respiratory burst and down-regulation of apoptotic caspases. *Molecular Immunology*, 44(15): 3751-3757.
- Shepherd, C. J., & Jackson, A. J. (2013). Global fishmeal and fish-oil supply: Inputs, outputs and markets. *Journal of Fish Biology*, *83*(4), 1046–1066.
- Sinha, A.K., Kumar, V., Makkar, H.P.S., De Boeck, G., Becker, K. (2011). Non-starch polysaccharides and their role in fish nutrition – A review. *Food chemistry*, 127(4): 1409-1426.
- Soleimani, N., Hoseinifar, S.H., Merrifield, D.L., Barati, M., Abadi, Z.H. (2012). Dietary supplementation of fructooligosaccharide (FOS) improves the innate immune response, stress resistance, digestive enzyme activities, and growth performance of Caspian roach (*Rutilus rutilus*) fry. *Fish Shellfish Immunol.*, 32: 316–321.
- Song, S.K., Beck, B.R., Kim, D., Park, J., Kim, J., Kim, H.D., Ringø, E. (2014). Prebiotics as immunostimulants in aquaculture: a review. *Fish & Shellfish Immunology*, 40: 40–48.
- Spinelli, J. (1980). Unconventional Feed Ingredients for Fish Feed. In *Fish Feed Technology*. Rome, Italy.
- Stevens, M.G., Kehrli, M.E., Canning, P.C. (1991). A colorimetric assay for quantitating bovine neutrophil bactericidal activity. *Vet Immunol Immunopathol*, 28: 45-56.
- Sugiura, S.H., Roy, P.K., Ferraris, R.P. (2006). Dietary acidification enhances phosphorus digestibility but decreases H+/K+-ATPase expression in rainbow trout. *J. Exp. Biol.*, 209(19): 3719-3728.
- Sun, M., Wu, W., Liu, Z., Cong, Y. (2017). Microbiota metabolite short chain fatty acids, GPCR, and inflammatory bowel diseases. *Journal of Gastroenterology*, 52: 1–8.
- Sunyer, J.O., Tort, L. (1995). Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. *Veterinary Immunology and Immunopathology*, 45: 333-345.
- Swain, P., Nayak, S.K., Nanda, P.K., Dash, S. (2008). Biological effects of bacterial lipopolysaccharide (endotoxin) in fish: A review. *Fish & Shellfish Immunology*, 25: 191-201.
- Szer, C.D., Kaaci, O.H., Saka, S., Firat, K., Otgucuoglu, O., Kucuksari, H. (2008). Lactobacillus spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: effects on growth performance and digestive enzyme activities. *Aquaculture*, 280: 140–145.
- Tan, J., McKenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R., Macia, L. (2014). Chapter three- The role of short-chain fatty acids in health and

disease. In: Alt FW (ed) *Advances in Immunology*, pp. 91–119. Academic Press, San Diego, CA.

- Tavares-Dias, M., Moraes, F.R. (2007). Leukocyte and thrombocyte reference values for channel catfih (*Ictalurus punctatus* Raf.), with an assessment of morphological, cytochemical, and ultrastructural features. *Vet. Clin. Pathol.*, 36: 49-54.
- Thirunavukkarasar, R., Kumar, P., Sardar, P., Prasad Sahu, N., Harikrishna, V., Pada Singha, K., Shamna, N., Jacob, J., Krishna, G. (2022). Protein-sparing effect of dietary lipid: Changes in growth, nutrient utilization, digestion and IGF-I and IGFBP-I expression of Genetically Improved Farmed Tilapia (GIFT), reared in Inland Ground Saline Water. *Animal Feed Science and Technology*, 284.
- Tiwari, C.B., Pandey, V.S. (2014). Studies of hematology and histology in *Labeo rohita* infected with cutaneous columnaris disease. *Zool. Surv. India*, 114: 151-157.
- Tocher, D.R. (2010). Fatty acids requirements in ontogeny of marine and freshwater fish. *Aquaculture Research*, 41: 717-73
- Torrecillas, S., Makol, A., Caballero, M.J., Montero, D., Gines, R., Sweetman, J., et al., 2011. Improved feed utilization, intestinal mucus production and immune parameters in sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides (MOS). *Aquacult. Nutr.*, 17 (2): 223–233.
- Tran, H. Q., Prokesova, M., Zare, M., Matousek, J., Ferrocino, I., Gasco, L., Stejskal, V. (2022). Production performance, nutrient digestibility, serum biochemistry, fillet composition, intestinal microbiota and environmental impacts of European perch (*Perca fluviatilis*) fed defatted mealworm (*Tenebrio molitor*). *Aquaculture*, 547
- Tran, N.T., Li, Z., Wang, S., Zheng, H., Aweya, J.J., Wen, X., Li, S. (2020). Progress and perspectives of short-chain fatty acids in aquaculture. *Reviews in Aquaculture*, 12: 283-298.
- Turnbaugh, P.J., Gordon, J.I. (2009). The core gut microbiome, energy balance and obesity. *J. Physiol.*, 587: 4153–4158.
- Tusche, K., Arning, S., Wuertz, S., Susenbeth, A., Schulz, C. (2012). Wheat gluten and potato protein concentrate - Promising protein sources for organic farming of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *344–349*, 120–125.
- Vandeputte, M., Gagnaire, P.-A., Allal, F. (2019). The European sea bass: a key marine fish model in the wild and in aquaculture. *Animal Genetics*, 50: 195-206.
- Vega-Bautista, A., de la Garza, M., Carrero, J.C., Campos-Rodríguez, R., Godínez-Victoria, M., Drago-Serrano, M.E. (2019). The Impact of Lactoferrin on the Growth of Intestinal Inhabitant Bacteria. *International Journal of Molecular Sciences*, 20(19): 4707.

