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&

BIOTECNOLOGIE

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Use of alternative protein sources and probiotics in the diet of farmed fish species: effects on the intestinal microbiome and health.

Utilizzo di fonti proteiche alternative e probiotici nella dieta di specie ittiche allevate: effetti sul microbioma e salute intestinale.

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Abstract

It is estimated that the global population might reach and overtake the mark of 9 billion people before 2050. Strictly linked to this growing trend is the food demand, which at current food production rates cannot satisfy such a large number of people all over the world. The future goal, in accordance with the 17 Sustainable Development Goals (SDGs), is to provide food and livelihoods to human population, in a sustainable manner, minimizing the environmental impact and improving the quality of life of the people. For this purpose, aquaculture, among the other agriculture and food-producing sectors, is the one that is still growing and expanding worldwide and is the most promising industry to meet the future demand for animal protein. To achieve this goal, the most important challenge facing the entire sector is the development of new fish feed formulations that fulfill fish nutritional requirements: the gold standard ingredients, fish meal (FM) and fish oil, represent finite resources as they heavily impact marine natural resources for production. Hence, the aim of the present PhD research project was to investigate the effects of different innovative strategies to replace the protein fraction of the feed from FM to alternative sources and also to evaluate the administration of a bacterial probiotic strain. The main focus of this experimentation was to assess how novel ingredients and feed additives modulate fish gut microbiota composition. Indeed, the microbial populations that inhabit the gastro-intestinal tract of animals play a fundamental role in the host physiology, too. For this reason, it is also called the "extra organ", as it takes part in numerous functions such as early-stage development, reproduction, immune response and nutrition, which is the primary interest of this study. Microbiota, divided into autochthonous and allochthonous populations, contribute to digestion thanks to the great versatility and potential metabolic pathways by which a plethora of nutritional compounds, such as complex

carbohydrates and fiber, which otherwise would remain indigestible for the host, are subject to hydrolysis and dissociation. In addition, the autochthonous microbial populations can produce a wide range of bioactive molecules, such as short-chain fatty acids (SCFA) and vitamins, which have an important impact on host intestinal physiology, and anti-microbial compounds, which also guarantee protection against the colonization of pathogens. Hence, the approach used in this project to investigate the effect of partial and total substitution of marine-based protein with two different insect larvae meals, and the administration of two doses of lactic-acid probiotic bacteria, on the fish intestinal microbiota, involved setting up three experimental trials using two species, a freshwater fish, rainbow trout (*Oncorhynchus mykiss*), and a marine Mediterranean species, gilthead sea bream (*Sparus aurata*). In the first study (**Chapter 2**), we investigated the effect of partial replacing dietary FM with 15% insect meal, specifically *Hermetia illucens*, on the microbiota composition of rainbow trout. The results demonstrate how this experimental diet could effectively modulate the intestinal microbiota of the fish, reducing Proteobacteria, which include several pathogenic genera, for example *Aeromonas sp.,* while increasing the percentage of beneficial bacteria such as *Lactobacillus* and *Bacillus*. In addition, the metagenomic analysis clearly demonstrates how insect diets enhance the metabolic capacity of the trout gut microbiota, improving dietary carbohydrate utilization. In the second trial (**Chapter 3**), we tested the effects of total replacement of FM with another insect species larvae meal: in particular we used *Tenebrio molitor* larvae, on rainbow trout skin and gut microbiota. After 22 weeks of experimentations, the results did not reveal any negative alterations in the bacterial populations between the two dietary groups, but only slight differences, mostly detected at the genus and family level both for skin and gut microbiota. Finally, in the last feeding trial (**Chapter 4**), we evaluated the effects of two doses (high and low dose) of lactic-acid bacteria (*Lactococcus lactis* subsp. *lactis*), used as a probiotic in gilthead sea bream. The analyses focused

on fish growth performance, morphological alterations of the intestine, gut microbiota composition, and the expression of a panel of 44 genes, including markers of epithelial integrity, nutrient transport, mucins, cytokines, immunoglobulins, cell markers and chemokines, and pattern recognition receptors. Interestingly, the results showed that the probiotic actually had an effect according to several of the aspects analysed: the final body weight of the fish fed the higher dose of probiotic was greater than that of the control group; in addition, though without appreciable structural modification of the gut, significant differences in the expression of key genes involved in innate and acquired immunity were detected, suggesting an enhancement of the immune system due to *L. lactis* administration. Regarding gut microbiota, the analyses revealed a lack of colonization of the probiotic in the host's intestinal mucosa; however, the probiotic did modulate the fish gut microbiota, confirming that colonization is not always necessary to induce host modification. Data obtained in this PhD project contribute to the knowledge gained so far on the application of different strategies to modulate gut microbiota so as to strengthen and enlarge the digestion capacity of fish in a framework of innovations in aquaculture that aim to promote positive effects on fish growth performance, metabolism, health, feed conversion ratio, and final product quality, in view of future growing food demand.

Chapter 1

Introduction

1.1 World Population and demand for livestock products

The demographic situation of the world has changed very rapidly in the last few decades. It is a fact that the world population is currently more than three times larger than in the mid-twentieth century. This trend, however, does not follow a linear progression. On the contrary, in 2020, the growth rate of the population was less than 1% per year for the first time since 1950, and the projection estimated that this level will continue to slow in the near future (Fig. 1). This phenomenon is occurring despite the fact that in some countries, such as those which compose Central-Southern Asia and sub-Saharan Africa, population size will continue to increase. In contrast, it is estimated that in Europe and Northern America the population will soon start to decline, negatively affecting the global growth rate.

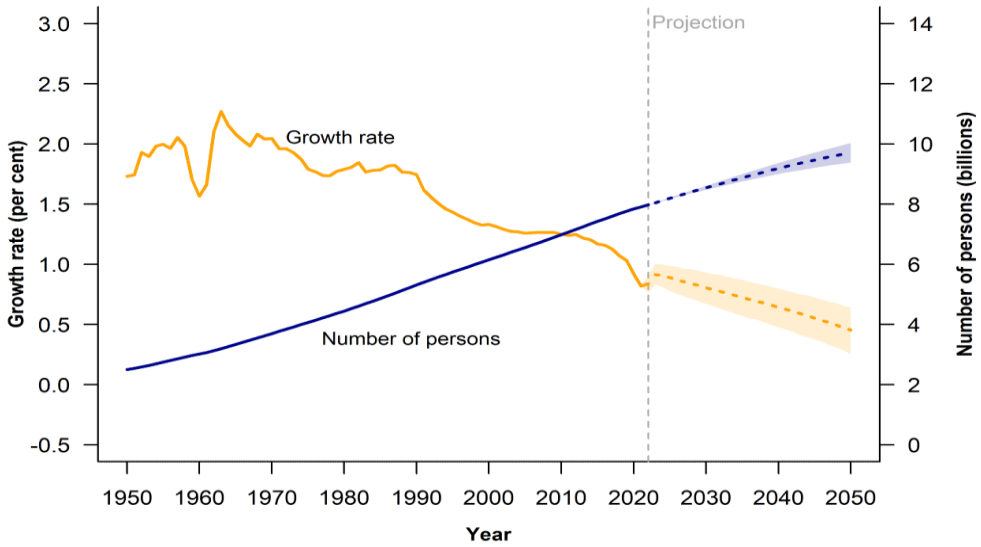


Figure 1. *Global population size and annual growth rate: estimates, 1950-2022, and medium scenario with 95 per cent prediction intervals, 2022-2050* (United Nations Department of Economic and Social Affairs, 2022)

However, despite this proclivity in the demographic situation, the world population could grow to around 9.7 billion in 2050 and 10.9 to 12.3 billion in

2100, according to the different scenarios and variables considered (Fig. 1) (Gerland et al., 2014; United Nations Department of Economic and Social Affairs, 2022). The impact of this slow but progressive trend is directly linked with the development and expansion of the food industry. In fact, the present and the future challenge of the world of food producers and scientists, in accordance with the 17 Sustainable Development Goals (SDGs), is to provide food and livelihoods for the ever-growing human population in a sustainable manner, minimizing the environmental footprint on the planet and improving the quality of life of the people that inhabit it (Glaser, 2012). Hence, the role of agriculture and in general food security, defined as "access to sufficient, safe, nutritious food to maintain a healthy and active life", is pivotal for achieving these goals (FAO (Food and Agriculture Organization of the United Nations), 2021). Considering the food derived from animal sources, the livestock system occupies 30% of the planet's ice-free terrestrial surface area today, and, contrary to crop production, whose growth is mostly related to yield increase, animal husbandry needs geographical expansion and an increase in the number of herds. The combination of these two factors will generate, in the near future, strong competition for the use of the arable lands(Thornton, 2010; Flachowsky et al., 2017). In addition, as reported by (Poore and Nemecek, 2018) (Fig.2), livestock farming has the greatest impact on the environment, due to greenhouse gases emission (GHG), disruption of nitrogen and phosphorus cycles and the impoverishment of biodiversity (Gilbert et al., 2018). However, the ineluctable growth of the world population will lead to an obvious increase in the demand for livestock products. It is worth mentioning that this pressure is not equally distributed in the population. Income and urbanization are the two main drivers determining the distribution of the animal-source food demand, and they will continue in the foreseeable future. Livestock products consumption, divided by the different types (Fig. 3A), is high in the richest countries (Fig. 3B) and particularly in the wealthier strata of societies, in low-

income countries, too, and as income will continue to increase in highly populated and developing countries, demand levels are likely to rise as well. Urbanization is the other factor that heavily impacts the patterns of food consumption, also because it often stimulates improvements in infrastructure, including in cold chains, which enables perishable goods to be traded more widely. It is estimated that in the next few decades more people will move to urban settings from rural areas at an unprecedented rate, particularly in Africa and Asia, determining a strong increase in demand in the most populated regions of the planet (Thornton, 2010; Béné et al., 2015).

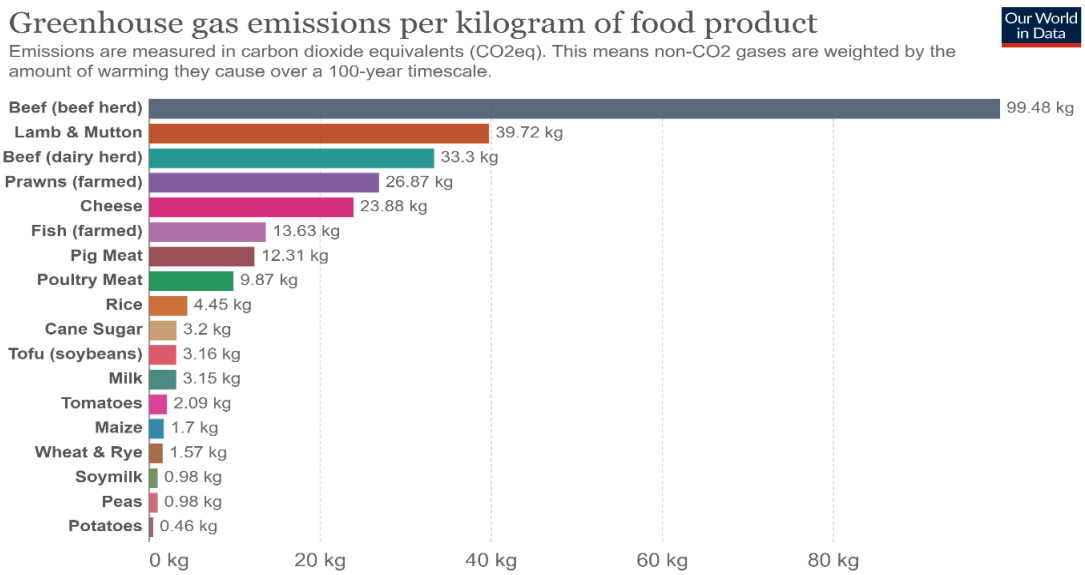


Figure 2. *Global GHG emission of different food product in 2010* (Poore and Nemecek, 2018) available at ourworldindata.org/environmental-impacts-of-food.

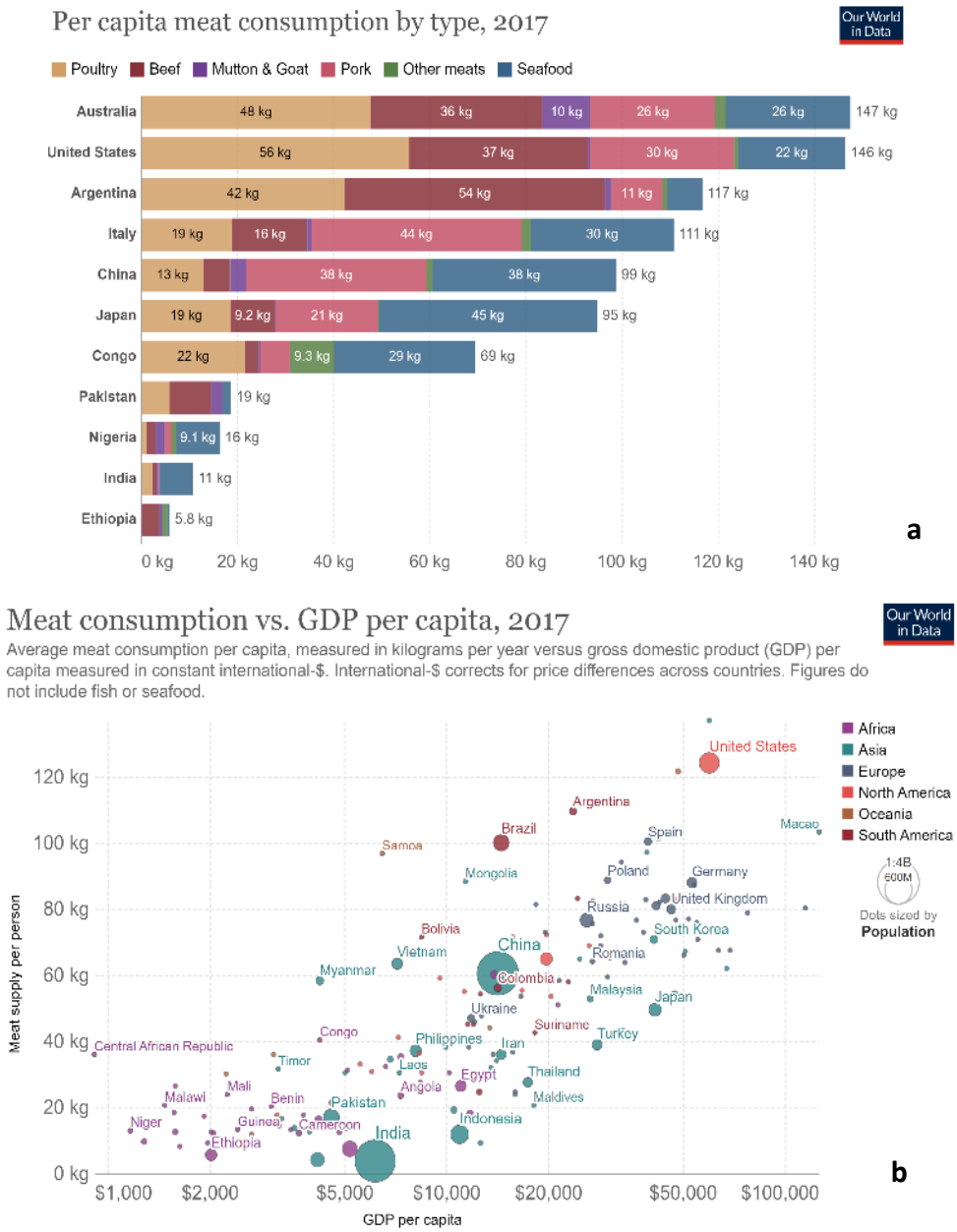


Figure 3. a Per capita meat consumption divided for 6 different types of animal-source food. **b** Meat consumption in relation to the income (GDP) and size population of different countries (FAO (2020) FAOSTAT database collections (Food and Agriculture Organization of the United Nations, Rome). Available at faostat.fao.org and ourworldindata.org/meat-production.

1.2 Modern Aquaculture

Among the different animal production industries that must meet the future growing demand of food, fisheries and aquaculture represent the most promising fields, as this is the fastest expanding source of animal protein in the world today. The global supply has grown by a factor of 8 since 1950, even greater than the improvements in rice production that followed the Green Revolution. In 2010 it was estimated that fish overshadowed the other animal-productive systems, double that of poultry and even triple that of cattle (Fig. 4a) (Béné et al., 2015). In 2020, the average per capita consumption of fish was around 20.2 kg year^{-1} and represents the end point of an ongoing growth in demand (1.5% per year) since the 1960s when consumption only amounted to 9.9 kg. Moreover, the distribution is not equal throughout the world. It is estimated that for 3.2 billion people capture fisheries and aquaculture provide almost 20% of their per capita intake of animal protein; otherwise, in some African and Asian countries, such as Cambodia, Bangladesh, Mozambique and Sierra Leone, often characterized by low-income and food-deficiency, this share can exceed 50-60% (Fig 4b) (FAO, 2022).

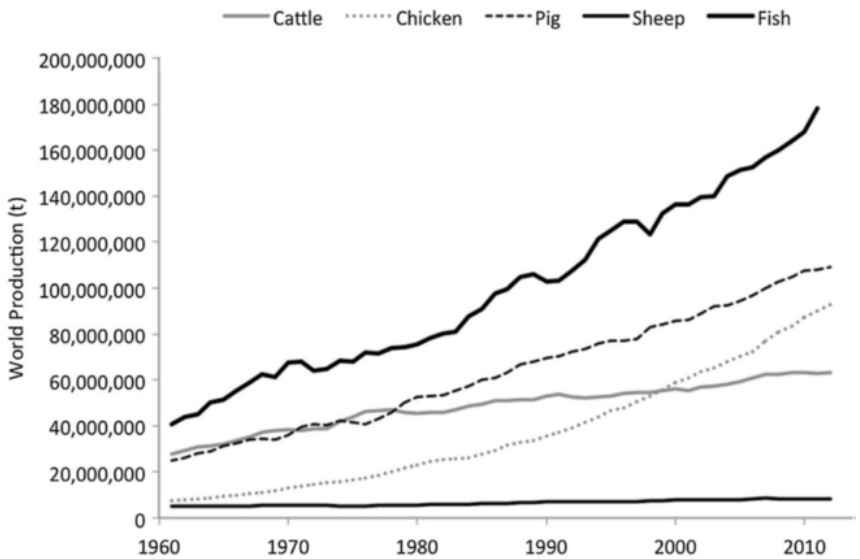


Figure 4. a World Production of the main sources of animal protein over the period 1960–2010 (Béné et al., 2015).

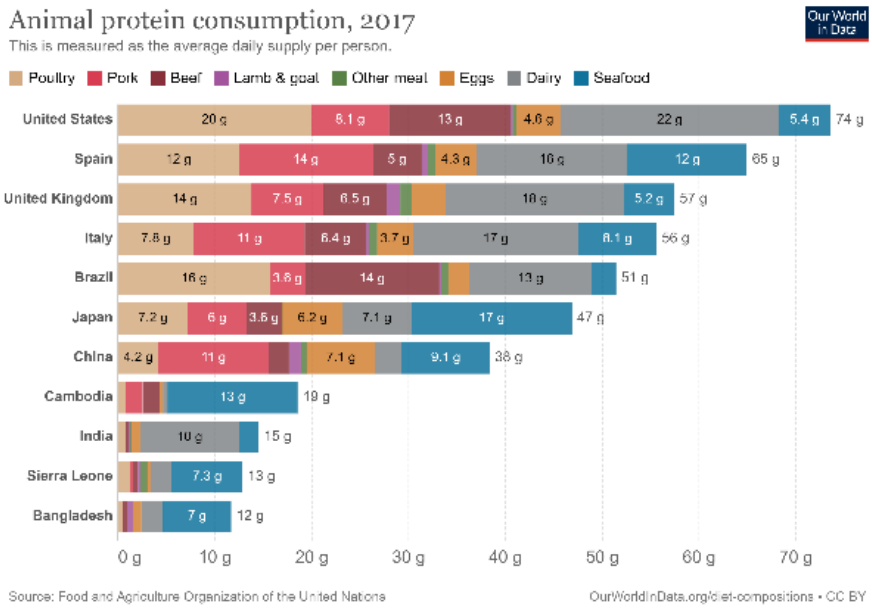


Figure 4. b Animal Protein consumption of different countries (FAO (2020) FAOSTAT database collections (Food and Agriculture Organization of the United Nations, Rome). Available at faostat.fao.org and ourworldindata.org/fish-and-overfishing.

In 2020 the global production of aquatic animals reached 178 million tons, of which 63% came from marine waters, but only 37% from inland waters, which is slightly lower than the two previous years (Fig. 5). This modest stagnation is mostly linked with a decline in capture fisheries, which is due to different factors, such as the fluctuation catches of pelagic species, for example, anchoveta, but also because of the recent reduction in China's catches and the disruptive impact of COVID-19 on the production sector. Nevertheless, fishery production remains the largest part (51% of the total volume, 90 million tons), with a stable fluctuation between 93-86 million tons per year since the late 1980s (FAO, 2022). It is worth mentioning that, although aquaculture volume production is slightly inferior to that of capture fishery (88 million tones, excluding algae production), it accounts for almost twice (65%) the value of capture over total estimates (USD 406 billion). Hence, aquaculture represents the main driver of total production growth, also because increasing the exploitation from oceans could aggravate the

environmental status of numerous endangered stocks. It is estimated that nowadays around 33-34% of all fish populations are overexploited, beyond their natural biological sustainability (Hilborn et al., 2020). As evidence of the boost that aquaculture gave to the total industry production, owing mostly to the development of inland production, growth production gradually increased from 12.6 (18%) in the 1990s to 54.4 million tons in 2020, representing more than half of the total (62.2%), as shown in Fig. 5.

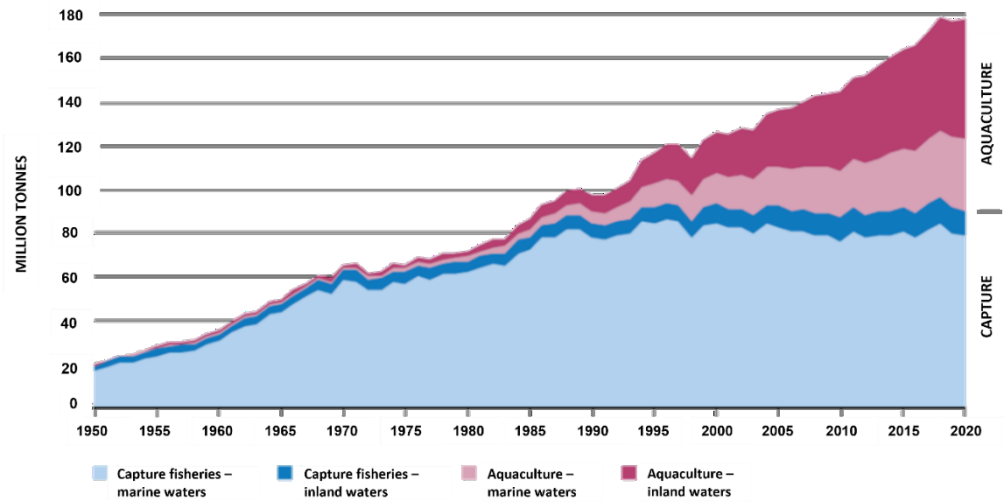


Figure 5. World Capture Fish and Aquaculture Production over the period 1950-2020 (FAO, 2022).