- Wahbeh, M.I. (1997). Amino acid and fatty acid profiles of four species of macroalgae from Aqaba and their suitability for use in fish diets. *Aquaculture*, 159: 101-109.
- Wang, Y., Guo, J. L., Bureau, D. P., Cui, Z. H. (2006). Replacement of fish meal by rendered animal protein ingredients in feeds for cuneate drum (*Nibea miichthioides*). *Aquaculture*, *252*(2–4), 476–483.
- Wang, Y., Li, J., Lin, J. (2008). Probiotics in aquaculture: challenges and outlook. *Aquaculture*, 281: 1-4.
- Wang, Y.B. (2006). Effect of probiotics for common carp (*Cyprinus carpio*) based on growth performance and digestive enzyme activities. *Anim. Feed Sci. Technol*., 127: 283–292.
- Wang, Y.B. (2007). Effects of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. *Aquaculture*, 269: 259–264.
- Wayman, C. (2016). Microbes and the gut–brain axis. *Lancet Gastroenterol. Hepatol.*, 1: 183.
- Welker, T.L., Lim, C., Yildirim-Aksoy, M., Klesius, P.H. (2012). Effect of short-term feeding duration of diets containing commercial whole-cell yeast or yeast subcomponents on immune function and disease resistance in channel catfish, *Ictalurus punctatus*. *J. Anim. Physiol. Anim. Nutr.*, 96 (2): 159–171.
- Windisch, W., Schedle, K., Plitzner, C., Kroismayr, A. (2008). Use of phytogenic products as feed additives for swine and poultry. *J. Anim. Sci.*, 86: E140–E148.
- Worm, B., Barbier, E.B., Beaumont, N., Emmett Duffy, J., Folke, C., Halpern, B. S., Jackson, J. B. C., Lotze, H. K., Micheli, F., Palumbi, S. R., Sala, E., Selkoe, K. A., Stachowicz, J. J., Watson, R. (2006). Impact of biodiversity loss on ocean ecosystem services. *Science*, 314: 787-790.
- Xiong, J., Nie, L., Chen, J. (2018). Current understanding on the roles of gut microbiota in fish disease and immunity. *Zoological Research*, 40(2): 70-76.
- Yuan, Y.V., Walsh, N.A. (2006). Antioxidant and antiproliferative activities of extracts from a variety of edible seaweeds. *Food and Chemical Toxicology*, 44(7): 1144-1150.
- Zare, R., Kenari, A.A., Sadati, M.Y. (2021). Influence of dietary acetic acid, protexin (probiotic), and their combination on growth performance, intestinal microbiota, digestive enzymes, immunological parameters, and fatty acids composition in Siberian sturgeon (*Acipenser baerii*, Brandt, 1869). *Aquaculture International*, 29: 891-910.
- Zhang, C.N., Li, X.F., Xu, W.N., Jiang, G.Z., Lu, K.L., Wang, L.N., Liu, W.B. (2013). Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on innate immunity, antioxidant capability and disease

resistance of triangular bream (*Megalobrama terminalis*). *Fish Shellfish Immunol.*, 35: 1380–1386.

- Zhang, H., Ding, Q., Wang, A., Liu, Y., Teame, T., Ran, C., Yang, Y., He, S., Zhou, W., Olsen, R.E., Zhang, Z., Zhou, Z. (2020). Effects of dietary sodium acetate on food intake, weight gain, intestinal digestive enzyme activities, energy metabolism and gut microbiota in cultured fish: Zebrafish as a model. *Aquaculture*, 523.

This work will be presented at the Aquaculture Europe 22 in Rimini as “SHORT CHAIN FATTY ACIDS AS FUNCTIONAL INGREDIENTS TO BOOST EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) GROWTH PERFORMANCE AND HEALTH STATUS.” (Fontinha et al.).

**Introduction**

Novel aquafeeds usually include high levels of plant feedstuffs (PF). However, most PF has antinutritional factors (ANF) that may jeopardize nutrient digestion and metabolic utilization, increasing fish susceptibility to oxidative stress, intestine inflammation, and disease susceptibility. Recent evidence showed that short chain fatty acids (SCFA) improve animals’ digestive function, immunological and oxidative responses, and modulate intestine microbiota. However, there is a lack of knowledge regarding single use or potential synergetic effects of SCFAs in fish fed with PF-rich diets. Therefore, the main goal of this work is to evaluate the potential of using SCFAs as functional ingredients in PF-rich diets on growth performance, immune status, and disease resistance of European sea bass one of the most important fish species in the Mediterranean aquaculture.

**Material and Methods**

European sea bass juveniles (initial body weight 15.2g) were randomly distributed into twenty-one tanks (twenty-five fish per tank) were fed for 56 days in triplicate tanks with seven isoproteic (44% crude protein) and isolipidic (18% crude lipids) diets with different short chain fatty acids and different percentage supplementation: Control diet; Diet SA 0.25 (sodium acetate at 0.25%), Diet SA 0.50 sodium acetate at 0.50 %), SP 0.25 (sodium propionate at 0.25%), SP 0.50 (sodium propionate at 0.50%) SB 0.25 (sodium butyrate at 0.25%), SB 0.50 (sodium butyrate at 0.50%). At the end of the growth trial in order to evaluate the immune status three fish per tank were collected.
After the growth trial fish were relocated to the challenge room. Six fish per diet (eighteen in total) were randomly selected and infected intraperitoneally (p.i) injected with 100ul of vibrio anguillarum (1.2x10 7 CFU/ mL -1 ). Four and twenty-four hours after the p.i infection, fish were sampled to assess the haematological profile and innate immune parameters.

To access the survival, fifteen fish per tank (thirty per diet) were injected. These fish were fed with the experimental diets and the mortality recorded for 3 weeks.
Data are presented as mean and the pooled standard error of the mean (SEM). Data were analysed for normality and homogeneity of

variance and, when necessary, transformed before being treated statistically.
Statistical analysis was performed with a three-way ANOVA, with time, fatty acid and levels of supplementation as the main factors. When significant interaction occurred one-way ANOVA for each factor was performed. The significant differences among means (p<0.05) were determined by Tukey's multiple range test.
Non-orthogonal contrasts between control and other treatments were performed in each sampling time.
All the statistical analysis was performed in the SPSS version 27.0 software package (IBM® SPSS® Statistics, New York, USA).

**Results**

Dietary treatment had no effects on the growth performance of European seabass juveniles.
Regarding blood parameters, all decreased with time regardless of fatty acid or its level of supplementation in relation to time 0 (prior infection), except for mean corpuscular haemoglobin concentration (MCHC). All parameters showed a tendency to recover at 24h.
Mean corpuscular volume (MCV) is higher in fish fed sodium butyrate than sodium acetate.
In innate immune parameters, all diets present a decrease in lysozyme activity and peroxidase activity at 4 and 24h post-infection. This decrease may be related to the lower WBC values at 4h.
Peroxidase activity showed recovery at 24h in sodium acetate at 0.25 and 0.5% and at sodium propionate at 0.25%.
Lysozyme activity was higher in fish fed sodium butyrate when compared with sodium acetate, despite the inclusion level.
The survival was highest in fish fed sodium butyrate 0.5% (93.3 vs. 66.7% SP 0.25%), there were no differences in survival between treatments and control.