Regarding the main producers, Fig. 6 clearly shows that Asia overwhelmingly dominates world aquaculture, producing approximately 91% of global aquatic animals and algae. However, there are huge differences within the continent, with many developing countries improving their infrastructure remarkably to fully express their potential. China produces more farmed aquatic organisms than the rest of the world, and in addition, the overall situation is characterized by a small number of other aquaculture producers. Many of them, including Chile, Brazil, Egypt, Bangladesh, and Vietnam, are highly populated developing countries. However, Norway also represents an example of a great producer owing to its

large areas of fjords and can boast a huge production of finfish in sea cages, mostly represented by salmon (FAO, 2022). For the aquatic species currently being cultivated, the conditions in which aquaculture is carried out vary highly and an enormous number of species are farmed, but a limited group of them ("staple species") dominate global production by far. Although it is difficult to make an exhaustive assessment, the total number of units that aquaculture has produced worldwide was calculated to be around 652 in 2020, including a certain level of taxonomic uncertainty and hybrids. However, as already mentioned, carp, Atlantic salmon, milkfish, tilapia, and catfish represent only a few examples of the approximately 20-25 dominant finfish species produced that account for over 75% of the total production. In addition, it is worth mentioning that, although marine and diadromous fish species and crustaceans are the main organisms farmed in certain geographical areas, for example, the Mediterranean basin, at the global level their number is dwarfed by the live-weight volume of freshwater aquaculture products, bivalves, and also seaweeds (Fig. 7) (Naylor et al., 2021; FAO, 2022).

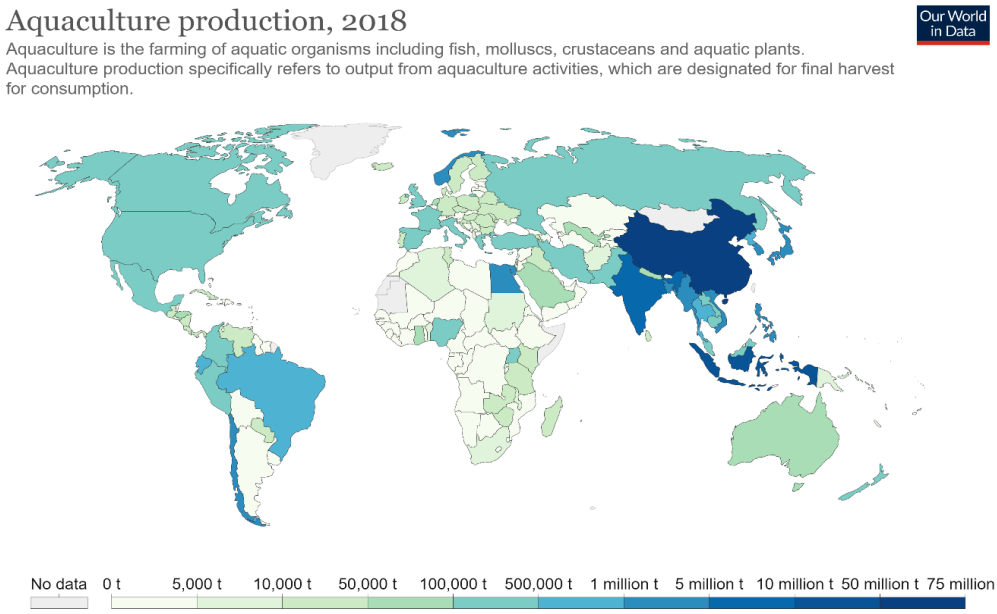


Figure 6 The distribution of the main aquaculture fish farming producers by country. Available at datacatalog.worldbank.org/search/dataset/0037712/World-Development-Indicators & ourworldindata.org/fish-and-overfishing.

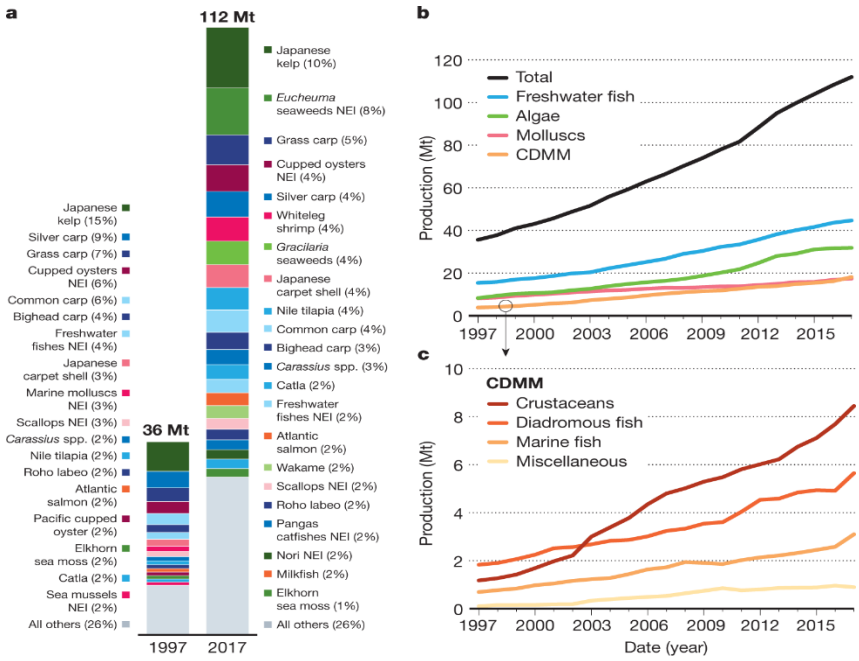


Figure 7. a Species composition shown for 1997 and 2017. **b, c** Growth shown from 1997 to 2017 for the following production categories (b): total, freshwater fish, algae, mollusks and CDMM, which comprises crustaceans, diadromous fish, marine fish, and miscellaneous species (Naylor et al., 2021).

1.3 Aquaculture Feeds

As already mentioned, aquaculture, which is the fastest growing food commodity sector and today accounts for an average of 17% of the total amount of animal protein intake globally, it is estimated that it will play an even more pivotal role in meeting the increasing demand of food in the future. To achieve this goal, the challenge will be fought on political, economic, and technological playing fields. Hence, the entire sector must accomplish the tasks of optimizing and introducing new reforms, diversifying the market demand on a global scale, and even more importantly, developing and implementing sustainable feed formulas and breeding techniques (Costello et al., 2020). The production of aquatic animals is largely dependent upon the external administration of feeds. According to the last estimates, about 70% of the farmed animals worldwide are "feeding" species, while the remaining part is composed of "filter-feeding" species. The manufactured diets, in addition to being one of the highest expenses for the farmers, constitute the vector for providing a properly balanced amount of nutrients, preserving fish health, and improving production. It is easy to understand why fish nutrition is the most innovative branch of the aquaculture sector (Tacon and Metian, 2015). Historically, fish meal (FM) and fish oil (FO) constitute the gold standard for feed production ingredients, as they have been used for decades, not only in the aquaculture sector, but also, in different proportions, for all the others animal-producing industries, such as pig farming (9%), pet food (4%) and poultry (1%) (Fig. 8). It was estimated for 2020 that, from all the fisheries and aquaculture production (178 million tons), about 89% was used for direct human consumption, and the remaining part (over 20 million tons) was converted for non-food purposes. Concerning the latter, excluding a small amount of about 4 million tons that is commonly utilized in ornamental fish trade, in pharmaceutical preparations, for pet food, or as a direct feeding source in aquaculture, the greater part is used to produce FM and FO. FM is a very protein-

rich flour, obtained by milling and drying fish, whereas FO is made by pressing cooked fish and then extracting oil by centrifugation. The typical fish species used for these purposes are mainly small pelagic fish such as anchoveta, mackerel, herring, sardine etc.: in the recent past, the annual fluctuation in the catches of those animals, together with the increasing demand for FM and FO, has brought about a high fluctuation in market prices with a progressively rising scenario, a trend which presumably will continue in the foreseeable future.

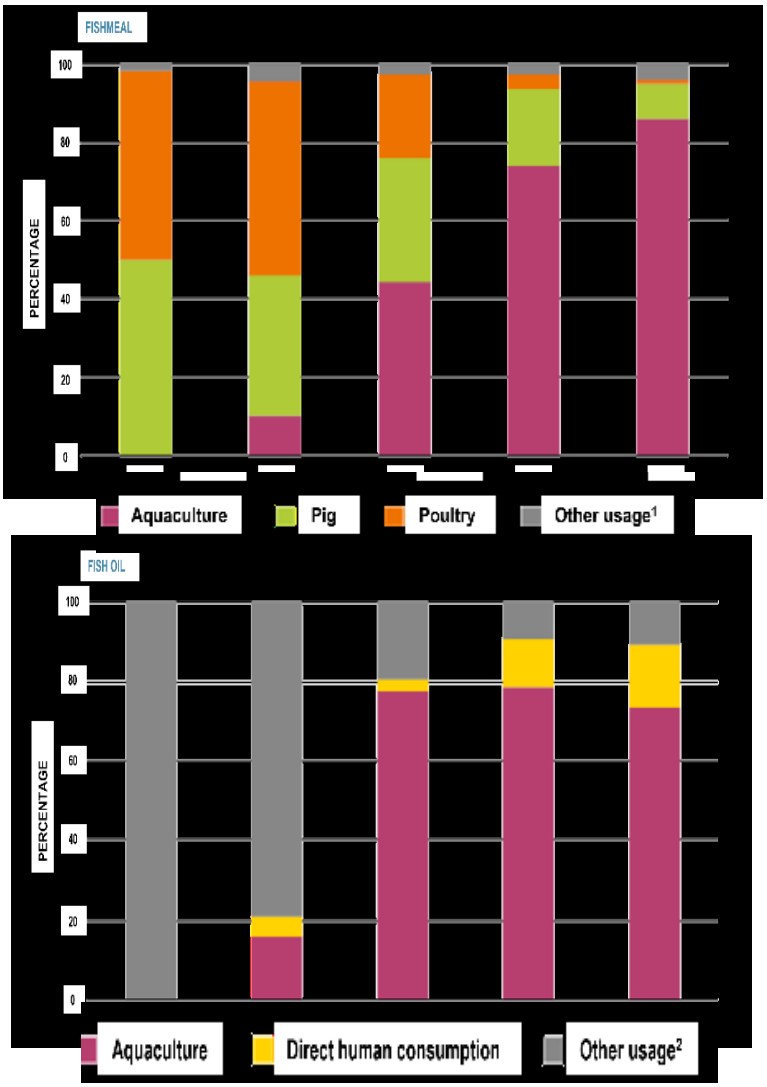


Figure 8. Fishmeal (**a**) and Fish oil (**b**) global utilization over the period 1960-2020. [1] Mainly pet feed; [2] Pet food, biofuel, cooking oil in Viet Nam. (FAO, 2022)

One of the consequences of this situation is the worldwide level of including these ingredients in feed formulations, which has decreased within the past few decades, even for those diets designed for marine piscivorous finfish, such as sea bream, sea bass, rainbow trout, and Atlantic salmon, which require 45-50% of crude protein and a high level of long-chain fatty acids (FAs) (Naylor et al., 2009). Nevertheless, FM and FO are still used and considered the most nutritious and digestible source of protein and lipids for farmed fish, as well as ideal resources to meet the essential amino acid (EAA) requirement and the major supply of omega-3 FAs (eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]). In fact, some specific production stages, such as hatchery, broodstock or in the finishing period before harvesting, continue to use them massively, due to their metabolic and nutritional importance (FAO, 2022). FM and FO oil represent ideal feed ingredients for aquaculture because they are not only an excellent source of dietary protein, EAAs, and essential FAs, but they possess a profile that can satisfy the nutritional requirements of most farmed aquatic species. Indeed, they are a good source of nucleotides, phospholipids, minerals, and trace elements (including calcium, phosphorus, magnesium, zinc, manganese, selenium, iodine, molybdenum, and chromium), fat-soluble and water-soluble vitamins (including vitamin A, D, E, choline, inositol, and B vitamins), and unique nutrients such as taurine, together with other components that have not been identified yet. In addition, they have no antinutritional factors, limited carbohydrates, and fiber content (Tacon and Metian, 2015; Turchini et al., 2019). However, as already mentioned, although FM and FO were originally used because they were, at the time, inexpensive and palatable sources of protein and lipid, today, the rate of including them in fish feed is decreasing on average by 1.7% per year due to their high fluctuating market value, but also for the awareness of environmental issues, underlying the production of these valuable ingredients (Bandara, 2018). The sustainability goal of modern aquaculture converges here with the need to reduce

the sector's dependence on marine resources, as they represent a finite supply, with at most a very small further exploitation for only some species, and with the aim of identifying valid and nutritionally adequate alternatives (Boyd et al., 2020). The efforts that will have to be made in the name of sustainability agree with the definition of the a "sustainable development" given by the United Nations World Commission on Environment and Development, which define it as "use of the environment and resources that meets the needs of the present without compromising the ability of future generations to meet their own needs" (World Commission on Environment and Development, 1987). During the past several years, numerous alternatives to the conventional marine ingredients have been implemented in feed formulations. The choice of candidate that represent a viable alternative is related to certain characteristics, such as nutritional suitability, ready availability, easy handling, shipping, storage etc. In addition, is very important that these new ingredients benefit the fish in terms of health maintenance, growth performance, and lower environmental impact, and, finally, the price must be competitive in order to overtake the other replacements. Nowadays, the principal sources currently included are vegetable meals, oilseed meals, and animal by-products, not only from fisheries and aquaculture sector, but also from other fields such as poultry livestock. Furthermore, more recently, interest in other organisms and biotechnological applications has been aroused for fish nutrition. Those new sources are insects, which possess very interesting metabolic abilities, but also Single-cell Ingredients (SCI), proteins and oils (SCP; SCO), produced and extracted from algae, bacteria, and yeasts. All these new possibilities are discussed extensively in the following paragraphs.

1.3.1 Vegetable meals and oils

Vegetable meals and oils represent the oldest and the principal alternatives tested as a basis for the animal feed in the last decades. Nowadays, the commonly

available commercial fish feeds, designed for most farmed aquatic animals, include a fair percentage of vegetable stuffs. The advantage of being readily available globally and the relatively low costs compared to products of animal origin, especially FM, represent strong points in their favor. The range of plant feedstuffs that are usually implemented in aquaculture commercial diets include barley, canola, corn, cottonseed, peas/lupins, soybeans, wheat, oilseeds (soybean, sunflower, rapeseeds, cottonseed) etc. (Naylor et al., 2009). From the nutritional point of view, the ideal ingredient for fish feed must possess certain characteristics, which include low level of fiber, non-soluble carbohydrates, and antinutrients. In addition, they must provide a high amount of protein, with a favorable amino acids profile, and an elevated digestibility and palatability. Unfortunately, including considerable levels of vegetable meals and oils could have adverse effects in fish, as this may affect feed intake, nutrient digestibility, immune response, stress, and histological alterations, expressed as enteritis (Mourente et al., 2007; Torrecillas et al., 2017). The negative consequences are the results of an imbalanced amino acid profile, insufficient to totally compensate for the EAAs, such as methionine, lysine, or cysteine, which are required by the animals, together with a lower concentration of omega-3 FAs, and instead these plant-based ingredients are high in medium-chain triglycerols (MCT), saturated fatty acids (SFAs), and omega-6 and omega-9 FAs, such as oleic (18:1n-9) and linoleic (18:2n-6) acids. In addition to that, the most challenging constraints to using a plant-based diet is the presence of anti-nutritional factors, which represent the ultimate defense of the plants against predators, but which, once consumed, could negatively affect the digestive capacity of the fish. They are in fact defined as “substances which by themselves, or through their metabolic products arising in living systems, interfere with food utilization and affect the health and production of animals” (Makkar, 1993). These compounds are chemically heterogenous and thus also have different modes of action, but they can be divided into heat-labile and heat-stable molecules. The

former, such as lectins, protease inhibitors, and amylase inhibitors, are heat-labile proteins, which can be inactivated by heat, while the latter, which cannot be destroyed by the high temperature, are typically phytic acids, saponins, phenols, and tannins (Francis et al., 2001). Although several undesirable features are associated with vegetable ingredients, they have largely been implemented in the diet formulations for aquaculture in the recent past. The strategy to circumvent these obstacles can be achieved by technological procedures. To increase the protein content, the carbohydrate fraction is removed from soybean, corn, or gluten meal in order to obtain protein-concentrated ingredients. As previously mentioned, some anti-nutritional factors are heat labile; thus, they can be eliminated by increased temperatures, such as during the extrusion process, with preliminary heat treatments, or by fractioning the crops. Finally, heat-stable compounds are eliminated by using enzymatic treatments or solvent purification to enhance the nutritional value of the feeds, avoiding the adverse effects (Bandara, 2018). In conclusion, terrestrial plant ingredients now comprise the largest FM and FO partial or total replacement used in fish feed formulations, mostly implemented not as a unique source, but rather in combination, to supply a correct balance of EAAs and FAs, which are fundamental for the species-specific fish requirements. In addition, the value of vegetable feedstuffs also resides in the possibility to reduce aquaculture's pressure on the fishery industry, and, regarding human health as well, to avoid the consumption of dioxins and PCBs, which are completely absent in terrestrial plants derivates.

1.3.2 Animal by-products

Another interesting source of proteins and lipids currently being used to partially substitute FM and FO in aquaculture is represented by the valorization of rendered products from terrestrial and aquatic animals. Commercially, the principal available ingredients are meat and bone meal, feather meal, blood meal, PAPs, and

seafood by-product meal. The importance of these resources also lies in the framework of mitigating the environmental impact of the industries. In fact, the animal by-products industry fits perfectly in the concept of a circular economy, where refuse from one industry is regenerated for other industries for feed and energy, reducing waste loss, the carbon footprint, and GHG emissions (Woodgate et al., 2022). Regarding terrestrial animal protein sources, animal by-products have a more balanced amino acid profile than the previously discussed vegetable feedstuffs, with higher contents of lysine and a considerable digestibility. In contrast, although the price of terrestrial animal-derived oils is very competitive compared to FO, these lipids sources are rich in SFAs, which strongly reduce the digestive capacity of the fish, especially at cold temperatures. Thus, as complete substitution cannot be achieved, they must be blended with polyunsaturated FAs (PUFAs) to be nutritionally adequate for the fish requirements. Despite this, animal lipids can surely contribute to reducing the over-exploitation of natural resources due to the use of marine ingredients (Naylor et al., 2009). The principal terrestrial animal-producing field providing such by-products is surely the poultry industry. The Association of American Feed Control Officials defines Poultry By-Products (PBM) as the 'ground, rendered, clean parts of the carcass of slaughtered poultry such as necks, heads, feet, undeveloped eggs, gizzards and intestines (provided their content is removed), exclusive of feathers (except in such amounts as might occur unavoidably in good processing practices)' (AAFCO 2010). Although PBM meal can change in nutritional value and quality due to the materials used and the production protocols, an average level of protein content is around 51-81% of dry matter, with a relatively good amino acid profile. However, as reported by Gasco et al., (2018) (Tab. 1), in comparing PBM, FM and soybean meal (SBM), major concerns are related to the low level of EAAs such as lysine and methionine, but also, compared to FM, the lower content of taurine, which, though not properly considered to be an EAA, it is fundamental for maintaining

good growth performance and avoiding susceptibility to disease and high mortality (Salze and Davis, 2015). In addition, as previously mentioned, PBM, like other land animal ingredients, has a range of 6.7-22.5% of lipids, but a very low content of omega 3 FAs, which can cause severe problems mostly for juveniles or marine fish species at high percentages of FM substitutions. Nevertheless, PBM are largely considered a cost-effective feed ingredient in fish feed formulations and can constitute a valid alternative to FM, and partially FO, for a very large number of fish species. In parallel to the terrestrial animal by-products, the other important group of rendered ingredients derives from the so-called seafood by-products. It is estimated that around 20 and 80% of fish is considered as waste by industries, depending on the fish species and the type of processing and elaboration of the resource. In this context, the refuse includes head, viscera, skin, bones, and scales (Fig. 9) (Caldeira et al., 2018). As a consequence of removing the fillet, the total amount of protein in the resulting meal is lower that of FM, but still presents a rich source of EAAs such as lysine and leucine, together with a huge amount of minerals, for example, hydroxyapatite, calcium, phosphate, zinc, selenium, and iron (Naylor et al., 2009). FO is extracted mostly from oily fish such as herring and mackerel, but valorization of the waste from other species still contributes to the total FO production, though with a with lower market value due to the reduced amount of omega 3 FAs. Fish waste is also an important source of value-added compounds. These molecules are a matter of interest not only for the fish feed industry, but also for the health-related sector, for example, cosmetics, the pharmaceutical industry, and medical care. Some examples are collagen, gelatin, obtained by thermal denaturation of collagen, and bioactive peptides, which consist in sequences of 2-20 amino acids and possess multiple biological activities, based on their composition. Another important molecule extracted from shellfish waste is chitin, the second-most abundant polysaccharide in the world, after

cellulose, and carotenoids, also used in fish feed as functional ingredient or additive (al Khawli et al., 2020).

	Unit	PBMa	FMb	SBMc
Dry Matter (DM)	% as fed	93.7 (82.4–97.4)	92.1 (90.0–94.4)	87.9 (85.0–92.1)
Crude protein	% DM	66.1 (51.6–81.0)	75.6 (70.2–80.7)	51.4 (48.3–54.5)
Lysine	% protein	4.4 (3.3–8.2)	6.1 (5.5–7.5)	6.1 (5.7–6.6)
Methionine	% protein	1.4 (1.0–2.0)	2.2 (2.0–2.6)	1.4 (1.2–1.6)
Methionine + Cistine	% protein	–	2.9 (2.6–3.2)	2.9 (2.5–3.3)
Tryptophan	% protein	0.5 (0–0.8)	0.8 (0.7–0.9)	1.3 (1.2–1.4)
Threonine	% protein	2.8 (1.9–3.9)	3.1 (2.9–4.3)	3.9 (3.5–4.3)
Leucine	% protein	5.0 (3.9–9.7)	5.9 (5.2–7.3)	7.5 (6.8–8.0)
Isoleucine	% protein	2.7 (1.8–4.7)	3.7 (3.3–4.4)	4.6 (4.3–5.0)
Valine	% protein	3.1 (2.2–5.2)	4.2 (3.9–4.8)	4.8 (4.3–5.4)
Histidine	% protein	1.9 (1.2–5.6)	1.8 (1.7–1.9)	2.6 (2.4–2.9)
Arginine	% protein	5.1 (3.2–8.8)	4.6 (4.0–6.0)	7.4 (6.8–8.1)
Phenylalanine	% protein	2.8 (2.2–4.0)	5.5 (5.2–6.5)	8.5 (7.7–9.4)
Ether extract	% DM	13.8 (6.7–22.5)	8.1 (2.0–12.0)	2.1 (2.0–2.2)
Crude fibre	% DM	1.1 (0.5–2.1)	–	6.7 (3.5–10.1)
Minerals (ash)	% DM	15.0 (5.1–29.7)	16.6 (12.0–23.3)	6.9 (6.8–7.0)
Calcium	% DM	5.1 (2.2–9.9)	36.3 (15.4–78.3)	3.9 (2.3–6.3)
Phosphorus	% DM	2.7 (1.6–5.0)	25.9 (19.0–40.4)	6.9 (5.8–8.6)
Sodium	% DM	0.6 (0.5–1.0)	10.0 (5.9–14.4)	0.1 (0.0–0.8)
Potassium	% DM	0.8 (0.4–1.8)	10.2 (5.9–14.4)	23.7(21.8–26.0)
Gross energy	MJ/kg	21.2 (16.2–24.9)	21.4 (19.6–23.8)	19.9 (19.8–20.0)

Table 1. Nutrient composition and nutritive value of poultry by-product meal (PBM) compared to fishmeal (FM) and soybean meal (SBM). Values are reported as mean of values found in the literature (with minimum and maximum values) (Gasco et al., 2018).

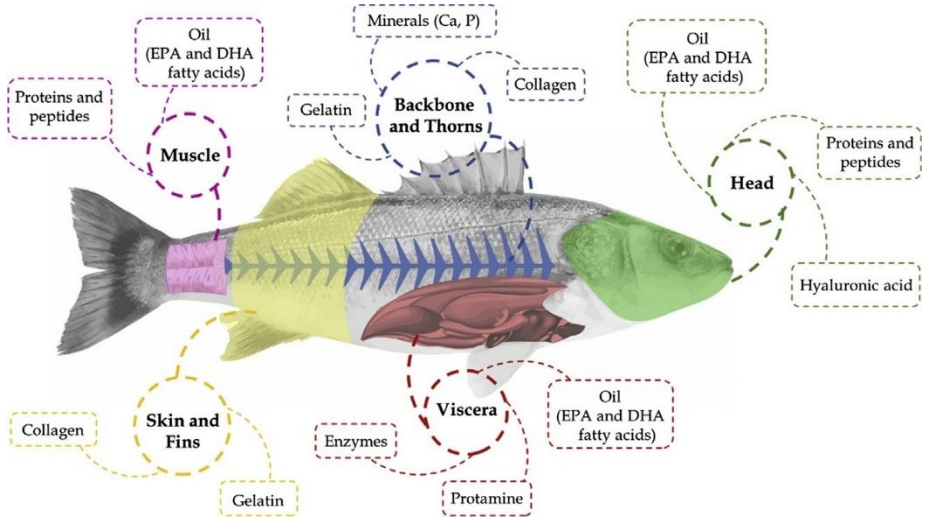


Figure 9. Fish by-products and main compounds obtained from them (al Khawli et al., 2020).

1.3.3 Single-cell Ingredients

Another innovative strategy to reduce FM and FO in the diet of farmed fish is to use microbial feed ingredients. These products have gained wider attention in the last few decades, as their production and use in the human food industry is far older than application in the aquaculture sector. In fact, these alternative sources have been used since the early 1950s, mostly with the purpose of finding a new way to produce protein, but only in 1966 the name Single Cell Protein (SCP) was coined, to describe the protein content obtained from a biomass composed of unicellular organisms, with few rare exceptions. The microbial sources commonly utilized to produce SCP are microalgae, yeast and other fungi, and bacteria. Each of them possesses unique advantages and challenges (Tab. 2), but generally, the goal of production is the maximization of cellular growth and co-products yields, with an economically and environmentally sustainable approach. Although the cellular harvest varies, the main advantages in using microbes to produce proteins over traditional methods lies in their short generation and duplication times, the easy transformation of the yields, and the ability and efficiency in use and in

converting a wide spectrum of growing substrates; and finally, they do not need to be planted or occupy arable land, and the seasons have no effect, which means a potentially enormous geographical dispersion (Nasseri et al., 2011). These favorable features are achieved thanks to the large number of usable microorganisms and, consequently, their strongly diversified metabolism and growth modalities, including autotrophs, photoautotrophs, chemoautotrophs, methylotrophs, heterotrophs and mixotrophs. Among the aforementioned organisms, yeasts and fungi have been used for a long time both in livestock and for direct human consumption (brewery and bakery); thus, they have a high grade of familiarity and acceptability among producers and consumers. The most widely known species are *Saccharomyces cerevisiae*, various *Aspergillus* sp. and *Fusarium venenatum*, but other strains are attracting growing interest for protein replacement. Typically, the protein content is lower than for other microbes (45-65%) and, in addition, even with high levels of threonine and lysine, the amount of methionine is relatively low. However, the high levels of B-complex vitamins, their larger size (easy to harvest) and the possibility of being used as a probiotic make them a widely used source of protein in aquaculture (Øverland et al., 2013; Bandara, 2018). Differently from yeasts, microalgae are currently used in aquaculture mainly as a supplement or functional ingredient, although the nutritional profile is very similar to that of FM (high protein content (60-70%) with a low nucleic acid content, vitamins A, B, C and E). This is due to their photoautotrophic metabolism. There are still some technical limitations for production, however, and further development is required to reduce costs on a large scale (Naylor et al., 2009). In addition, microalgae possess a cellulosic cell wall, which represents about 10% of the dry weight, which, if not disrupted or eliminated, limits the bioavailability of the nutrients and the general digestibility of the ingredient. However, even with some differences among the species (e.g., *Chlorella* sp., *Scendesmus* sp., *Spirulina* sp., *Dunaliella* sp.) the greatest potential

for using microalgae in aquaculture resides in their ability to produce highly nutritionally valuable oils (single-cell oils, SCOs), rich in omega 3 PUFAs, such as EPA and DHA, together with other compounds, including carotenoid pigment, widely used in several industries (Sprague et al., 2017). In the same way, heterotrophic marine protists, previously classified as microalgae or fungi, such as *Schizochytrium limacinum*, are of particular interest because of their ability to produce omega 3 FAs (Ye et al., 2015). Finally, similarly to yeasts, bacteria also have a long history of being used to produce protein and oil. They are obviously the most diversified group of organisms, compared to those mentioned previously, but generally they contain a very high amount of protein (50-80%, or even >80% on dry weight basis), high levels of EAA (only slightly low level of lysine, compared to FM), along with vitamins, especially those of the B group, phospholipids, and other functional compounds. Despite this, bacterial SCP present a lipid profile dominated by C 16:0 and C 16:1 omega 7, and a high nucleic acid content (8–12%), especially RNA, and thus, as already reported for yeasts, they require processing prior to usage as food/feed. Bacterial SCPs have been receiving more and more attention in the last few years thanks to their incredible metabolic plasticity, which allows them to be used as different substrates (Nasseri et al., 2011). Most of these microbial ingredients can be obtained by treating waste or using refuse from refinery processes, with only minimum dependence on soil, water, and climatic conditions. Materials considered wastes or by-products retain a high commercial value as energy sources: for example, gas oil, methane, CO_2 and H_2, second-generation sugars, methanol, and alkanes are all potential substrates for unicellular fermenters organisms (Ritala et al., 2017). Additionally, the agricultural and forestry industries can massively contribute to provide convertible materials. Cellulose is the most abundant polysaccharide in the world, but in nature it has a complex structure, as in like lignin, starch etc.; if chemically or enzymatically pretreated, this enormous resource could be used as fermentable

sugar to produce microbial biomass. The revaluation of waste materials will play a pivotal role in the future economy, as it serves multiple functions. It can reduce pollution by transforming environmental burdens into edible protein and lipids, in the framework of a circular economy, reducing, in addition, the industrial production costs. SCIs have been demonstrated to have the potential to provide a sustainable, renewable feed ingredient to make up for the deficiencies of plant-based meals and reduce the need for FM in diets, as reported in numerous feeding trials conducted with the most common farmed fish (Jones et al., 2020).

	Protein content	Special characteristics	Example of specific organisms	Challenges
Microalgae	60-70%	- Phototrophic growth - Production of omega 3 fatty acids	- *Chlorella vulgaris* - *Desmodesmus* sp.	- Economical scale-up - Cell disruption to release nutrients
Yeasts	30-50%	- Use of a variety of feedstocks - Production of vitamins and micronutrients	- *Saccharomyces cerevisiae* - *Candida utilis*	- Improve protein and EAA content
Bacteria	50-80%	- High protein content - Growth on C1 substrates	- *Methylococcus capsulatus* - *Cupravidus nectar*	- Palatability issues
Protists	10-20%	- Production of omega-3 fatty acids	- *Schizochytrium Limacinum*	- Improve protein content

Table 2. Summary of SCP sources with protein content range, Special characteristics, most used organisms, and challenges (Jones et al., 2020).

1.3.4 Insects

A large number of insects are part of the natural diet of numerous freshwater species including tilapia, carp, and trout, in contrast to marine environments, in which, apart from very rare cases, insects are practically absent. Hence, the use of this source as part of fish feed diets seems to be a reasonable approach, and, in fact, interest in testing and using it in aquaculture has grown significantly in recent years. The use of insect-derived PAP in aquafeeds in Europe has been permitted since July 2017 (Commission Regulation (EU) 2017/893 of 24 May 2017). The

list of insects currently used as feed worldwide is long, but in Europe, the authorized insect meal may only include 7 species: *Hermetia illucens* (HI, Black Soldier Fly), *Musca domestica* (MD, Housefly), *Tenebrio molitor* (TM, Yellow Mealworm), *Alphitobius diaperinus* (Lesser Mealworm), *Acheta domesticus* (House cricket), *Gryllodes sigillatus* (Banded cricket), and *Gryllus assimilis* (Field Cricket); two belonging to the order of Diptera, two Coleoptera and three Orthoptera, respectively (Barroso et al., 2014). Even considering all the available scientific literature, only few species have the potential to be used and produced on a large scale, thanks to their particular metabolism, alimentary behaviors, and life cycle. The principal species currently receiving considerable attention for aquaculture feed formulations are HI, TM, and MD (Gasco et al., 2018). Generally, the nutritional value of these insects largely depends on several factors, for example, the stage of development of the animal (larva, pupa, prepupa, imago, or adult) and the growing substrates used to rear the larvae, considering both the diet administered through it, and the rearing conditions. This is particularly true not so much for the protein content, which can vary in the range of 10-70% of dry matter, but for maintaining on average an amino acid profile similar to that of the FM and SBM, even with deficiencies in lysine and/or methionine, depending on the insect source. Instead, the larvae substrates can strongly influence the lipid fraction of the animal, both in terms of quantity and quality (Nogales-Mérida et al., 2019). The fatty content of the larvae is usually around 6-40% of dry matter and is characterized by a high percentage of SFAs and omega 9 and omega 6 unsaturated FAs, such as oleic, lauric, linoleic and palmitic acids; however, like other terrestrial-based products, insects are devoid of omega 3 PUFAs, which in contrast are fundamental for marine fish species, as they are almost unable to synthesize the required amount by themselves. For this reason, the use of defatted insect meals obtained with physical or chemical extraction methods is common. However, another possible solution exploits the metabolic plasticity of the larvae,

which, as previously mentioned, can modify the lipid profile of insects reared on different growing substrates. For instance, it has been reported that replacing the substrate from cow manure to a mix (50:50) of cow manure and fish offal increased the level of omega-3 FAs in HI larvae from 0.2% to 2%, and the total lipid concentration from 20 to 31% (Tran et al., 2015). This ability can also be optimized to reduce contamination and to convert undesirable by-products, the elimination of which would involve an economic and environmental effort. The carbohydrates content of the insect is generally low, around 20%, and contains fiber, sugars, starches, and chitin (a nitrogen-containing polysaccharide), which represent the peculiar molecules of arthropods and the principal constraints to using the insect in fish diet formulations. Chitin is a polysaccharide of glucosamine and N-acetylglucosamine joined by a b-1,4 glycosidic bond, which constitute a very strong link and, as a consequence, render the chitin fibers not completely digestible by monogastric animals. The chitin percentage and composition can vary according to the life stage of the animals, but it is generally around 10% of dry weight. In addition, chitin fibers are directly connected to structural proteins, which define the final strength of the cuticle; hard cuticles have high protein contents between 70% and 85% and low chitin contents of 15–30%, whereas soft cuticles contain approximately 50% each of chitin and proteins (Sánchez-Muros et al., 2014; Nogales-Mérida et al., 2019). Although the presence of chitinase, chitobiase, and lysozyme has been reported in numerous fish species, the complex matrix of the chitin fibers limits the efficiency of the enzymes, reducing nutrient digestibility and protein bioavailability (Gasco et al., 2018). In contrast, it is worth mentioning that low levels of chitin in the diet can increase activity of the innate immune system, stimulating macrophage activity, act as a prebiotic by selectively stimulating the growth of beneficial gut bacteria and promoting their colonization, and improve growth performance in different farmed species. In conclusion, the ability of the insect to optimize wastes, organic side

streams, render them very good recyclers that can transform refuse from different industries into sustainable, high-protein ingredients that can be incorporated in fish feed, replacing more expensive compound ingredients, such as FM (Sánchez-Muros et al., 2014; Guerreiro et al., 2018).

1.3.5 Feed Additives

Along with macro-ingredients that constitute the gross composition of the commercial aquaculture diets designed to supply the nutritional requirements of the animals and guarantee the normal physiological functions and the healthy status of the fish, an increasing number of feed additives have been being used in the last few years. The nature and the spectrum of action of these additives are very diversified, but generally, adding them aims to preserve or increase the bioavailability of certain feed characteristics, improve fish performances or ability, or, if necessary, inactivate or eliminate the presence of certain molecules. Strictly related to maintaining fish health, more and more studies are demonstrating how the gut microbiota of the fish plays a fundamental role, not only in digestive and absorption functions, but also in animal welfare and growth performances. Hence, the correct management of the fish microbiome is crucial, and feed additives are very good candidates for modulating and restoring the eubiotic state of the intestinal environment (Encarnação, 2016). The first group of additives are phytogenics (PFA), which have a long history of being used in swine and poultry, but their use in the aquaculture sector is increasing. PFAs are composed of a very heterogeneous group of molecules, mostly commercialized as essential oils, including terpenoids, phenol-derived aromatic components, and aliphatic components. Their effects on animals depends on the nature of the chemical compounds, but, generally, PFAs are utilized to stimulate the appetite, modulate gut microbiota, and stimulate gastric juices, enhancing the immune system, and they also have antimicrobial, antioxidant, and anti-inflammatory

properties (Can Baser, 2008; Nya et al., 2010). Remaining focused on gut health, other feed additives commonly used are organic acids, such as acetic, butyric, citric, formic, lactic, propionic, malic, and sorbic acids. Their application is strictly linked with their positive influence on the digestive system of the animals. By administering these organic acids, the pH of stomach and small intestine decreases, contributing to improve the activity of digestive enzymes, meanwhile inhibiting the growth of potential pathogens bacteria directly by penetrating the bacterial cells and altering the cytoplasmic pH and cellular homeostasis and indirectly by reducing the growth rate of Gram-negative bacteria due to the acidification of the gastric environment (Zhou et al., 2007). As already reported and discussed in the previous chapter, one of the problems associated with feed ingredients, mainly vegetable-based, to replace FM and FO is the presence of anti-nutritional factors. In order to avoid this problem, it has been demonstrated that adding enzymes to the feed formulation can improve digestion and nutrient utilization in farmed animals (Encarnação, 2016). Due to the wide use of vegetable meals, phytates represent a common constraint for fish nutrition; hence, phytases are largely used to free the phosphate groups and disaggregate the phytate complex, which include numerous minerals, proteins, and amino acids, significantly improving their bioavailability. Other microbial enzymes are also commonly used in aquaculture; proteases and non-starch polysaccharide (NSP) enzymes have been tested in several fish species with success as they improve feed efficiency and apparent digestibility of crude protein, also degrading NSPs such as cellulose, xylans, and mannans, which are known to dramatically reduce the nutritive value of many plant ingredients (Boyd et al., 2020). Finally, the last two groups of the principal additives are strictly related to each other, and, in fact, they are often administered synergistically. The first category is represented by prebiotics, which are defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a

limited number of bacteria in the colon" (Gibson and Roberfroid, 1995). This definition, coined for humans, can be obviously extended to all animals, considering the whole intestine the main target. Prebiotics are basically dietary fibers composed of complex carbohydrates that act as a substrate for the fermentation, selection, and proliferation of probiotic bacteria. These molecules are typically oligosaccharides that contain a small number of monosaccharides (3-10). Most prebiotics are derived from plant cell walls, bacteria, or yeast. Among the numerous candidates used in aquaculture, mannan-oligosaccharides (MOS) and fructo-oligosaccharides (FOS), together with inulin and glucans, are the most widely used and have been studied in several species, obtaining numerous benefits, as shown in Tab.3, taken from (Boyd et al., 2020). In contrast, probiotics are live microbial supplements that, if administered in adequate amounts, have the potential to benefit the host intestine by restoring microbial balance, reestablishing a physiological condition after an insult, or simply modulating the microbiota composition in order to improve digestive capacity and nutrient assimilation (FAO (Food and Agriculture Organization of the United Nations), 2016). The mechanisms of action depend on the species used as a probiotic, but, in general, these microorganisms are able to hamper pathogens through direct competition for nutrients and adhesion space or through the production of inhibitory molecules, such as lactoferrin, lysozyme, bacteriocins, siderophores, and enzymes; in addition, they can hinder pathogens by producing hydrogen peroxide or decreasing the pH of the intestinal lumen. In addition, probiotic administration can improve fish growth and feed conversion rate as these microorganisms can increase host digestion capacity through the production of secrete enzymes such as proteases, amylases, and lipases that hydrolyze molecules that the fish intestine cannot otherwise digest. In aquaculture, a great number of bacterial species are currently used as probiotics (Newaj-Fyzul et al., 2014a). The most popular probiotics for aquaculture purposes, which include improved growth and nutrient utilization, are

lactic acid bacteria (e.g., *Lactobacillus* spp., *Pediococcus* spp., *Enterococcus* spp.) and *Bacillus* spp., but also a few yeasts species such as the most well-known and widely studied *Saccharomyces cerevisiae*. All these microorganisms are part of the autochthonous population that commonly inhabits the gastro-intestinal tract (GIT) of most farmed fish, and this aspect is fundamental for modulating fish microbiome, as they can potentially establish themselves as a resident or at least transient population, greatly influencing the intestinal environment (Encarnação, 2016).

Prebiotic	Species	Effects	References
Beta glucans	European sea bass	↑ growth, ↓ FCR, ↑ immunity	Bagni et al. (2005)
Beta glucans and yeast extract	Nile tilapia	↑ immunity, ↑ disease resistance	El-Boshy, El-Ashram, Abdelhamid, and Gadalla (2010)
Beta glucans and MOS	Atlantic salmon	↑ growth (only MOS), ↑ diseaseresistance (only glucans)	Refstie, Baeverfjord, Seim, andElvebø (2010)
FOS and MOS	Atlantic salmon	↔ growth, ↑ E retention, ↑ immunity (only MOS)	Grisdale-Helland, Helland, andGatlin (2008)
FOS	Whiteleg shrimp	↑ immunity, ↕ gut bacterial composition	Li et al. (2007)
Inulin	Atlantic salmon	↓ bacterial counts, ↓ gut bacterial	Bakke-McKellep et al. (2007)
Inulin	Whiteleg shrimp	↔ growth, ↑ immunity, ↑ disease resistance	Luna-González et al. (2012)
Inulin and FOS	Rainbow trout	↑ growth, ↕ gut bacterial composition	Ortiz et al. (2013)
MOS	Atlantic salmon	↔ growth, ↑ N retention, ↑ disease resistance	Dimitroglou, Reynolds, Ravnoy, and Johnsen (2011)
MOS	Gilthead sea bream	↑ growth, ↑ N and C digestibility	Gultepe, Salnur, Hossu, and Hisar (2011)
MOS	Rainbow trout	↑ growth, ↓ FCR, ↑ N retention, ↑	Rodriguez-Estrada, Satoh, Haga, Fushimi,and Sweetman (2013)
MOS	European sea bass	↔ growth, ↓ FCR, ↑ immunity	Torrecillas et al. (2011)
MOS	European sea bass	↑ immunity, ↑ disease resistance	Torrecillas et al. (2007)
MOS and yeast	Rainbow trout	↕ bacterial composition, ↑ gut bacterial diversity (NGS)	Gonçalves & Gallardo-Escárate, 2017
Yeast extract and MOS	Rainbow trout	↔ growth, ↔ FCR, ↕ gut bacterial composition (NGS)	Betiku et al., 2017
Yeast extract	Rainbow trout	↑ immunity, ↑ disease resistance	Tukmachi and Bandboni (2014)

Table 3. Examples of prebiotics and their effects on common aquaculture species. Abbreviations: N, nitrogen (protein); E, energy; C, carbohydrate; FCR, feed conversion ratio; FOS, fructo-oligosaccharides; MOS, mannan-oligosaccharides; NGS, next-generation sequencing (Boyd et al., 2020).

Probiotics can be administered as multi-species (multi-strain) or single-species (single-strain) (FAO (Food and Agriculture Organization of the United Nations), 2016). However, each organism possesses different and peculiar characteristics, so it is unlikely to find a candidate that will fulfill all of the requirements. One of the best options is actually to use several probiotics species simultaneously or to combine the use of probiotics with prebiotics (termed synbiotics) in order to produce the greatest benefit for the host. The other important aspect related to probiotic administration, which over the years has been widely studied in depth, is its ability to modulate the host immune system and improve disease resistance. Numerous infection trials demonstrated how different bacterial species used as probiotics can increase fish survival rates against pathogens such as *Aeromonas anguillarum, A. hydrophila, A. salmonicida, Streptococcus iniae,* and *Yersinia ruckeri* (Tab. 4). The underlying molecular mechanism is not always clear, but it has been extensively reported that probiotics can interact with the immune system by generating systemic and/or local responses, which include activating various antioxidant pathways, producing cytokines, and increasing the activity of immune cells, such as mononuclear phagocytic cells (monocytes, macrophages), polymorphonuclear leukocytes (neutrophils), and natural killer (NK) cells to enhance the innate response as well as interact with the gut-associated lymphoid tissue (GALT) (Nayak, 2010).

Probiotic	Species	Effects	References
Bacillus amyloliquefaciens	Nile tilapia	↑ immunity, ↑ disease resistance	Selim and Reda (2015)
Bacillus coagulans	Whiteleg shrimp	↑ growth, ↓ FCR, ↑ immunity, ↑ disease resistance, ↑ gut bacterial diversity, ↕ gut bacterial composition (NGS)	Amoah et al. (2019)
Bacillus licheniformis	Whiteleg shrimp	↑ growth, ↑ immunity, ↑ water quality, ↑ survival	Franco et al. (2017)
Bacillus subtilis	Gilthead Sea bream	↓ gut bacteria diversity, ↕ gut bacteria	Cerezuela et al. (2013)
Bacillus subtilis* and *Bacillus licheniformis	Rainbow trout	↑ growth, ↓ FCR, ↑ N retention, ↑ gut bacterial counts, ↑ survival, ↕ gut bacterial composition	Bagheri, Hedayati, Yavari, Alizade, and Farzanfar (2008)
Enterococcus faecium	Nile tilapia	↑ growth, ↑ immunity	Wang, Tian, Yao, and Li (2008)
Enterococcus casseliflavus	Rainbow trout	↑ growth, ↓ FCR, ↑immunity, ↑ gut bacterial counts, ↑ disease resistance	Safari, Adel, Lazado, Caipang, and Dadar (2016)
Enterococcus faecalis	Rainbow trout	↑ growth, ↓ FCR, ↑ N retention, ↑ immunity, ↑ disease resistance	Rodriguez-Estrada et al. (2013)
Lactobacillus acidophilus	Nile tilapia	↑ immunity, ↑ disease resistance	Villamil, Reyes, and Martínez-Silva (2014)
Lactobacillus plantarum	Nile tilapia	↑ growth, ↓ FCR, ↑ N retention, ↑ immunity, ↑disease resistance	Hamdan, El-Sayed, and Mahmoud (2016)
Lactococcus lactis	Whiteleg shrimp	↑ growth, ↓ FCR, " N retention, ↑ gut bacterial counts, ↑ survival, ↑ disease resistance	Adel, El-Sayed, Yeganeh, Dadar, and Giri (2017)
Lactobacillus rhamnosus	Rainbow trout	↑ immunity (only SD and FD), ↓ gut bacterial counts (only SD and FD)	Panigrahi et al. (2005)
Mix of Bacillus subtilis, B. licheniformis, and Lactobacillus	Whiteleg shrimp	↑ growth, ↓ FCR, ↑ immunity, " gut bacterial diversity, ↕ gut bacterial composition (NGS)	Xie et al. (2019
Mix of Bacillus subtilis, Enterococcus faecium, Lactobacillus reuteri, and Pediococcus acidilactici	Nile tilapia	↑ growth, ↔ FCR, ↑ immunity, ↑ gut bacterial counts, ↕ gut bacterial composition	Standen et al. (2016)
Pediococcus acidilactici	Atlantic salmon	↔ growth, ↔ FCR, ↑ immunity, ↑ gut bacterial diversity, ↓ gut bacterial counts	Abid et al. (2013)
Pediococcus acidilactici	Rainbow trout	↔ growth, ↑ immunity, ↕ gut bacteria composition, ↔ gut bacterial diversity (NGS)	Ingerslev et al. (2014)
Yeast	Rainbow trout	↑ gut bacterial diversity, ↕ gut bacterial composition (NGS)	Gonçalves & Gallardo-Escárate, 2017
Yeast	Rainbow trout	↔ bacterial counts, ↑ bacterial diversity, ↕ bacteria composition (NGS)	Huyben et al. (2018)

Table 4. Examples of probiotics and their effects on common aquaculture species. Abbreviations: CFU, colony-forming unit; FD, freeze dried; N, nitrogen (protein); E, energy; FCR, feed conversion ratio; NGS, next-generation sequencing; SD, spray dried (Boyd et al., 2020).

1.4 Fish Microbiota

The scientific world has still not reached agreement on the definition of "microbiota". The term "microbiome" was introduced by Whipps and colleagues in 1988 as an association of a "characteristic microbial community in a reasonable well-defined habitat which has distinct physio-chemical properties". Nowadays, this definition, although commonly accepted, has been elaborated and refined in some of its nuances. For our purposes, it would be wasted effort to try and distinguish the terminology for an ecological or genetic point of view. Ver important, however, is what the words "microbiota" or "microbiome" identify, and, as reported in the Fig. 10, the first refers to the assemblage of living microorganisms present in a defined environment, and the latter includes not only the community of the microorganisms, but also their "theater of activity", which considers the whole spectrum of molecules produced by the microorganisms, including their structural elements (nucleic acids, proteins,

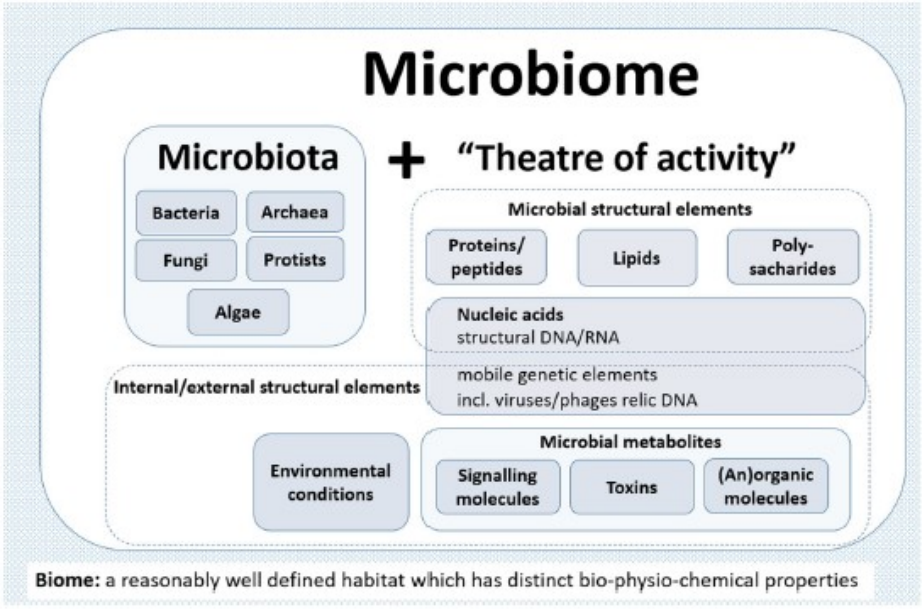


Figure 10. A schematic highlighting the composition of the term microbiome containing both the microbiota (community of microorganisms) and their "theatre of activity" (structural elements, metabolites/signal molecules, and the surrounding environmental conditions) (Berg et al., 2020).

lipids, polysaccharides), metabolites (signaling molecules, toxins, organic, and inorganic molecules), and molecules produced by coexisting hosts and structured by the surrounding environmental conditions. In addition, as phages, viruses, plasmids, prions, viroids, and free DNA are usually not considered as living microorganisms, they are included in the microbiome definition, but not in the microbiota set (Berg et al., 2020). Microbiota is therefore a vast group of microorganisms including bacteria, archaea, and also eukaryotes. These microbes colonize every part of the host, both the surfaces that are in contact with water and the external environment, and the internal organs. Typically, each district has a peculiar bacterial community that adapts its physiology and contributes to create a complex habitat-specific niche. Fish microbiota is often defined as "extra organ" due to its great contribution to important physiological host functions. One of its principal tasks, especially for those communities that inhabit the areas in contact with the outside environment, is to improve the host health by collaborating with its immune system. For each mucosal surface tissue, fish exhibit an associated adaptive immune system. The major mucosal-associated lymphoid tissues (MALT) are shown in Figure 5. There are gut-associated lymphoid tissue (GALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and nasopharynx-associated lymphoid tissue (NALT) (Fig. 11) (Bjørgen and Koppang, 2021). Microbial populations can vary greatly between these mucosal sites, suggesting that specialized symbiotic relationships are established between microbes and the host. In this way, by properly maintaining immune homeostasis, the microbiota constitutes a proper extension of teleost physiology, as it provides essential functions in nutrient metabolism, maintenance of mucosal barriers, and protection from pathogens. However, it is worth mentioning that this complex system represents a dynamic equilibrium, in which the microbes must "evade" the host immune system defence in order to build a structured community, and the latter, in turn, although remaining tolerant of the microbiota communities that

inhabit mucosal microenvironments, must be ready to prevent possible infection by opportunists.

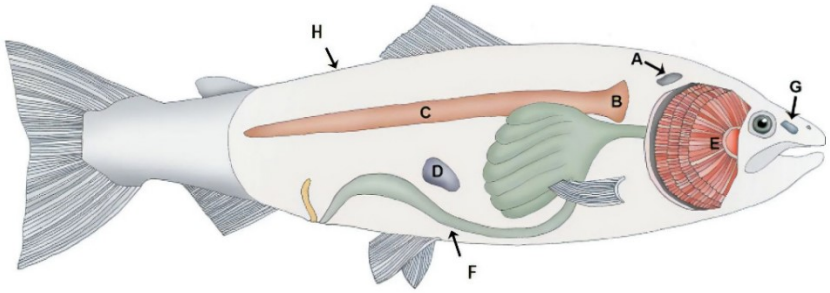


Figure 11. Schematic representation of the four major mucosal associated lymphoid tissues (MALTs) in Atlantic salmon. A) Thymus, B) head kidney; C) trunk kidney; D) spleen; E) gills with the interbranchial lymphoid tissue (ILT); F) the intestine with lymphoid tissue associated (GALT); G) olfactory organ with the nasopharynx-associated lymphoid tissue (NALT); H) lymphoid tissue associated with the skin (SALT) (Bjørgen and Koppang, 2021).

The crosstalk between microbiota and the immune system is crucial for maintaining the health status of the whole host-microbes system. In fact, numerous molecules produced by microbiota can influence the immune system cells of the fish, in either an immunostimulatory or immunosuppressive fashion. It is well known that microbial products, such as sphingolipids or entericidin, can act as promotors or inhibitors of the growth of other pathogens and symbionts, both locally and systemically, if they enter the host's bloodstream (Schubiger et al., 2015; Sepahi et al., 2016). In addition, confirming the importance of the interaction between microbiota and the immune system, numerous studies demonstrated how dysbiosis, an imbalance of microbial equilibrium that could be caused by several factors, including stress, can lead to proliferation of diseases linked to opportunistic pathogens that take over the others and destroy systemic homeostasis. Stress indirectly affects the composition of the microbiota as it alters the normal physiological, hormonal, and cellular functions of the body. Hence, it can be assumed that changes in the microbiota composition in response to stress

are the direct result of modification of the innate immune responses. From this perspective, dysbiosis becomes a prelude to a pathological state; therefore, the composition of the microbiota can be seen as a marker of fish health, even if the molecular mechanisms have not yet been elucidated (Llewellyn et al., 2014). Another important task of the microbiota is to improve the digestive ability of the host, greatly increasing the bioavailability of the molecules contained in the diets. These microorganisms, which of course inhabit the GIT of the fish, represent the majority of the microbe count as a whole, and in fact, gut microbiota is also defined as an 'extra organ' due to its significant contribution to important physiological functions of the host, especially with respect to nutrition, development, reproduction, and immune and stress responses (Nayak, 2010). The gut microbiota is typically divided into two populations: allochthonous and autochthonous. Those microbes that belong to the first category are also defined as transient, as they are associated with digestion or are present in the lumen, without clear contact or interaction with the host intestinal mucosa. The second population, in contrast, represents microorganisms that are residents in the host gut; they colonize the epithelial surface or are associated with the mucosal folds. Among them, despite there being certain species-specific differences, it is possible to define a so-called "core gut microbiota", which represents the most abundant taxa shared between specimens of the same species or even between different species. The first evidence was reported by Roeselers and colleagues, who analysed the intestinal microbiota composition of lab-reared zebrafish and zebrafish collected from the natural habitat (Roeselers et al., 2011). As already mentioned, there are a few exceptions, but overall, the phyla Proteobacteria, Bacteroidetes, and Firmicutes comprise together 90% of the core gut microbiota of fish species analyzed to date (Ghanbari et al., 2015a; Givens et al., 2015; Egerton et al., 2018). This complex symbiotic microbial association fulfills different roles in the digestion process: many bacterial populations are beneficial as they are involved in the acquisition

of nutrients, allowing a more efficient extraction and energy of nutrients from food as well as in xenobiotic processing. The gut microbiota possesses versatile metabolic genes and provides specific enzymes and biochemical pathways that make it possible to the digest substances otherwise indigestible by the host, for instance, complex carbohydrates of plant origin (cellulose, hemicellulose, pectin, and oligosaccharides) by anaerobic fermentation, as well as simple carbohydrates such as starch and glucose that escape digestion and absorption in the intestine. The products of microbial fermentation are mainly short-chain fatty acids (SCFA), such as acetate, propionate and butyrate, which are indispensable for maintaining the host's state of health because they are also implicated in the modulation of the body weight and gluconeogenesis. In addition, intestinal bacteria are also responsible for the fermentation of proteins and polysaccharides contained in intestinal mucus, and can produce peptides, EAA, and vitamins that can be used for energy production or biosynthetic processes (Balcázar et al., 2006). The gut microbiota composition can be affected by a plethora of factors, both biotic and abiotic factors, which will be further discussed extensively. Understanding and optimizing the gut microbiota represents a challenging task but constitutes the strategy to maximize feed efficiency and utilization in order to achieve a more sustainable aquaculture with regards to new feed sources and formulations.

1.4.1 Factors affecting fish gut microbiota composition

The intestinal microbiota community is estimated to be populated by 10^8 bacteria, divided into approximately 500 different species with a specific metabolism, aerobes, and facultative or strictly anaerobes. As previously mentioned, the composition of this complex group of microbes is influenced by several factors, both exogenous and endogenous, which can be summarized in three main categories: environmental, host-related, and dietary factors.**Environmental factors**

In addition to the aforementioned small species-specific differences at the phyla level in the composition of the intestinal microbiota, the dissimilarities appear more evident at a lower taxonomic level. Environmental factors include salinity, season, geographic location, rearing conditions, and water quality. In particular, the aquatic medium represents the principal source of differentiation of a microbial community since the fish is in constant contact with the environment; consequently, the microbiota populations that inhabits the external surface of the host must also adapt to changes in the surrounding environment. Interestingly, although they have great influence, the gut microbial populations generally do not reflect the same taxa that abound in the water, suggesting that the environment is not the only factor that defines the intestinal microbiota. Numerous studies have highlighted the difference that an ambient element can trigger in the gut microbial community. Zhang and colleagues, and separately, Llewellyn et al., tested how salinity influences this aspect, obtaining similar results in Nile tilapia (*Oreochromis niloticus*) and Atlantic salmon (*Salmo salar*), respectively. Both articles reported an increase in Firmicutes, Bacteroidetes, and Actinobacteria phyla in fish reared in a freshwater environment (Llewellyn et al., 2016; Zhang et al., 2016). Furthermore, Llewellyn et al., in another publication investigated in depth which taxa dominated the gut microbiota of freshwater species, identifying *Acinetobacter sp.*, *Aeromonas sp.*, *Flavobacterium sp.*, *Lactococcus sp.*, and *Pseudomonas sp.*, obligate anaerobes *Bacteroides sp.*, *Clostridium sp.*, and *Fusobacterium sp.*, and members of family Enterobacteriaceae, whereas in the marine fish it is more common to find *Aeromonas sp.*, *Alcaligenes sp.*, *Alteromonas sp.*, *Carnobacterium sp.*, *Flavobacterium sp.*, *Micrococcus sp.*, *Moraxella sp.*, *Pseudomonas sp.,* and *Vibrio sp.* (Llewellyn et al., 2014). Seasonality changes in the gut microbial population is another factor that has been taken into consideration. In fact, both Dulski et al., and Zarkasi et al., reported a change in the gut microbe's profile in different seasons in tench (*Tinca tinca*) and

Atlantic salmon (*Salmo salar*), respectively (Zarkasi et al., 2014; Dulski et al., 2020). However, it is worth mentioning that, even if other aspects can influence gut microbiota composition, it has been observed that greater changes due to the environmental pressure have been documented in the skin microbial population than in the fish intestine.

Host-related factors

As previously mentioned, environmental factors alone are insufficient to explain the differences between the gut microbiota population of the fish and those who are abundant in the environment. The already discussed "core microbiota", explains this tendency exactly, which can be considered as a general rule for all fish species, but it is actually more precise to consider this set of the most abundant taxa as a species-specific characteristic. Indeed, these similarities confirm that host genotype is a very important factor in determining and shaping the gut microbiota composition. Numerous studies were carried out supporting this theory. In particular, it has been demonstrated that, even with slight differences at lower taxonomic level, the core microbiota was found to be conserved between reared animals and wild specimens, suggesting a genetic shaping of the microbial populations rather than an environmental one. These results were obtained in fine flounder (*Paralichthys adspersus*), in which the presence of Alphaproteobacteria, Gammaproteobacteria, Bacilli, Clostridia, and Actinobacteria was reported in 80% of the samples, considering both wild and aquaculture fish. Similar outputs were documented for laboratory and cage-reared Atlantic salmon (*Salmo salar*) (Dehler et al., 2017; Ramírez and Romero, 2017). In addition, Li and colleagues demonstrated the strong tendency of the host genetic to influence microbiota, comparing the microbial intestinal populations of different fish species, reared under the same conditions and fed with similar diets. The results, once again, showed a great similarity in the microbial profile of fish belonging to the same species as compared to others (Li et al., 2015). Furthermore, the development stage

of the fish also determines the shape of the intestinal microbiota. The change begins with the first ingestion of food by the larvae, because until that time, the microbial community is far less diversified than in the adult stage, and the composition is completely determined by the environment (Yukgehnaish et al., 2020). In contrast, when larvae start to eat, the microbiota changes as a result of complex interactions between host genetics and bacteria that determine a natural selection of specific microorganisms. As a consequence of this parallel development, some biologists have put forth the hypothesis that the organisms can be defined as holobiont, combining the host and its associated microbiome as a single meta-organism. The host acts as a selection environment for the microbiota, filtering the favorable variants, and then together they evolve as single unit (Guerrero et al., 2013).

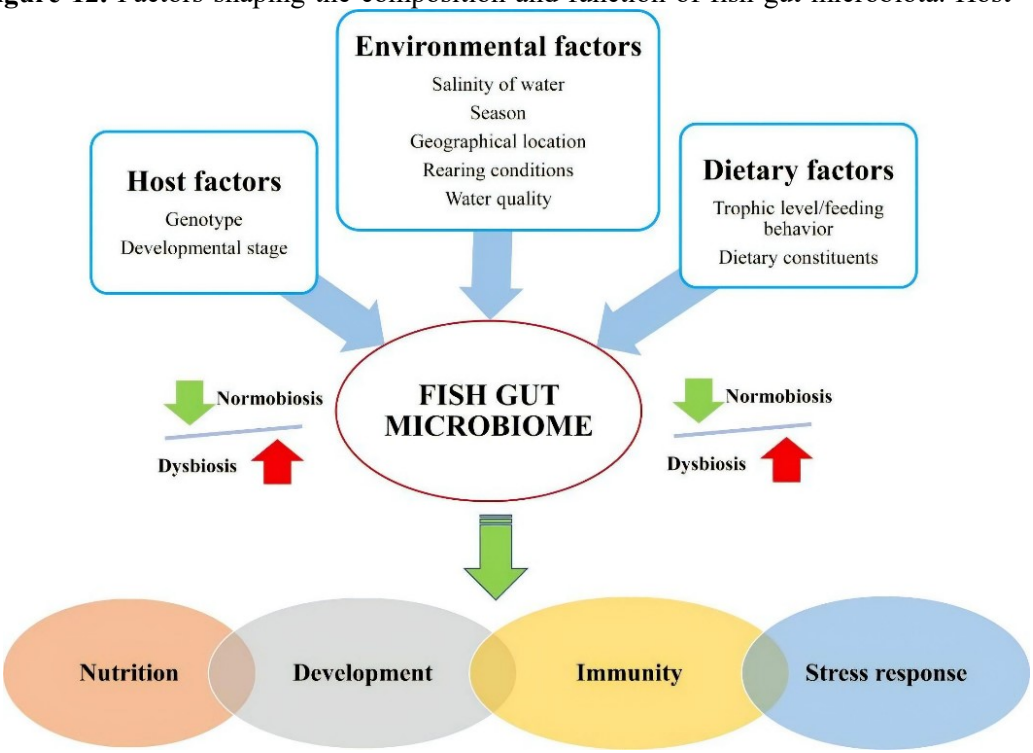
Dietary factors

The last aspect that was taken into account, which is also the most relevant according to the goal of this thesis, is the influence of the diet in modulating the fish gut microbiota. In absolute terms, diet is undoubtedly a primary factor affecting the diversity of the community structure of fish gut microbiomes. The trophic level of the fish and their feeding behaviour are collateral factors that can also have a certain degree of influence. Most of the publications focused on these differentiations reported a growing level of microbial diversity from carnivore fish to omnivores and finally to herbivores, which are the richest in number of bacterial species (Egerton et al., 2018). Starvation is an additional factor, which is related to alimentary behaviour and has the power to modify the microbial community. Xia et al., demonstrated a shift in gut microbiota populations, due to a period of starvation, in cultured Asian seabass (*Lates calcarifer*). Compared to the bacterial profile during the normal feeding routine, starvation led to an increased abundance of Bacteroidetes and Betaproteobacteria. One possible explanation is that Bacteroidetes, which are often dominant in the gut of fish, include some genera of

bacteria that are able to aid in the digestion of polysaccharides, through the production of particular digestive enzymes. Hence, during starvation, it is reasonable to assume that Bacteroidetes can harvest additional energy from food, gaining a competitive advantage over other phyla and allowing for their proliferation (Xia et al., 2014). In addition to these particularities in shaping the microbiota compositions, change in the diet formulations from conventional feed sources to those considered innovative and with less environmental impact, such as replacing of FM and FO, are the main factors in gut microbiome modifications. As a matter of fact, to confirm the importance of the nature of the ingredients and their nutritional composition, diet effect is the most studied aspect in this field. A vast literature can be found in support of the different effects of dietary changes, for example, different sources of proteins, carbohydrates and lipids that actually affect the bacterial composition of fish. As stated, the number of publications is enormous, and impossible to report here; however, the evaluation of alternatives to FM and FO in relation to intestinal microbiota modulations constitutes the main topic of this thesis, and therefore an in-depth analysis of this issue will follow in the next chapters. In summary, as previously discussed, the intestinal microbial composition of fish is determined by a wide range of factors and each of them contributes to modulate and define it. Although it is difficult to identify and distinguish the role of each factor, their influence is clear. Thus, as shown, the environment determines the initial microbiota composition, but during the developmental path, fish physiology starts to interact with the microbial community, influencing and defining it to obtain the best and most beneficial bacteria populations, a state that can be called "normobiosis" (Fig. 12). Hence, in line with the principal goal of defining the future of aquaculture, which aims to increase the feed efficiency and optimize fish growth performance, replacing progressively FM and FO, to pursue a more sustainable industry, an understanding of the mechanisms underlying the modifications of the microbiota populations will

play a crucial role in avoiding microbial alteration "dysbiosis", which leads to pathogen diffusion and diseases, and in improving the digestive capacity of the animals.

Figure 12. Factors shaping the composition and function of fish gut microbiota. Host-related factors, environment and diet may either lead to the development of a healthy microbiota (normobiosis) or an altered microbiota (dysbiosis), both of which affect the physiological functions of the host (Johny et al., 2021).



1.5 Aim of the work

In a scenario of growing food demand due to the rapid increase in the world's population, which is estimated to reach 9.7 billion people by the end of 2050, agriculture is facing an enormous challenge. In this context, aquaculture represents the fastest growing food provider industry, but also one which only has a minor impact in terms of environmental footprint. However, a limitation to expanding the sector lies in the need for advancing technology, management, regulations, and, to the largest degree, in the necessity to reduce or eliminate the dependence of the sector on marine ingredients. Conventional ingredients, FM and FO, although they represent the best nutritional source for fish are finite and no longer sustainable, as their use still requires a large amount of wild marine resources. Hence, for the continuation and further development of the sector, it is essential to replace them. The number of possible alternatives has increased in the past few decades. The most widespread alternatives include vegetable feedstuffs, both meals and oils, that are currently used as partial or total replacement of marine-based ingredients. Insects, single-cell proteins, and feed additives, such as probiotics represent other possibilities that are attracting more and more interest in aquaculture nutrition owing to their balanced profile, availability, and promising results. Despite the advantages of these alternatives, a single substitute ingredient cannot satisfy all the nutritional and physiological requirements of fish; therefore, different application approaches and, consequently, numerous studies will have to be conducted to obtain the best feed formula for the numerous fish species now being farmed in aquaculture. One of the strategies to assess the effects of an innovative diet on the physiology and growth parameters of the fish is to investigate changes in the microbiota community that inhabit the intestine of the animal, as it represents the main factor in digestion and fermentation processes. Interpretations of the molecular mechanisms underlying the interaction between host, microbial populations, and fish feed represent the keystone to modulating

fish gut microbiota and future, more sustainable diets, in order to enhance the digestive capacity and performance of these animals. Hence, the aim of the present PhD research project was to study how the composition of the intestinal microbiota is affected by using alternative protein sources and probiotics in the diet of freshwater and marine fish.

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Chapter 2

Intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) may be improved by feeding a *Hermetia illucens* meal/low-fishmeal diet

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Intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) may be improved by feeding a *Hermetia illucens* meal/low-fishmeal diet

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Abstract With demands and reliance on aquaculture still growing, there are various challenges to allow sustainable growth and the shift from fishmeal (FM) to other protein sources in aquafeed formulations is one of the most important. In this regard, interest in the use of insect meal (IM) in aquafeeds has grown rapidly. Accordingly, the aim of the present study was to assess the effects of dietary IM from *Hermetia illucens* (Hi) larvae included in a low-FM diet on gut microbial communities of rainbow trout (*Oncorhynchus mykiss*), in terms of both composition and function of microbiome. A feeding trial was conducted using 192 trout of about 100-g mean initial weight. Fish were fed in quadruplicate (4 tanks/diet) for 131 days with two diets: the control (Ctrl) contained 20% of FM as well as other protein sources, whereas the Hi diet contained 15% of Hi larvae meal to replace 50% of the FM contained in the Ctrl diet. High-throughput sequencing of 16S rRNA gene was used to identify the major feed and gut bacterial taxa, whereas Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis was performed on gut bacterial genomes to identify the major active biological pathways. The inclusion of IM led to an increase in Firmicutes, mainly represented by Bacilli class and to a drastic reduction of Proteobacteria. Beneficial genera, such as *Lactobacillus* and *Bacillus*, were enriched in the gut of fish fed with the Hi diet, whereas the number of bacteria assigned to the pathogenic *Aeromonas* genus was drastically reduced in the same fish group. The metagenome functional data provided evidence that dietary IM inclusion can shape the metabolic activity of trout gut microbiota. In particular, intestinal microbiome of fish fed with IM may have the capacity to improve dietary carbohydrate utilization. Therefore, *H. illucens* meal is a promising protein source for trout nutrition, able to modulate gut microbial community by increasing the abundance of some bacteria taxa that are likely to play a key role in fish health.

Keywords Aquaculture · Intestinal microbiota · Metagenomics · Insect meal · *Hermetia illucens* · Rainbow trout

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Introduction

Aquaculture is growing rapidly and becoming integral in global food resources, supplying around half of the world's seafood supply. One of the most important challenges that aquaculture sector is currently facing is the shift from fishmeal (FM) to other protein sources in aquafeed formulations and considerable efforts have been made so far to achieve this (Oliva-Teles et al. 2015). In this regard, interest in insect meals (IM) has

grown rapidly within both scientific and fish farmer communities.

The high potential of insects as an alternative protein source to substitute FM in aquafeeds is related to their nutritional value and life cycle process. Insects are rich in proteins (45–75% dry matter), essential amino acids, lipids, minerals, and vitamins, having a nutritional profile similar to FM (Gasco et al. 2020). Being a part of the natural diet of wild fish, insects have several ecological and economic advantages, too. They easily grow and reproduce on organic waste having a high substrate conversion efficiency. Furthermore, insect mass production generates low greenhouse gas and ammonia emissions thus meeting the recycling principles of the circular economy promoted by EU (van Huis and Oonincx 2017).

The EU Regulation No. 2017/893 (Annexe II of 24th May 2017) authorises the use in fish feeds of insect-derived proteins originating from seven species, namely, black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*).

Of these, black soldier fly (*Hermetia illucens*) is considered one of the most promising species to be used in feeds for salmonids, i.e. rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) (Henry et al. 2015; Renna et al. 2017; Belghit et al. 2018, 2019; Józefiak et al. 2019a; Li et al. 2020b; Fisher et al. 2020). High levels of dietary protein and lipid and low levels of carbohydrates are requested to meet the nutritional requirements of these fish species (Lock et al. 2018), and *H. illucens* (Hi) larvae satisfy these requirements as they contain a very high percentage of protein (36–48% DM) and fat (31–33% DM) and an essential amino acid profile similar to FM (Henry et al. 2015).

In the last years, a high number of scientific contributions on the use of IM in aquafeeds have been published demonstrating the great potential of Hi as a feed ingredient for cultured fish. The most recent evidences indicate that up to 50% of FM can be replaced by Hi larvae meal in Atlantic salmon and rainbow trout diet without any negative effect on growth performances or fillet quality (Renna et al. 2017; Bruni et al. 2018, 2020; Belghit et al. 2019).

In addition to the aforementioned nutrients, insects contain bioactive compounds that seem to have beneficial effects on animal health (Gasco et al. 2018). For instance, insects are rich in chitin and lauric acid that positively modulate host gut microbiota. Chitin is the primary constituent of the exoskeleton of arthropods, structurally analogous to cellulose, and therefore considered an insoluble fibre with potential prebiotic properties (Goycoolea et al. 2000). Lauric acid (C12:0), instead, is a medium-chain fatty acid (MCFA) known for its antimicrobial effects on Gram-positive bacteria (Spranghers et al. 2018).

However, few information is available about IM modulatory effect on fish intestinal microbiota (Parma et al. 2016; Bruni et al. 2018; Huyben et al. 2019; Belghit et al. 2019; Rimoldi et al. 2019; Terova et al. 2019; Józefiak et al. 2019a, b; Osimani et al. 2019; Li et al. 2020a). Indeed, only few studies have investigated the effect of dietary Hi meal inclusion on the gut bacterial communities of rainbow trout using high-throughput sequencing technologies (Huyben et al. 2019; Rimoldi et al. 2019; Terova et al. 2019).

The existing data suggest that fish gut microbiota is plastic and can be modulated by dietary insect meal that affects gut microbial diversity by enhancing the colonization of beneficial bacteria, such as lactic acid bacteria, which are widely used as probiotics in animal nutrition (Bruni et al. 2018; Rimoldi et al. 2019; Terova et al. 2019; Józefiak et al. 2019a, b). Such modulation of fish intestinal microbiota is reasonably expected since chitin, in addition to prebiotic properties, has antimicrobial and bacteriostatic effects on several harmful Gram-negative bacteria (Nawaz et al. 2018). Furthermore, the principal end products of chitin bacterial fermentation are short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, which serve as the main energy sources for enterocytes.

Although the composition of fish intestinal bacterial community and the principles of its preservation are nearly known, we are still far from understanding how to manipulate gut microbiota through the diet to improve fish health. Intestinal microbiota, indeed, affects the immune response and digestive functions of the host through bacterial digestive enzyme production (Ghanbari et al. 2015). The commensal microorganisms can confer resistance by direct competition with pathogen for nutrients or may also produce bactericidal or bacteriostatic substances, such as lactic acid, hydrogen peroxide, bacteriocins, or biosurfactants (Corr et al. 2007; Gudiña et al. 2015).

Accordingly, the aim of the present study was to assess the effects of dietary inclusion of *H. illucens* larva

meal as a replacer of FM on the gut microbial community of rainbow trout in terms of both microbiota's composition and function. Furthermore, since previous studies of our group (Rimoldi et al. 2019; Terova et al. 2019) were focused on testing different inclusion levels of Hi in a high-FM diet, the aim of the present research was to investigate the inclusion of Hi in a practical (low FM) formulation context.

High-throughput sequencing of 16S rRNA gene was used to identify the dynamics of major gut bacterial taxa in response to diet. An in silico analysis through bioinformatics software package PICRUSt was performed on bacterial genomes to identify the major active biological pathways of gut bacteria.

Materials and methods

Ethics statement

The trial was conducted at the DISAFA Experimental Facility of the University of Turin (Italy). All procedures involving fish comply with the guidelines of the European Union Council (2010/63/EU) for the use and care of experimental animals. The Ethical Committee of the University of Turin (protocol no. 143811) approved the experimental protocol.

Diets

Two diets were formulated to be isonitrogenous, isolipidic, and isoenergetic (Table 1). The first diet (control (Ctrl)) contained 20% of FM as well as other protein sources (wheat gluten, soybean meal, and haemoglobin), whereas the second diet (Hi15) contained 15% of *Hermetia illucens* (Hi) larva meal to replace 50% of the FM contained in the Ctrl diet. *Hermetia illucens* larva meal was provided by MUTATEC (Caumont-sur-Durance, France; https://mutatec.com/). Due to differences in chemical composition between Hi and FM and to ensure isonitrogenous, isolipidic, and isoenergetic diets, the level of inclusion of porcine haemoglobin and wheat starch slightly changed.

All feeds were prepared through cold pelleting at the experimental facility of the Department of Agricultural, Forest and Food Science (DISAFA) of the University of Turin (Torino, Italy). Briefly, all grounded ingredients were mixed with oil and desired consistency for pelleting was gained by adding water to the mixture. Each diet was cold pelleted using a 2.5-mm die meat grinder and the obtained pellet was dried at 50 °C for 48 h. Diets were stored in dark bags at a controlled temperature and humidity conditions.

Feeding trial and fish sampling

A total of 192 rainbow trout with an initial mean body weight of about 100 g were randomly distributed in 8 outdoor fibre glass tanks of 0.4 m^3 connected to a flow through an open system supplied with artesian well water (constant temperature of 13 ± 1 °C, 8 L min^{-1}, DO 7.6–8.7 mg L^{-1}). Fish were manually fed with two experimental diets in quadruplicate (four tanks/diet). The feeding rate was restricted to 1.4% of biomass for all the duration of the trial (131 days). Fish mortality was checked and recorded every day. At the end of the feeding trial, eight fish/dietary groups (2 fish/tank) were sacrificed by over anaesthesia with MS-222 (PHARMAQ Ltd., UK; 500 mg/L). The intestine was aseptically isolated from each fish, and the faecal matter

Table 1 Ingredients (g kg^{-1}) and proximate composition of the experimental diets

Ingredients	Ctrl	Hi15
Fishmeal[a]	200.0	100.0
Hermetia illucens larva meal[b]	0.0	150.0
Wheat gluten	130.0	130.0
Soybean meal	200.0	200.0
Porcine haemoglobin	92.0	82.0
Wheat starch	233.9	193.9
Fish oil	69.8	69.8
Soybean oil	69.8	69.8
Minerals[c]	2.5	2.5
Vitamins[d]	2.0	2.0
Chemical analysis		
Dry matter (g 100 g^{-1})	97.15	96.56
Ash (g 100 g^{-1}, as fed)	5.83	5.45
Crude protein (g 100 g^{-1}, as fed)	45.60	46.14
Ether extract (g 100 g^{-1}, as fed)	14.91	14.32
Gross energy (MJ kg^{-1}, as fed)[e]	22.43	22.56

[a] Purchased from Corpesca S.A. (Santiago, Chile). [b] Provided by MUTATEC, Caumont-sur-Durance, France (https://mutatec.com/). [c] Mineral mixture: provided by Skretting. [d] Vitamin mixture provided by Skretting. [e] Determined by calorimetric bomb

Chemical analysis values are reported as mean of duplicate analyses

was obtained by squeezing out and scrapping the intestinal mucosa with a sterile spatula, in order to collect both the digesta- and the mucosa-associated microbiota (transit and resident microbiota). The microbiota samples were then transferred into a sterile 2-mL tube containing 800 μL of Xpedition™ Lysis/Stabilization Solution (Zymo Research, Irvine, CA, USA) and then stored at room temperature, until DNA extraction (within 48 h).

Bacterial DNA extraction from feeds and fish gut and 16S rRNA gene amplicon library construction

The amplification of the V4 region of the bacterial 16S rRNA gene and amplicon library construction were conducted as previously reported by our group (Rimoldi et al. 2018, 2019). In brief, DNeasy PowerSoil® Kit (Qiagen, Milan, Italy) was used to extract DNA from 250 mg of intestinal contents and from 200 mg of feed (3 replicates for each diet). The V4 hypervariable region of the 16S rRNA gene was amplified by PCR using forward primer 515F: 5′-GTGYCAGCMGCCGCGGTAA-3′ and reverse primer 806R: 5′-GGACTACNVGGGTWTCTAAT-3′. Amplicons were cleaned up followed by PCR to attach unique paired-end adapters with unique indices using Nextera XT Index Kit Library, in accordance with the Illumina protocol "16S Metagenomic Sequencing Library Preparation for Illumina MiSeq System" (#15044223 rev. B). Libraries were then quantified by qRT-PCR and pooled in one tube at equimolar concentrations. The amplicon library was pair-ended sequenced (2 × 250) on a MiSeq sequencing platform (Illumina). All sequences were submitted to European Nucleotide Archive (EBI ENA).

Metabarcoding data analysis

The raw sequences were processed and analysed using QIIME™ 2 (v. 2018.4) at the default setting (Bolyen et al. 2019). The reads were trimmed at both 3′ and 5′ ends using Cutadapt v.2018.4.0 software, filtered for base quality ($Q > 30$), and merged. Filtered reads were dereplicated; singletons and chimeric sequences were removed using QIIME DADA2 denoise-paired command. All sequences were then clustered into operational taxonomic units (OTUs) at a 97% similarity cut-off. OTUs were classified using the reference Greengenes v. 13.8 as reference database (http://greengenes.lbl.gov/) down to genus level. Chloroplasts as well as sequences that were eukaryotic were removed. Sequences that had a frequency lower than 0.005% were removed from the dataset. Alpha rarefaction curves were plotted to determine the adequacy of sequencing depth. Alpha diversity indexes (Chao 1, observed OTUs, Shannon, Faith-PD, and evenness) were calculated to explain the species richness and diversity in each sample. Good's coverage estimator was used to assess the percentage of the total species that are represented in a sample. Principal coordinates analysis (PCoA) was conducted to visualize similarities or dissimilarities of data based on unweighted UniFrac and weighted UniFrac distance metric (Lozupone and Knight 2005; Lozupone et al. 2007).

Functional analysis of intestinal microbiota

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al. 2013) was used to perform the predicted functional analysis (Langille et al. 2013). Taxonomic classification was performed using QIIME2 feature-classifier classify-sklearn function, a Naive Bayes classifier that was trained on the Greengenes v. 13.8 as reference database (http://greengenes.lbl.gov/) at 99% of similarity. The corresponding biom table was generated using the tools export function and used as input for the PICRUSt pipeline. In brief, PICRUSt was first used to correct biom tables for 16S rRNA copy numbers and subsequently used to predict KEGG (Kyoto Encyclopedia of Genes and Genomes) orthologues (KO). The maximum allowed Nearest Sequenced Taxon Index (NSTI) value was set to 2 to control for the overall accuracy of the metagenomic predictions. The output data generated with PICRUSt were subsequently uploaded to the Statistical Analysis of Metagenomic Profiles (STAMP) software package (Parks et al. 2014) for further downstream statistical analyses. A two-sided Welch t test with 95% confidence was applied to identify differences in microbial metabolic pathways between two groups.

Statistical analysis

Normality and homogeneity of variance of data were checked by Shapiro-Wilk and Levene's test, respectively. To test null hypothesis ($p < 0.05$), Student's t test or nonparametric Mann-Whitney U test was applied

depending on normality and homoscedasticity of the data. All analyses were performed using Past3 software (Hammer et al. 2001). To perform statistics on microbial relative abundance data, the percentage values were firstly angular transformed. Only those taxa with an overall abundance of more than 1% (up to order level) and 0.5% at family and genus levels were considered for the analysis. The significance of the calculated beta-diversity dissimilarities was assessed by nonparametric analysis of similarities (ANOSIM) and PERMANOVA tests based on 999 permutations using QIIME script "compare_categories.py".

Results

Metabarcoding sequencing outcome

Sixteen intestinal and six feed samples were efficiently and correctly sequenced on an Illumina MiSeq platform. An overall sequences of 1,652,358 corresponding to an average of 75,107 ± 16,411 sequences per sample, was retained after the quality filtering and processing of sequencing reads.

Dataset was representative of bacterial communities due to Good's coverage estimators for all samples that were greater than 99%.The sequencing depth was set based on the saturation phase of the alpha diversity rarefaction curves at 10,780 sequences in both feed and intestinal content samples (Supplementary Fig. 1, Online Resource 1). All sequencing data were submitted to the European Nucleotide Archive (EBI ENA) public database, under the accession code PRJEB38953.

Characterization of feed-associated bacterial communities

A total of 38,073 and 34,672 high-quality reads was taxonomically classified for Ctrl and Hi15 feed samples, respectively. The high-throughput sequencing analysis revealed that the microbial profiles of feed were mainly comprised of 2 phyla, 4 classes, 6 orders, 12 families, and 8 genera. The most abundant taxa of bacteria at the phylum, family, and genus levels are shown in Fig. 1. The complete list of OTUs found in feeds with their relative abundances is given in Online Resource 2.

The microbial community diversity of feeds was evaluated by alpha diversity analysis, and indices are shown in Table 2. No differences in terms of species richness (Chao 1) and biodiversity (Shannon diversity index) or any other considered alpha diversity indexes were found between feed-associated communities. The relative abundances (%) of the most abundant taxa found in feed samples are listed in Supplementary Table 1 (Online Resource 3).

At phylum level, Hi15 feed was characterized by higher percentage of Firmicutes (47%), mainly represented by Bacilli class than the Ctrl feed (40%). Conversely, microbiota associated with Ctrl showed a higher relative abundance of Proteobacteria (58%), principally belonging to Alpha- and Gammaproteobacteria classes (Fig. 1a, Supplementary Table 1). Accordingly, a high amount of the Enterococcaceae (34%), Erysipelotrichaceae (2.5%), and Bacillaceae (0.8%) families was found in the diet with insect meal. Ctrl feed was instead rich in Lactobacillaceae (15.8%), Leuconostocaceae (1.3%), Fusobacteriaceae (0.7%), and Shewanellaceae (1.7%) (Fig. 1b; Supplementary Table 1). At genus level, Ctrl feed had higher relative abundance of *Lactobacillus*, *Weissella*, and *Shewanella* than Hi15 feed, which was instead rich in *Vagococcus*, *Erysipelothrix*, and *Vibrio* genera (Fig. 1c, Supplementary Table 1). Genus *Oceanobacillus* was found only associated to insect-based feed.

Microbial profile and dietary modulation of trout gut communities

Overall high-quality reads of 144,164 and 178,036 were taxonomically classified for Ctrl and Hi15 trout feeding groups, respectively. After removing the OTUs assigned to eukaryotic sequences, the most abundant bacterial taxa were mainly comprised of 6 phyla, 9 classes, 14 orders, 19 families, and 10 genera. The profiles of microbial communities at the phylum, family, and genus taxonomic levels for each trout group are shown in Fig. 2. The complete list of OTUs detected in intestinal samples is available as additional data in Online Resource 4.

The alpha rarefaction analysis of gut bacterial communities showed that indexes of species richness "Chao 1" and "Observed OTUs" were significantly higher in fish fed with Hi15 diet than in the control fish. Conversely, diet type did not affect either phylogenetic diversity (Faith PD) or entropy (Shannon and evenness) (Table 3).

Analysis of beta-diversity revealed an overall effect of diet on microbial communities in the presence/

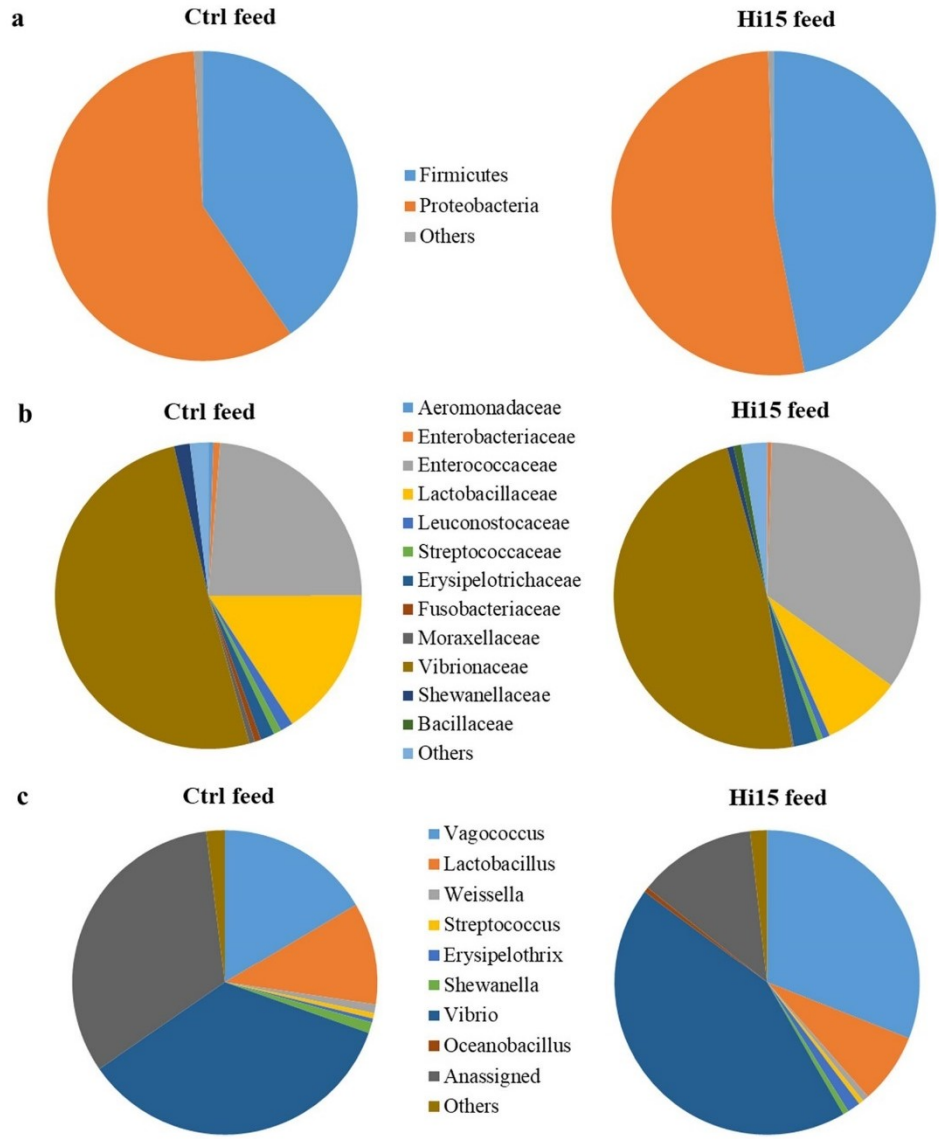

Fig. 1 Relative abundance (%) of the most prevalent bacteria in Ctrl and feeds at phylum (**a**), family (**b**), and genus (**c**) taxonomic level. Only bacteria with an overall abundance of 0.5% were reported. Bacteria with lower abundance were pooled and indicated as "others"

absence (unweighted UniFrac) (Fig. 3a), but not in relative abundance (weighted UniFrac), of specific OTUs (Fig. 3b). Principal coordinates analysis (PCoA) of unweighted UniFrac distances clearly showed that the intestinal microbiota of the Hi15 feeding group clustered separately from the Ctrl group; the two main components explain 53% of the observed variance (Fig. 3a). Additionally, intestinal communities were remarkably different from feed-associated bacterial ones, thus indicating that observed differences at the gut level were not simply a consequence of undigested feed that might have been present in the gastrointestinal tract. The PERMANOVA and ANOSIM tests confirmed the PCoA results, showing significant differences ($R =$

0.46, pseudo-$F = 3.32$, $q < 0.05$) in the composition of the microbiota between Ctrl and Hi15 feeding groups only in the unweight UniFrac analysis (Table 4). The relative abundances (%) of the most abundant taxa found in fish intestinal samples are reported in Table 5.

The gut microbial community of trout was dominated, regardless of the diet, by four phyla: Proteobacteria, Firmicutes, Tenericutes, and Fusobacteria (Fig. 3a). Of these, the amount of Firmicutes was positively influenced ($p < 0.05$) by dietary insect meal (Hi15 54%, Ctrl 7.6%) (Table 5). This was essentially due to the enrichment in bacteria belonging to the Clostridia (3.6%) and Bacilli (50%) class. On the contrary, the average relative abundance of Proteobacteria, mainly represented by Gammaproteobacteria, was significantly higher in Ctrl fish (43%) than in the Hi15 feeding group (7.6%). At order level, the only difference between two groups was in the amount of Aeromonadales and Bacillales (Table 5). The first taxon was more abundant in Ctrl samples, whereas Bacillales were enriched in fish fed Hi15 diet. Accordingly, Aeromonadaceae were particularly abundant in the gut of controls (18%), whereas Bacillaceae (25%) and Paenibacillaceae (7.4%) were solely found in trout receiving Hi15 diet (Fig. 3b, Table 5). The *Oceanobacillus*, *Bacillus*, *Paenibacillus*, and *Cetobacterium* genera were exclusive of the intestine of fish fed Hi meal. In the same dietary group, the amount of *Aeromonas* and *Lactobacillus* genera was significantly less and more abundant, respectively, in comparison to controls (Fig. 3c, Table 5).

Prediction of metabolic pathways of gut bacterial communities

PICRUSt was applied to predict the functional potential of the intestinal microbiome of rainbow trout. Level 3 KEGG orthologue function prediction was used. Our analysis revealed 217 predicted metabolic pathways (Online Resource 5). Among them, 28 were significantly different between the two dietary groups (Fig. 4). Metabolic inference from 16S rRNA gene sequencing data showed that dietary inclusion of Hi meal upregulated the abundance of genes responsible of pathways involved in starch and sugar metabolism and in the transcription processes. On the contrary, genes involved in the peptidoglycan biosynthesis and recycling and in the protein folding and biofilm formation were enhanced in the microbiome of control fish (Fig. 4).

Table 2 Alpha diversity metrics (rarefied at 10,780 sequences) of feed microbial communities. All data are reported as mean values ($n = 3$) ± SD

Item	Ctrl feed	Hi15 feed	*p* value
Observed OTUs	340.67 ± 3.51	346.33 ± 9.24	0.48
Chao 1	368.91 ± 4.95	368.00 ± 21.28	0.94
Faith-PD	5.11 ± 0.32	5.60 ± 0.37	0.12
Shannon	6.00 ± 0.08	5.82 ± 0.06	0.05
Evenness	0.71 ± 0.01	0.69 ± 0.01	0.05

Discussion

The use of insect meal in fish feed is a way to respond to the problems of aquaculture industry related to the stability and reduction of feeding costs and to promote sustainable aquatic environment management. So far, several researches have shown that insect meal can partially replace fishmeal and completely replace soya bean meal that are commonly used in aquafeeds, without affecting fish growth performances, feed utilization, digestibility, and fillet quality (Magalhães et al. 2017; Renna et al. 2017; Bruni et al. 2018, 2020; Iaconisi et al. 2018; Terova et al. 2019). Indeed, the present research confirms what has been stated in previous studies on rainbow trout; i.e. defatted Hi meal is well accepted by trout and does not negatively affect fish growth and survival if it is included at levels up to 40% in the diet (Renna et al. 2017; Stadtlander et al. 2017; Bruni et al. 2018; Terova et al. 2019). Because fish are natural predators of insects, it is reasonable to assume that they are evolutionarily adapted for consuming them.

Nevertheless, fish growth performance is not the only outcome that defines a successful aquaculture practice; fish welfare has to be taken into account, too. In this prospect, intestinal microbiota, which directly affects the digestive functions and the immune response of the host should be considered a key indicator of a healthy fish (Ghanbari et al. 2015).

In line with our previous researches, the present study showed that Hi meal inclusion in the diet can modify fish gut microbiota, thus improving the health status of trout. In two recent studies in trout, we have reported that the partial substitution of dietary FM with 10%, 20%, or 30% of a defatted Hi meal had an important effect in modulating both the intestinal transient and resident bacterial communities (Rimoldi et al. 2019;

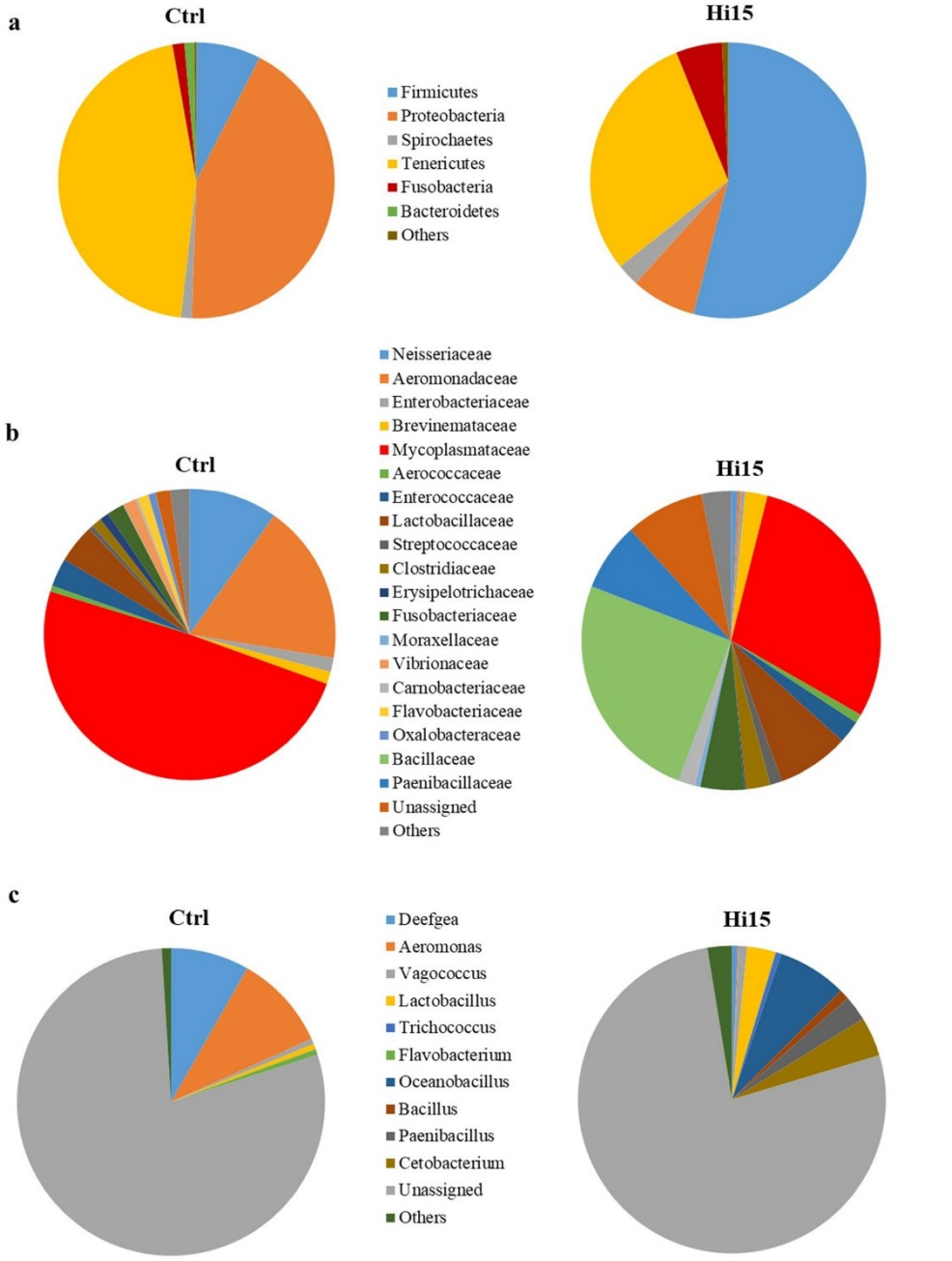

Fig. 2 Relative abundance (%) of the most prevalent intestinal bacterial phyla (**a**), families (**b**), and genera (**c**) in each trout dietary group. In the figure, all taxa with an overall abundance of ≥ 0.5% were reported. Bacteria with lower abundance were pooled and indicated as "others"

Table 3 Alpha diversity metrics (rarefied at 10,780 sequences) of gut microbial communities of trout fed with Ctrl or Hi15 diets. All data are reported as mean values ($n = 8$) ± SD. Significant p values are in italic

Item	Ctrl	Hi15	p value
Observed OTUs	229.25 ± 57.68	370.50 ± 131.84	*0.02*
Chao 1	259.13 ± 70.03	421.93 ± 142.40	*0.01*
Faith-PD	4.52 ± 1.21	5.48 ± 1.77	0.11
Shannon	4.85 ± 0.42	5.39 ± 0.82	0.09
Evenness	0.62 ± 0.05	0.64 ± 0.05	0.60

Terova et al. 2019). As expected, the present metabarcoding analysis revealed that Firmicutes, Proteobacteria, and Tenericutes phyla were dominant in the gut of rainbow trout, regardless of the diet (Lyons et al. 2017a, b; Terova et al. 2019). The phylum Tenericutes is considered specifically adapted to the gastrointestinal environment of farmed rainbow trout. Several studies have reported that this phylum, with *Mycoplasma* being the dominant genus, is prominent in the distal intestine of rainbow trout as well as in other farmed salmonids (Lyons et al. 2017a; Huyben et al. 2018; Fogarty et al. 2019; Terova et al. 2019). Therefore, our data provide a further evidence of the importance of this genus in trout, thus corroborating the idea that this fish species could be a specific host for *Mycoplasma*.

Although gut bacterial communities were dominated by the same phyla irrespective of the diet, species richness (Chao 1 index, observed OTUs) was significantly increased by dietary supply of 15% of insect meal in our study. Previously, we found an increase of species richness only in the digesta-associated (allochthonous), but not in mucosa-associated (autochthonous), gut microbiota of rainbow trout fed with increasing levels of Hi

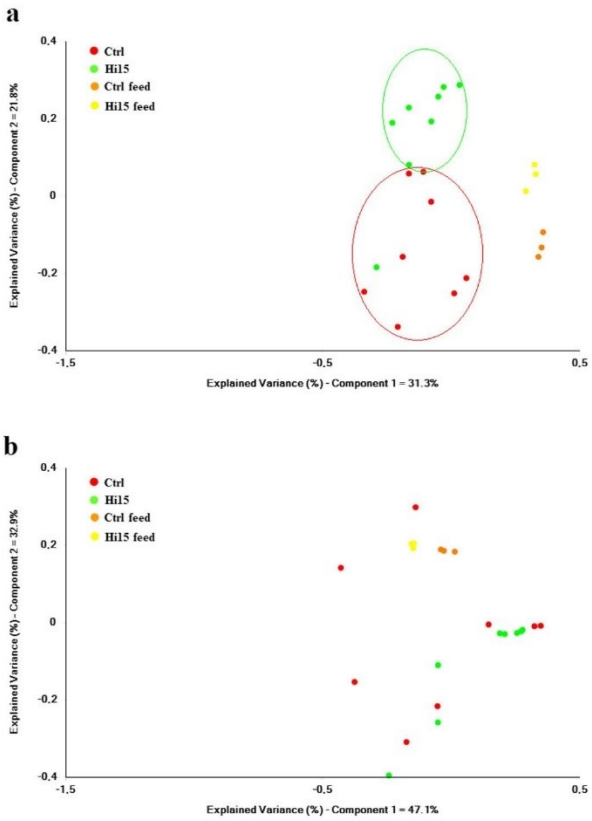

Fig. 3 PCoA plot of unweighted (**a**) and weighted (**b**) UniFrac distances of gut microbial communities associated to two experimental dietary groups. Each dot represents an individual sample according to its microbial profile at genus level

Table 4 ANOSIM and PERMANOVA test results for comparisons of gut microbiota composition between Ctrl and Hi15 feeding groups. Significant q-values (< 0.05) are shown in italic

Statistical test	Unweighted		Weighted	
ANOSIM (permutation *N* = 999)	***q*-value**	***R***	***q*-value**	***R***
Ctrl vs Hi15	*0.015*	0.42	0.247	0.06
Ctrl vs Ctrl diet	*0.042*	0.45	0.915	− 0.22
Hi15 vs Hi15 diet	*0.046*	0.46	0.174	0.47
Ctrl diet vs Hi15 diet	0.095	1.00	0.247	1.00
PERMANOVA (permutation = 999)	***q*-value**	**Pseudo-*F***	***q*-value**	**Pseudo-*F***
Ctrl vs Hi15	*0.009*	3.32	0.279	1.46
Ctrl vs Ctrl diet	*0.012*	4.87	0.346	1.22
Hi15 vs Hi15 diet	*0.009*	4.26	*0.036*	6.21
Ctrl diet vs Hi15 diet	0.119	9.02	0.228	58.18

meal (10–30%) (Rimoldi et al. 2019; Terova et al. 2019). Bruni et al. (2018) found instead, a higher species richness in autochthonous intestinal microbiota of trout fed a diet containing 20% of Hi meal. In any case, a higher microbial richness should be considered a positive effect, since it may potentially provide further metabolic capabilities to the host thus improving its health status (Borrelli et al. 2017).

Insect meals are rich in chitin, a form of insoluble fibre, which may act as prebiotic by selectively stimulating the growth of beneficial gut bacteria and promoting their colonization (Guerreiro et al. 2018). In the same way, biodiversity parameters were increased by dietary administration of krill or inclusion of 5–20% chitin in the diet of salmonids (Askarian et al. 2012; Ringø et al. 2012). Furthermore, chitin and its deacetylate derivate chitosan have antimicrobial properties and a bacteriostatic effect against several harmful Gram-negative bacteria (Nawaz et al. 2018).

Multivariate analysis of bacterial community's diversity, based on unweighted UniFrac dissimilarity data, displayed a strong clustering of fish groups fed with Hi meal and with the control diet that were cleanly separated into uniformly distant regions. Our data confirm previous researches showing that the Hi meal inclusion in the diet causes a significant reduction of gut Proteobacteria, predominantly belonging to the Gammaproteobacteria class, in comparison to the control diet without insect meal (Huyben et al. 2019; Rimoldi et al. 2019; Terova et al. 2019). In particular, in line with those studies, our metagenomic analysis highlighted the dramatic shift from an high Proteobacteria to Firmicutes ratio in the gut of fish fed with the Ctrl diet to a low ratio in fish fed with the insect meal diet. The most dominant genus in the control fish gut was *Aeromonas*, which includes several Gram-negative bacteria commonly present in fresh water and potentially pathogenic for fish, as they can cause skin ulcerations. In the current study, intestinal abundance of *Aeromonas* in trout fed Hi15 was significantly reduced and this is in line with our findings on autochthonous intestinal microbiota of trout fed with Hi meal.

In another study of our group, microbiota of trout fed with Hi meal showed a reduction of Gammaproteobacteria, mainly represented by genera *Shewanella*, *Aeromonas*, *Citrobacter*, and *Kluyera* (Rimoldi et al. 2019). Similarly, Bruni et al. (2018) found a high abundance of OTUs related to the *Aeromonas* genus only in the control fish group, but not in the intestine of the insect-fed groups. An increase amount of *Aeromonas* genus with the Hi treatment has been recently reported only in Siberian sturgeon (*Acipenser baerii*) (Józefiak et al. 2019b).

We recorded an increase in the number of *Bacillus* and *Lactobacillus* genera in response to dietary insect meal. Proliferation of lactic acid bacteria (LAB) may be due to the prebiotic effect of chitin, and, as proposed by Bruni et al. (2018), it may indicate that chitin was a preferential growth substrate for LAB. Indeed, LAB play an important role in degrading fibres. Furthermore, they have an active role in host defence against pathogens, by producing bactericidal compounds, such as lactic acid, hydrogen peroxide, bacteriocins, and biosurfactants, which prevent pathogen colonization of the intestinal epithelial surface (Ringø and Gatesoupe 1998; Corr et al. 2007; Gudiña et al. 2015;

Table 5 Mean relative abundance (%) ± SE ($n = 8$) of the most prevalent phyla, orders, classes, families, and genera found in the intestine of trout fed with two experimental diets. Significant p values (< 0.05) are shown in italic

	Ctrl	Hi15	p value
Phylum			
Firmicutes	7.58 ± 4.32	54.08 ± 14.58	*0.024*
Proteobacteria	42.95 ± 10.61	7.58 ± 1.42	*0.041*
Spirochaetes	1.29 ± 0.83	2.63 ± 1.64	0.563
Tenericutes	45.39 ± 11.44	29.56 ± 13.50	0.411
Fusobacteria	1.39 ± 0.68	5.42 ± 4.28	0.958
Bacteroidetes	1.14 ± 0.72	0.00 ± 0.00	
Class			
Clostridia	0.86 ± 0.63	3.59 ± 0.98	*0.031*
Alphaproteobacteria	10.77 ± 5.86	4.65 ± 1.25	0.793
Betaproteobacteria	10.92 ± 6.65	1.35 ± 0.92	0.103
Gammaproteobacteria	21.09 ± 11.45	1.59 ± 0.69	*0.018*
[Brevinematae]	1.29 ± 0.98	2.63 ± 1.84	0.563
Mollicutes	45.39 ± 11.56	29.55 ± 14.60	0.411
Bacilli	5.98 ± 3.53	50.35 ± 13.73	*0.024*
Fusobacteriia	1.39 ± 0.93	5.41 ± 4.82	0.958
Flavobacteriia	1.12 ± 0.93	0.00 ± 0.00	
Order			
Clostridiales	1.40 ± 1.10	3.81 ± 1.07	0.083
Neisseriales	9.84 ± 6.74	0.72 ± 0.56	0.178
Aeromonadales	17.76 ± 11.21	0.31 ± 0.20	*0.009*
Enterobacteriales	1.57 ± 0.98	0.49 ± 0.22	0.371
[Brevinematales]	1.36 ± 1.01	2.40 ± 1.57	0.636
Mycoplasmatales	49.22 ± 10.94	29.37 ± 14.71	0.320
Lactobacillales	9.27 ± 6.10	14.62 ± 4.60	0.339
Erysipelotrichales	1.02 ± 0.70	0.14 ± 0.06	0.220
Fusobacteriales	1.92 ± 1.35	4.74 ± 4.09	0.958
Vibrionales	1.44 ± 0.85	0.10 ± 0.07	0.215
Stramenopiles	0.77 ± 0.76	1.88 ± 1.88	0.543
Flavobacteriales	1.21 ± 1.01	0.00 ± 0.00	
Burkholderiales	1.13 ± 0.63	0.23 ± 0.08	0.956
Bacillales	0.02 ± 0.02	38.90 ± 11.18	*0.001*
Family			
Neisseriaceae	9.84 ± 6.74	0.72 ± 0.56	0.149
Aeromonadaceae	17.76 ± 11.21	0.31 ± 0.20	*0.009*
Enterobacteriaceae	1.57 ± 0.98	0.49 ± 0.22	0.371
Brevinemataceae	1.36 ± 1.01	2.40 ± 1.57	0.636
Mycoplasmataceae	49.23 ± 10.95	29.37 ± 14.71	0.173
Aerococcaceae	0.65 ± 0.58	0.97 ± 0.66	0.122
Enterococcaceae	3.16 ± 1.64	2.45 ± 0.80	0.902
Lactobacillaceae	4.30 ± 3.01	7.78 ± 2.48	0.226
Streptococcaceae	0.54 ± 0.49	1.30 ± 0.41	0.067
Clostridiaceae	1.09 ± 0.88	2.66 ± 0.68	0.083
Erysipelotrichaceae	1.02 ± 0.70	0.14 ± 0.06	0.220
Fusobacteriaceae	1.92 ± 1.35	4.74 ± 4.09	0.958
Moraxellaceae	0.05 ± 0.04	0.60 ± 0.52	0.717
Vibrionaceae	1.44 ± 0.85	0.08 ± 0.05	0.215
Carnobacteriaceae	0.26 ± 0.20	1.69 ± 0.95	*0.031*
Flavobacteriaceae	1.21 ± 1.01	0.00 ± 0.00	
Oxalobacteraceae	0.81 ± 0.46	0.04 ± 0.02	0.629
Bacillaceae	0.00 ± 0.00	25.17 ± 7.16	
Paenibacillaceae	0.00 ± 0.00	7.40 ± 2.25	
Genus			
Deefgea	8.26 ± 6.57	0.61 ± 0.56	0.178
Aeromonas	10.04 ± 6.66	0.11 ± 0.07	*0.007*
Vagococcus	0.56 ± 0.20	0.89 ± 0.35	0.439
Lactobacillus	0.55 ± 0.22	3.02 ± 1.03	*0.028*
Trichococcus	0.02 ± 0.02	0.60 ± 0.31	*0.018*
Flavobacterium	0.60 ± 0.43	0.00 ± 0.00	
Oceanobacillus	0.00 ± 0.00	7.27 ± 2.12	
Bacillus	0.00 ± 0.00	1.09 ± 0.28	
Paenibacillus	0.00 ± 0.00	2.65 ± 0.76	
Cetobacterium	0.00 ± 0.00	4.12 ± 4.11	

Ringø et al. 2018). Even the increased amount of *Bacillus* represents a positive effect of dietary chitin deriving from insect meal. Chitin, indeed, may have increased the proliferation of chitinolytic bacteria, since several *Bacillus* species have been shown to secrete chitinase (Cody 1989). Together with LAB, the *Bacillus* genus is one of the most common probiotics used in aquaculture to enhance host immune response and disease resistance. Up to date, several studies have demonstrated the immunomodulatory effects of *Bacillus subtilis* in fish (Salinas et al. 2005; Newaj-Fyzul et al. 2007; Cerezuela et al. 2013) and there are several evidences documenting that the use of insect meals from *H. illucens* may positively modulate trout gut microbiota, increasing LAB and Bacilli amount in both mucosa- and digesta-associated microbiota (Bruni et al. 2018; Huyben et al. 2019; Terova et al. 2019; Józefiak et al. 2019a).

In addition to taxonomic characterization of gut microbiota in response to dietary insect meal, this study investigated the functional potential of the intestinal microbiome of rainbow trout using the computational

approach PICRUSt (Langille et al. 2013). Indeed, the use of dietary insect meal clearly affected the structure of trout intestine–associated microbial community (what's there?) but, to understand the intrinsic processes that lead to similar functionality, it is necessary to search the connections between individual microbiota (what are they doing?) and the corresponding metabolic phenotype (Piazzon et al. 2017).

Gut microbes carry out a multitude of biochemical reactions, which play a critical role in host nutrition by contributing to the digestion of several dietary ingredients. In agreement with Lyons et al. (2017a), we found that the principal functional pathways associated with bacterial communities of trout intestine, regardless of the diet, were metabolism, cellular processes, membrane transport, and genetic information processing.

However, based on metagenome prediction, trout fed with insect meal showed an enhancement of pathways involved in sugar and starch metabolism. Members of the phylum Firmicutes are known to play a pivotal role in the fermentation of dietary carbohydrates (Corrigan et al. 2015). In our case, the increase of sugar metabolism observed in the Hi group of trout could be reasonably correlated to the higher presence of Bacilli that typify the intestinal microbiota of these fish. The fermentation of dietary carbohydrates and resistant starches by the intestinal microbiota leads to the formation of a variety of beneficial substances, including short-chain fatty acids (SCFAs). It is well established that SCFAs (mainly acetate, propionate, and butyrate), in addition to being energy sources for colonocytes, promote fish intestinal health (Hamer et al. 2008; Koh et al. 2016; Rimoldi et al. 2016). Furthermore, the increased ability of gut microbiome to utilize dietary carbohydrates could be an interesting approach to improve feed digestibility in trout that is known as a poor user of dietary carbohydrates and fibres (Wilson 1994; Polakof et al. 2012). In fact, *Bacillus* genera are widely used as probiotics in aquaculture to increase feed absorption and digestion (Soltani et al. 2019).

On the contrary, intestinal microbiome of trout fed with the Ctrl diet showed an increased capacity for

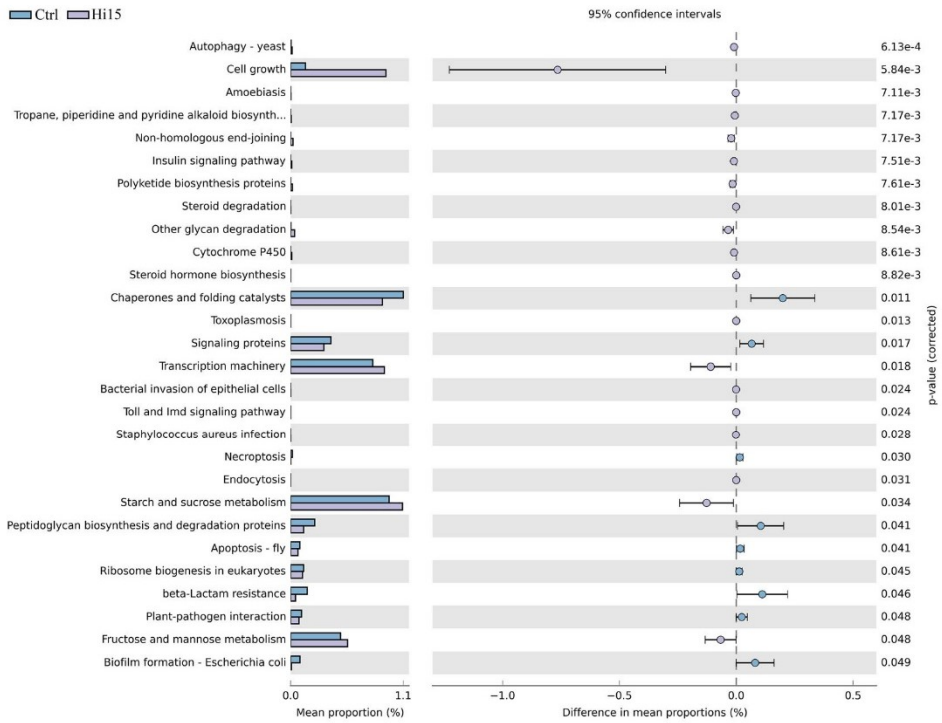

Fig. 4 Predicted functional metagenomic pathways of trout gut microbiome, as identified by PICRUSt. The extended error bar graph and statistical analysis were made using STAMP bioinformatics software

peptidoglycan synthesis. Peptidoglycan is the major structural component of the cell wall of both Gram-positive and Gram-negative bacteria. It is the major wall structural component of the most pathogenic bacteria and it is considered a proinflammatory molecule that stimulates host innate immune response (Mogensen 2009). In human, for instance, functional analysis of the faecal microbiome of healthy individuals and atherosclerosis patients revealed an increase in the peptidoglycan synthesis gene in the afflicted population (Karlsson et al. 2012). It means that the increased capacity for peptidoglycan synthesis might contribute to the chronic inflammation of the atherosclerotic arterial walls.

The hypothesis that control fish in the present study were affected by an inflammatory status seems to be supported by the increase of gene pathways of chaperones and protein-folding catalysts found in their intestinal microbiota. Indeed, secretion of chaperones and protein-folding catalysts (foldase) from prokaryote cells acts as intercellular signal, principally for leukocytes. Chaperones and foldase have been defined "moonlighting" proteins since they may act as homeostatic immune regulators and, under certain circumstances, contribute to tissue pathology as well (Henderson and Pockley 2010).

Effectively, Proteobacteria dominated intestinal microbiome of control trout, whereas Firmicutes were scarcely represented. This phylum was mainly represented by Gammaproteobacteria class, which includes important disease-causing pathogens of fish. Among these, *Aeromonas* resulted particularly abundant in the intestine of fish fed with Ctrl diet, possibly as a sign of intestinal dysbiosis or disease.

In summary, the present research reinforces the insights of previous studies conducted by us and other groups showing that insect proteins can have beneficial effects on intestinal microbiota of fish. The inclusion of 15% of *H. illucens* led to an increase in the total number of Firmicutes, mainly represented by Bacilli class, and to a drastic reduction of Proteobacteria. Beneficial genera, such as *Lactobacillus* and *Bacillus*, were enriched in the gut of fish fed with an insect-based diet, while the number of bacteria assigned to the pathogenic *Aeromonas* genus was drastically reduced in the same fish group. The metagenome functional data provided evidence that dietary IM inclusion can shape the metabolic activity of trout gut microbiota. In particular, intestinal microbiome of trout fed with insect meal may have the capacity to complement the endogenous digestive enzymes, thus improving dietary carbohydrates utilization. Therefore, *H. illucens* meal is a promising alternative protein source for trout nutrition, able to modulate gut microbial community by increasing the abundance of some bacteria taxa that are likely to play a key role in fish health.

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Authors' contributions Conceptualization, G.T. and L.G.; methodology S.R., M.A., and F.M.; data collection, curation, and analysis, S.R., L.G., M.A., and F.M.; writing—original draft preparation, S.R. and G.T.; writing, review, and editing, S.R., G.T., and L.G.; funding acquisition, G.T. All authors have read and agreed to the published version of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All procedures involving fish comply with the guidelines of the European Union Council (2010/63/EU) for the use and care of experimental animals. The Ethical Committee of the University of Turin (protocol no. 143811) approved the experimental protocol.

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Chapter 3

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Effects of full replacement of dietary fishmeal with insect meal from *Tenebrio molitor* on rainbow trout gut and skin microbiota

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Effects of full replacement of dietary fishmeal with insect meal from *Tenebrio molitor* on rainbow trout gut and skin microbiota

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Abstract

Background: Aquaculture must continue to reduce dependence on fishmeal (FM) and fishoil in feeds to ensure sustainable sector growth. Therefore, the use of novel aquaculture feed ingredients is growing. In this regard, insects can represent a new world of sustainable and protein-rich ingredients for farmed fish feeds. Accordingly, we investigated the effects of full replacement of FM with *Tenebrio molitor* (TM) larvae meal in the diet of rainbow trout (*Oncorhynchus mykiss*) on fish gut and skin microbiota.

Methods: A feeding trial was conducted with 126 trout of about 80 g mean initial weight that were fed for 22 weeks with two isonitrogenous, isolipidic, and isoenergetic extruded experimental diets. Partially defatted TM meal was included in one of the diets to replace 100% (TM 100) of FM, whereas the other diet (TM 0) was without TM. To analyse the microbial communities, the Illumina MiSeq platform for sequencing of 16S rRNA gene and Qiime pipeline were used to identify bacteria in the gut and skin mucosa, and in the diets.

Results: The data showed no major effects of full FM substitution with TM meal on bacterial species richness and diversity in both, gut mucosa- and skin mucus-associated microbiome. Skin microbiome was dominated by phylum Proteobacteria and especially by Gammaproteobacteria class that constituted approximately half of the bacterial taxa found. The two dietary fish groups did not display distinctive features, except for a decrease in the relative abundance of *Deefgea* genus (family Neisseriaceae) in trout fed with insect meal. The metagenomic analysis of the gut mucosa indicated that Tenericutes was the most abundant phylum, regardless of the diet. Specifically, within this phylum, the Mollicutes, mainly represented by Mycoplasmataceae family, were the dominant class. However, we observed only a weak dietary modulation of intestinal bacterial communities. The only changes due to full FM replacement with TM meal were a decreased number of Proteobacteria and a reduced number of taxa assigned to Ruminococcaceae and Neisseriaceae families.

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Conclusions: The data demonstrated that TM larvae meal is a valid alternative animal protein to replace FM in the aquafeeds. Only slight gut and skin microbiota changes occurred in rainbow trout after total FM replacement with insect meal. The mapping of the trout skin microbiota represents a novel contribution of the present study. Indeed, in contrast to the increasing knowledge on gut microbiota, the skin microbiota of major farmed fish species remains largely unmapped but it deserves thorough consideration.

Keywords: Aquaculture, Circular economy, Gut microbiome, Insect meal, Metagenome, Next-generation sequencing, Rainbow trout, Skin microbiome, *Tenebrio molitor*

Introduction

Aquafeeds have largely been relied on fishmeal (FM), which is an optimal protein source to ensure fast growth and good health of farmed fish. However, most wild capture fisheries are operating at or above maximum sustainable yield; therefore, fish farming can no longer rely on oceanic resources for manufacturing aquafeeds and such feed options are simply not sustainable. This has promoted the search for more sustainable alternative ingredients to reduce the inclusion of FM in aquafeeds.

In this regard, insects can represent a new world of sustainable and protein-rich ingredients for farmed fish feeds. Breeding insects has low environmental footprint and this makes them even more interesting as protein source for aquafeeds [1]. Furthermore, insects are very efficient and quick bio converters – which makes them excellent organic waste recyclers. They can grow on agricultural wastes [2, 3], such as expired fruit and vegetables from packaging facilities and convert them into their own biomass, i.e., a high-value protein resource for farmed animals (pig, chicken, and fish) [1]. There is a real potential here to convert millions of tons of agricultural waste produced globally each year, into tones of high quality proteins for fish feeds [4], which in turn can increase fish production for human consumption, thus improving food and nutrition security, promoting economic growth and protecting our environment and natural resources

Demonstrating the emergence of a new sector, in recent years, a bulk of research has focused on insects [5–9] and dozens of companies all over the Europe have started breeding insects.

In this view, the yellow mealworm, *Tenebrio molitor* (TM) (Coleptera: Tenebrionidae), is a great match because it is very efficient at bio converting organic waste - the ideal circular insect! Furthermore, the percentage of edible biomass in larval and pupal stages of TM is only slightly less than 100% [10]; therefore, low extra waste (insect excreta called frass), is produced following its rearing. Mealworm frass is considered a sustainable resource for managing plant nutrition in cropping systems and a promising alternative to conventional fertilizer [4, 11]. Frass can also be employed to grow earthworms such as *Lumbricus terrestris or Eisenia fetida*, which may improve the efficiency of organic fertilizers [4, 11].

T. molitor is one of the seven insect species (2 flies, 2 mealworms, and 3 cricket species) that has been recently authorized by an EU commission regulation (2017/893–24/05/2017) for fish feed. Larval and pupal stages of TM are rich in protein and lipids whose levels range from 47% to 60% and from 31% to 43% (on a dry weight basis), respectively. In terms of protein quality, meal from TM larvae has a well-balanced amino acid profile and the content of some indispensable amino acid is higher (as % of protein) than in land plants and slightly lower than in FM [12].

Different studies have successfully incorporated TM as a protein source in the diet of different fish species. In rainbow trout (*Oncorhynchus mykiss*), feeding trial using diets with different FM/TM meal replacement levels have shown optimal fish performance [13–15]. In red seabream (*Pagrus major*), significant growth enhancement was obtained in fish fed on diets with 65% defatted TM larvae meal, i.e., complete replacement of FM [16]. Furthermore, in a study conducted on Nile tilapia (*Oreochromis niloticus*), TM had the highest apparent digestibility coefficient in comparison to other four insect meals that were tested, validating TM larvae as a good protein source alternative to FM for fish diets [17].

Insects contain bioactive compounds that are able to modulate the vast consortiums of microorganisms that inhabit fish gut. Therefore, diets in which FM was replaced by insect meal from either *Hermetia illucens* or *T. molitor*, have led to changes in the diversity and abundance of fish gut bacteria [18–20]. Studies indicate that chitin, a major structural component of the insect cuticles, is a potential modulator of fish gut microbiota [21], as it acts as a substrate for chitinase producing bacteria that are not commonly found in the fish gut [22, 23]. Supplementation of chitin or krill (chitin-rich) in the diet of Atlantic salmon (*Salmo salar*) changed the membership and structure of intestinal microbiota with over a hundred autochthonous bacterial strains identified [24].

Much of the current research on fish microbiota has focused on the microbial communities present in the gut, but fish harbor distinct microbial communities across other major anatomical regions, too. Of these anatomical sites, the skin contains the highest microbial

diversity, followed by gills and gut [25–30]. The skin of fish is covered with thin and partially overlapping scales for protection and secretes an aqueous mucus layer that coats the epidermal surface. All these structures and appendages, with an abundance of folds and invaginations provide many specialized skin niches that harbour a wide range of microorganisms [27]. Furthermore, skin mucus is a biochemically complex fluid that includes a number of nutrients that favour a high bacterial diversity.

In contrast to the increasing knowledge on gut microbiota, the skin microbiota of major farmed fish species remains largely unmapped but it deserves thorough consideration [31]. Indeed, skin is one of the main mucosal barriers between fish and its external environment, constituting the first line of defense from pathogens or toxic substances [27]. Fish inhabit an aqueous environment very rich in highly diverse planktonic microbes, including bacteria, fungi and viruses. Such microbial-rich surrounding environment has potential to colonize fish skin and cause infections [31]. Consequently, fish have evolved mechanisms to gain benefits from harmless symbiotic bacteria, which help them to fight against invasion by pathogenic or harmful microorganisms. For instance, fish skin mucus host commensal bacterial species, which are able to protect their host against pathogens by inhibiting enzymatic activities and secreting antimicrobial compounds [32]. Skin microbiota plays thus a critical role in the control of fish diseases. Therefore, an enhanced understanding of host-symbiont-pathogen nexus is necessary not only to gain insight into microbial involvement in fish diseases, but also to enable novel promicrobial and antimicrobial approaches for their treatment.

To the best of our knowledge, there are no articles in the literature dealing with the effects of diet on skin microbiota of farmed fish. However, since the feed catabolites are dispersed in the water, and the quality of water is one of the factors that can change the composition of fish microbiota [33–35], it would be interesting to see the dynamics of both gut and skin microbiota in fish fed diets with insect meal.

Accordingly, the present research aimed at investigating the effects of full replacement of FM with TM larvae meal in the diet of rainbow trout (*Oncorhynchus mykiss*) on fish growth performance, and microbiota of gut and skin. The feed microbiota was analyzed, too.

Methods

Feeding trial, diets and fish sampling

Details of the feeding trial have been described by Chemello et al. [36]. In brief, SPAROS LDA (Olhão, Portugal) and Ÿnsect (Evry, France) formulated two isonitrogenous, isolipidic, and isoenergetic extruded experimental diets named TM 0 and TM 100. Partially defatted TM meal was included in one of the diets to replace 100% (TM 100) of FM, whereas the other diet (TM 0) was without TM. Main ingredients and proximate composition of the diets are shown in Table 1. The

Table 1 Main ingredients and proximate composition of the diets

Item	TM 0	TM 100
Ingredients, %		
Fishmeal 65 (Peruvian)	20	-
Tenebrio molitor larvae meal	-	20
Soy protein concentrate	18	18
Wheat gluten	7.75	7.06
Corn gluten	8	8
Soybean meal (48%)	7	7
Wheat meal	14.23	13.8
Sardine oil	4.3	4.1
Soybean oil	8.6	8.2
Rapeseed oil	8.6	8.2
Soy lecithin	0.5	0.5
Vitamin[a] and mineral premix[b]	1	1
Antioxidant	0.2	0.2
Sodium propionate	0.1	0.1
Monocalcium phosphate	0.52	1.73
L-Arginine	-	0.1
L-Lysine	-	0.6
L-Tryptophan	0.05	0.12
DL-Methionine	0.15	0.3
Celite®	1	1
Proximate composition, % as fed		
Dry matter	93.77	94.41
Crude protein	42.08	44.25
Ether extract	22.63	22.36
Ash	7.57	5.6
Chitin	-	1.49
Nitrogen-free extract[c]	21.49	20.71
Gross energy, MJ/kg as fed[d]	22.24	22.55

This table has been modified from previously published data in Chemello et al. [36]

[a]Vitamin mixture (IU or mg per kg diet): *DL*-atocopherolacetate, 60 IU; sodium menadione bisulfate, 5 mg; retinylacetate, 15,000 IU; *DL*-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; vitamin B_{12}, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (Granda Zootecnici, Cuneo, Italy)

[b]Mineral mixture (g or mg per kg diet): bicalcium phosphate 500 g, calcium carbonate 215 g, sodium salt 40 g, potassium chloride 90 g, magnesium chloride 124 g, magnesium carbonate 124 g, iron sulfate 20 g, zinc sulfate 4 g, copper sulfate 3 g, potassium iodide 4 mg, cobalt sulfate 20 mg, manganese sulfate 3 g, sodium fluoride 1 g (Granda Zootecnici, Cuneo, Italy)

[c]Calculated as 100 – (crude protein + ether extract+ ash + chitin)

[d]Determined by calorimetric bomb

processing and storage conditions of the two diets were the same. The feeds were stored in a refrigerated room (6 °C) for the entire duration of the feeding trial.

Rainbow trout of 78.3 ± 6.24 g mean initial weight were randomly distributed into six 400-L tanks (3 tanks/diet, 21 fish/tank). Tanks were supplied with artesian well water at 13 ± 1 °C in a flow-through open system (tank water inflow: 8 L/min). The dissolved oxygen levels were measured every 2 weeks and ranged between 7.6 and 8.7 mg/L, whereas the pH was 7.5–7.6. The feeding trial lasted 22 weeks. The first 8 weeks, fish were fed at 1.6% of the tank biomass and then, according to the fish growth and water temperature, the daily quantity of distributed feed was decreased to 1.4%. Fish were fed twice a day (at 8:00 and at 15:00), 6 d per week. Feed intake was monitored at each administration. In order to update the daily feeding rate, fish in the tanks were weighed in bulk every 14 days. Mortality was checked every day.

At the end of the trial, six fish/diet were sampled and the whole intestine was aseptically dissected out. The animals used for sampling were sacrificed by an overdose of anaesthetic (MS-222; PHARMAQ Ltd., UK; 500 mg/L) using water bath immersion and all efforts were made to minimize pain, stress, and discomfort in the animals. The skin mucus microbiota was obtained by gentle scraping of fish body with a cotton swab (individually wrapped sterile cotton swab with a polystyrene handle), whereas the gut autochthonous microbiota was obtained by scraping the mucosa of the entire intestine (excluding pyloric caeca). Each swab head was immediately cut off and placed inside a sterile 1.5 mL Eppendorf tube containing 200 μL of Xpedition Lysis/Stabilization Solution. The tube was then vortexed for shaking out the bacteria from the swab tip [18] and stored at room temperature for up to 24 h until bacterial DNA extraction. Trained researchers performed all collection procedures.

Bacterial DNA extraction

The bacterial DNA was extracted from four aliquots from each feed, six samples of skin mucus, and six samples of intestinal mucosa per each dietary fish group. The DNA extraction from feeds was done in parallel to biological samples, right after the end of feeding trial.

DNeasyPowerSoil® Kit (Qiagen, Italy) was used to extract DNA, following the manufacturer's instructions with only few modifications at the lysis step, as previously described by Rimoldi et al. [37]. In brief, 200 mg of feed or 200 μL of skin and gut bacteria suspension were lysed in PowerBead Tubes by means of a TissueLyser II (Qiagen, Italy) for 2 min at 25 Hz. A sample with only lysis buffer was processed in parallel to the biological samples as a negative control of the extraction procedure. The concentration of extracted DNA was measured using NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Italy). Then, bacterial DNA was stored at − 20 °C until the microbiota sequencing.

Illumina 16S metagenomic sequencing library construction

16S ribosomal RNA gene amplicon libraries were prepared using a pair of primers specific for the V3-V4 region applying the Illumina protocol "16S Metagenomic Sequencing Library Preparation for IlluminaMiSeq System" (#15044223 rev. B). Amplicons of 16S rRNA gene were generated starting from 10 μL of microbial genomic DNA by PCR using Platinum®-Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Italy) and tailed forward and reverse primer Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′-GACTACNVGGGTATCTAATCC-3′) selected by [38] The expected size of PCR products on Agilent 2100 Bioanalyzer trace was ~550 bp. The entire procedure for 16S rRNA gene library preparation and sequencing is described in [18] In brief, Illumina paired-end adapters with unique Nextera XT indexes were ligated to 16S amplicons using Nextera XT Index Kit (Illumina, San Diego, CA, USA). A quality control of all libraries was then performed by qPCR using KAPA Library Quantification Kits Illumina® Platforms (KapaBiosystems Ltd, UK). Libraries were then pooled at equimolar concentrations and diluted to 6 pM. Pooled libraries were then multiplexed and sequenced on an Illumina MiSeq platform (Illumina) with paired-end 2 × 300 bp sequencing chemistry.

Metagenome data analysis

Raw sequencing data were processed by QIIME 2 (2018.8) pipeline [39] at the default setting. Barcode sequences and primers were removed using the Cutadapt software v.2018.8.0 from raw reads. The sequences were filtered for quality (Q > 30), trimmed at the 3′ end and merged with default values of DADA2 software package. The remaining high quality reads were then dereplicated to obtain the unique sequences (*uniques*) and the chimeras were eliminated using qiime DADA2 denoise-paired command. The sequences were clustered in operational taxonomic units (OTUs) at 99% of similarity. The OTUs were filtered at 0.005% of frequency and two OTU-tables (one per each macro-group of samples: skin mucus+feeds and gut mucosa+feeds) were created. The rarefaction analysis was performed on the OTU-tables (biom format) to verify the minimum number of reads to normalize all samples. Each OTU was taxonomical assigned using GreenGenes v.13-8 as reference database. Reads assigned to chloroplasts and mitochondria were removed from the analysis since

of eukaryotic origin. Alpha-diversity analysis was performed based on rarefied OTU tables considering Observed OTUs, Shannon, Pielou's evenness, and Faith PD indices. To compute microbial beta diversity both weighted and unweighted UniFrac analyses were performed and sample UniFrac distances were visualized on 3D PCoA plots.

Statistical analysis

The number of reads across samples was normalized by sample size and the relative abundance (%) of each taxon was calculated. Only those taxa with an overall abundance of more than 1% (up to order level) and 0.5% at family and genus level were considered for statistical analysis. Before being statistically analysed, the resulting microbial relative abundances were calculated as the angular transformation (arcsine of the square root). All data were checked for normality and homoscedasticity by Shapiro-Wilk's and Levene's test, respectively. Depending if normality of the data was satisfied or not, differences between groups were analysed by *t*-test or by nonparametric Mann-Whitney test. Statistical significance was set at $P< 0.05$. All the statistical analyses were performed using Past4 software version 4.02 [40] . Kruskal-Wallis test was applied to verify differences in alpha-diversity indices between treatments. Multivariate analysis of beta diversity was verified using non parametric permutational multivariate analysis of variance (Adonis) and analysis of similarity (ANOSIM) with 999 permutations ($P<0.05$). Both alpha and beta metrics, including their related statistics, were computed using QIIME 2's diversity analysis commands "qiime diversity alpha-group-significance" and "qiime diversity beta-group-significance" available through the q2-diversity plugin.

Results

Fish growth performance

Our previous publication by Chemello et al. [36] reported all data on fish growth performances and feed utilization efficiency. In brief, at the end of the feeding trial, all fish tripled their mean body weight, but there were no significant differences between the dietary groups for any of the considered growth performance indexes ($P > 0.05$). The mean individual weight gain was 312 g and 353 g for fish fed with TM 0 and TM 100 diets, respectively, whereas feed conversion ratio was 1.07 and 1.02, respectively. Protein efficiency rate was 2.09 for both dietary groups.

Evaluation of microbiome diversity

Thirty-two microbiome profiles (from 8 feeds, 12 skin mucus, and 12 gut mucosa samples) were successfully obtained by high throughput sequencing of 16S rRNA gene amplicons on Illumina MiSeq platform. A total of 1,701,326 of reads were achieved, corresponding to 575 OTUs and 158 OTUs for skin mucus+feeds and gut mucosa+feeds macro-groups, respectively.

To calculate alpha diversity indices, samples were rarefied to 21,146 reads for gut mucosa+feeds macro-group and to 16,752 reads for skin mucus+feeds macro-group, but maintaining an adequate Good's coverage (> 0.99). The number of OTUs ranged from 84 to 107 for feed-associate bacterial communities, from 9 to 13 for gut mucosa, and from 153 to 187 for skin mucus microbial community (Table 2). No statistically significant differences were found for any of the alpha diversity index considered, within the same starting sampling substrate, in response to diet ($P \geq 0.05$). The only exception was represented by Shannon index value, which resulted significantly higher in TM 100 feed samples ($P = 0.021$). Although due to the different level of rarefaction, it is not statistically acceptable to compare the two anatomical districts (gut and skin) to each other, skin microbiome clearly showed higher bacterial species richness (Observed OTUs) and biodiversity (Shannon and Faith PD indices) than intestine. All sequencing data were deposited as FASTQ files at the European Nucleotide Archive (EBI ENA) public database under the accession code: PRJEB38845.

Table 2 Alpha diversity. Number of reads per group-treatment assigned to OTUs and alpha diversity metrics values of feed, gut mucosa (GMMC), and skin mucus microbial communities (SMMC) of rainbow trout fed TM 0 and TM 100 diets

Items	TM 0	TM 100	*P*-value
Feed (rarefied at 21,146 reads)			
Reads	54,465 ± 18,561	44,708 ± 19,771	0.498
Observed OTUs	107 ± 20	84 ± 25	0.248
Shannon	3.73 ± 0.05	3.29 ± 0.07	**0.021**
Pielou's evenness	0.55 ± 0.02	0.52 ± 0.03	0.083
Faith PD	7.79 ± 0.68	6.70 ± 1.05	0.149
GMMC (rarefied at 21,146 reads)			
Reads	63,530 ± 31,477	61,665 ± 16,983	0.901
Observed OTUs	13 ± 3	10 ± 4	0.231
Shannon	1.32 ± 0.76	0.28 ± 0.24	0.054
Pielou's evenness	0.36 ± 0.21	0.09 ± 0.07	0.055
Faith PD	1.34 ± 0.23	1.26 ± 0.42	0.872
SMMC (rarefied at 16,752 reads)			
Reads	49,824 ± 21,594	39,064 ± 16,875	0.359
Observed OTUs	187 ± 40	154 ± 71	0.336
Shannon	4.43 ± 1.05	4.29 ± 1.05	0.749
Pielou's evenness	0.59 ± 0.13	0.61 ± 0.06	0.521
Faith PD	17.30 ± 4.52	13.86 ± 7.18	0.423

All data are expressed as means ± SD ($n = 4$ for feed and $n=6$ for GMMC and SMMC). $P<0.05$ are in bold

The multivariate analysis Adonis of feed microbial communities based on UniFrac distance matrix, showed differences between TM 0 and TM 100 diets in terms of presence/absence (unweighted UniFrac), and relative abundance (weighted UniFrac) of taxa (Adonis unweighted $P = 0.038$ and weighted $P = 0.034$) (Table 3). Significant differences were also found between microbial communities of gut mucosa in function of the diet, but in this case only for weighted UniFrac analysis (Adonis $P = 0.025$ and ANOSIM $P = 0.038$) (Table 3). On the contrary, the diet type seemed to exert no effect on microbial communities associate to skin mucus (Table 3). Accordingly, for both macro-groups of analysis, PCoA plots clearly showed that feed samples clustered separately from biological samples, thus indicating that observed differences were not simply a consequence of feed contamination that might have been present in the gastrointestinal tract or water (Fig. 1). Weighted Unifrac PCoA confirmed that the gut mucosa communities were the only affected by diet type (Fig. 1b).

Table 3 Beta diversity. Permutational multivariate analysis of variance (Adonis) and Analysis of similarity (ANOSIM) on weighted and unweighted UniFrac distances of feed, gut mucosa (GMMC), and skin mucus microbial communities (SMMC) at genus level

Items	Adonis				ANOSIM			
	Unweighted		Weighted		Unweighted		Weighted	
	P-value	R^2	*P*-value	R^2	*P*-value	*R*	*P*-value	*R*
Feeds								
diet TM 0 vs. diet TM 100	**0.038**	0.33	**0.034**	0.42	**0.023**	0.38	**0.029**	0.53
GMMC								
TM 0 vs. TM 100	0.315	0.11	**0.025**	0.37	0.339	0.04	**0.038**	0.25
TM 0 vs. diet TM 0	**0.004**	0.72	**0.007**	0.64	**0.005**	1.0	**0.008**	0.92
TM 100 vs. diet TM 100	**0.006**	0.66	**0.003**	0.99	**0.004**	1.0	**0.007**	1.0
SMMC								
TM 0 vs. TM 100	0.525	0.08	0.274	0.11	0.456	0.00	0.140	0.11
TM 0 vs. diet TM 0	**0.006**	0.73	**0.011**	0.86	**0.007**	1.0	**0.004**	1.0
TM 100 vs. diet TM 100	**0.004**	0.48	**0.003**	0.82	**0.011**	0.58	**0.005**	1.0

$P < 0.05$ are in bold

Characterization of microbial community associated to feeds

Considering only the most representative taxa, the overall feed microbial community consisted of 2 phyla, 3 classes, 4 orders, 7 families, and 6 genera (Fig. 2; Table 4). At phylum level Firmicutes and Proteobacteria constituted together approximately 99% of bacteria population (Fig. 2a). Differences in taxa abundance were found at lower taxonomical levels. Feed TM 0 had more abundance of Gammaproteobacteria (3-fold increase, $P = 0.030$) compared to feed TM 100 containing insect meal (Fig. 2b, Table 4). At order level, Vibrionales were only found at consistent in percentage associate to diet TM 0 ($P = 0.030$), whereas, Lactobacillales were significantly (0.13-fold increase, $P < 0.001$) more abundant in feed TM 100 (Fig. 2c, Table 4). Accordingly, at family level, Vibrionaceae were practically undetectable in feed TM 100 ($P = 0.030$), resulting together with Fusobacteriaceae (6-fold increase, $P = 0.026$) and Staphylococcaceae (0.5-fold increase, $P = 0.026$) more abundant in control feed TM 0 (Fig. 2d; Table 4). Lactobacillaceae were enriched in feed TM 100 (0.21-fold increase, $P = 0.006$) (Fig. 2d; Table 4). The relative abundance of genus *Lactobacillus* was higher in TM 100 than in control feed (0.2-fold increase, $P = 0.006$), which was instead characterized by higher amount of *Photobacterium* (5-fold increase, $P = 0.030$) and *Staphylococcus* (0.5-fold increase, $P = 0.038$) genera (Fig. 2e; Table 4).

Characterization of gut microbial community

By taking into account all samples and considering only the most representative taxa, the gut microbial community of trout consisted of 3 phyla, 4 classes, 5 orders, 6 families, and 2 genera (Fig. 3; Table 5). Regardless of the diet, the most abundant phylum was Tenericutes, followed by Proteobacteria and Firmicutes in descending order of abundance. Among them, relative amount of Proteobacteria, mainly represented by Beta- and Gammaproteobacteria, was significantly influenced by diet ($P = 0.047$) resulting higher in control group (3-fold increase) (Fig. 3b, Table 5). At order level, trout fed with diet TM 100 showed a significantly four-fold decrease ($P = 0.033$) in Neisseriales, represented by Neisseriaceae family, compared to control trout (Fig. 3c; Table 5). The Ruminococcaceae family of Clostridiales order resulted detectable only in intestine of TM 0 fish (Fig. 3d; Table 5). No differences in relative abundances of intestinal bacterial genera were found in response to diet (Table 5).

Characterization of skin microbial community

The skin microbial community was mainly consisted of 4 phyla, 11 classes, 17 orders, 25 families, and 20 genera (Fig. 4; Table 6). Regardless of the diet, the skin

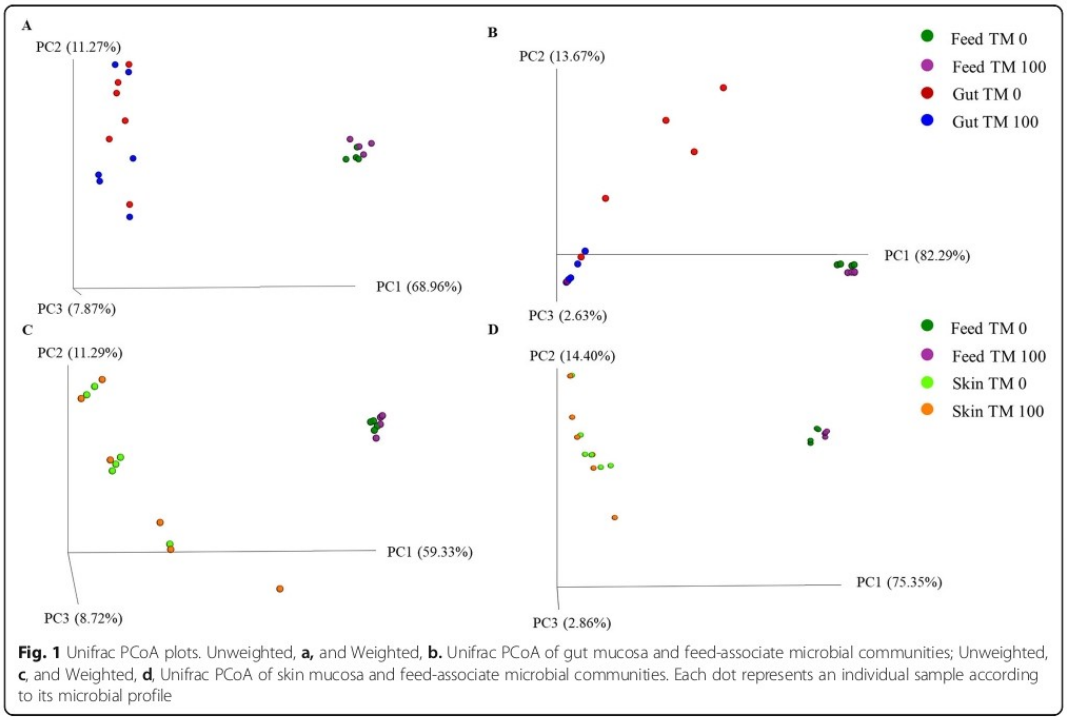

Fig. 1 Unifrac PCoA plots. Unweighted, **a,** and Weighted, **b.** Unifrac PCoA of gut mucosa and feed-associate microbial communities; Unweighted, **c**, and Weighted, **d**, Unifrac PCoA of skin mucosa and feed-associate microbial communities. Each dot represents an individual sample according to its microbial profile

microbiome of trout was dominated by four phyla: Proteobacteria, Firmicutes, Tenericutes, and Bacteroidetes (Fig. 4a). At order level, the only difference between two groups was for Neisseriales, mainly represented by Neisseriaceae family, that were significantly higher (2-fold increase, P=0.013) in fish fed control diet (Fig. 4c; Table 6). At family level, Clostridiaceae resulted enriched (4-fold increase, P=0.013) in skin microbiota of trout fed with insect-based diet TM 100 (Fig. 4d; Table 6). Only genus Deefgea resulted significantly affected by diet (P=

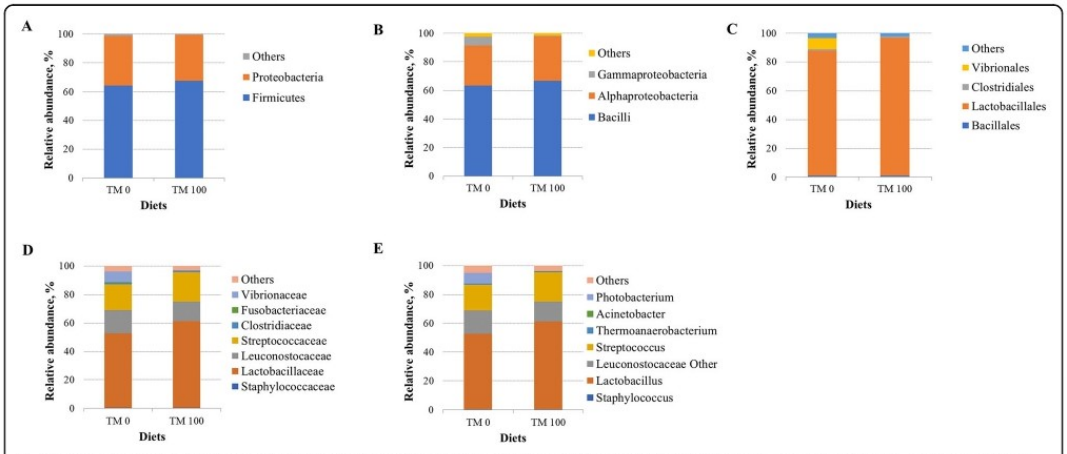

Fig. 2 Relative abundance (%) of the overall most prevalent bacterial phyla (**a**), classes (**b**), orders (**c**), families (**d**), and genera (**e**) in feeds. All bacteria with an overall abundance of ≥1% for **a**, **b** and **c**; and ≥ 0.5% for **d** and **e** were reported. Bacteria with lower abundance were pooled and indicated as "Others"

Table 4 Mean of relative abundance (%) ± SD of the most prevalent phyla, classes, orders, families, and genera found in feeds

Items	TM 0	TM 100	*P*-value
Phylum			
Firmicutes	64.20 ± 4.37	67.48 ± 3.14	0.271
Proteobacteria	34.54 ± 4.27	31.85 ± 3.15	0.351
Class			
Bacilli	63.39 ± 4.30	66.79 ± 3.24	0.254
Alphaproteobacteria	27.90 ± 3.66	30.96 ± 3.18	0.251
Gammaproteobacteria	6.30 ± 0.71	0.65 ± 0.12	**0.030**
Order			
Lactobacillales	86.77 ± 1.65	95.73 ± 0.28	**<0.001**
Vibrionales	7.50 ± 1.18	0.17 ± 0.29	**0.030**
Clostridiales	1.12 ± 0.20	0.99 ± 0.72	0.483
Bacillales	1.02 ± 0.01	0.98 ± 0.28	0.691
Family			
Lactobacillaceae	52.33 ± 2.97	60.95 ± 2.91	**0.006**
Streptococcaceae	17.95 ± 1.59	20.52 ± 4.35	0.470
Leuconostocaceae	16.25 ± 0.52	13.88 ± 1.88	0.055
Vibrionaceae	7.50 ± 1.18	0.17 ± 0.08	**0.030**
Clostridiaceae	0.99 ± 0.18	0.90 ± 0.26	0.553
Fusobacteriaceae	0.70 ± 0.21	0.01 ± 0.03	**0.026**
Staphylococcaceae	0.51 ± 0.06	0.39 ± 0.07	**0.038**
Genus			
Lactobacillus	52.22 ± 3.01	60.85 ± 2.89	**0.006**
Streptococcus	17.78 ± 1.60	20.24 ± 4.36	0.665
Photobacterium	7.44 ± 1.13	0.17 ± 0.08	**0.030**
Thermoanaerobacterium	0.70 ± 0.16	0.68 ± 0.19	0.885
Staphylococcus	0.51 ± 0.06	0.39 ± 0.07	**0.038**
Acinetobacter	0.15 ± 0.06	0.09 ± 0.07	0.312

$P < 0.05$ are in bold

0.017), being two fold increased in control feeding group TM 0 (Fig. 4e; Table 6).

Discussion

In the last decades, research on the use of insects as FM replacers in aquafeed is rapidly evolving. Several reviews have been published on insects nutritional value, environmental low impact, and food safety, all attributes that could contribute to make aquaculture system more productive and sustainable [6, 8, 9, 41].

In terms of fish growth, the research of our group, as also reported by Chemello et al. [36], confirms what has been found in previous studies, i.e. the complete or partial substitution of dietary fishmeal with TM does not affect rainbow trout growth performance and fillet quality [13–15]. Similarly, TM was successfully utilised and well accepted by several marine fish species [42–44]. While the effects of dietary FM/TM replacement on fish growth performances have been widely investigated, less evidence is available on the effects on host commensal bacterial communities. In particular, skin microbiome is underexplored in fish as well as in most farmed animals.

The data showed no major effects of FM substitution with TM meal on species richness and diversity of both gut mucosa- and skin mucus-associated bacteria. In line with our results, the inclusion of hydrolysed TM meal did not affect the total number of digesta-associated bacteria in sea trout (*Salmo trutta* m. *trutta*) [45]. In contrast, in the study of Józefiak et al. [46], the total number of intestinal bacteria increased in rainbow trout fed a diet in which FM was partially replaced by TM in comparison to control fish that were fed a FM-based diet.

Interestingly, Antonopoulou and colleagues [20] reported that the dietary inclusion of *T. molitor* larvae meal led to a five-fold increase of Simpson dominance D index, and to a two-fold decrease of the Shannon H index in rainbow trout gut microbiota, but not in sea bream and sea bass microbiota in which the same diversity indices remained practically unchanged. This evidence suggests a species-specific impact of insect meal on gut bacterial communities. Equally, in our previous studies, we found an increase of bacteria species richness and diversity in intestinal microbiome of trout fed diets with partial replacement of FM with *Hermetia illucens* meal [18, 19].

Regardless of the diet type, marked differences in terms of alpha diversity were found between gut and skin microbiota, being the latter characterized by higher microbial diversity and richness. Although these divergences could be partly due to the different rarefaction depth applied to compute alpha diversity, it is also true that previous studies on trout and other fresh water species displayed a similar trend with a lower alpha diversity in the gut than in the skin mucosal surface [27, 47, 48]. Unfortunately, in contrast to high number of studies focused on fish gut microbiome, the skin mucus microbiome remains largely underexplored.

Initially, fish skin is colonized by bacteria present in the water, but over time, the superficial mucus harbors an increasingly divergent microbial community [47, 49]. Like in intestine, the balance between members of skin microbial community, i.e., commensals, symbionts or pathogenic bacterial strains, collectively forming skin microbiome, is important to preserve fish health. It is well known that factors such as diet, water quality, seasonality, host physiology, infections, and stress can shape the composition of fish microbiomes and influence the balance of the microbic ecosystems [33–35].

Our metabarcoding analysis showed that rainbow trout skin microbiome was largely dominated by

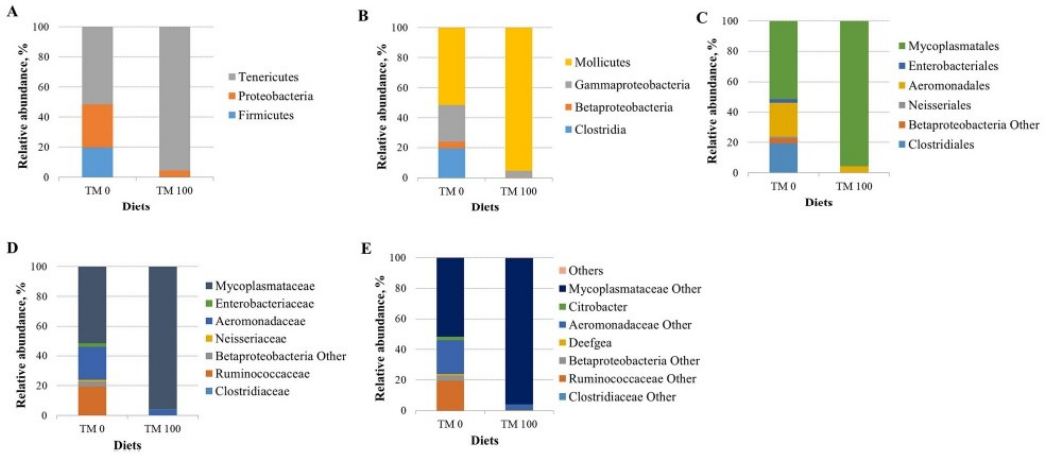

Fig. 3 Relative abundance (%) of the overall most prevalent bacterial phyla (**a**), classes (**b**), orders (**c**), families (**d**), and genera (**e**) in GMMC. All bacteria with an overall abundance of ≥1% for **a**, **b** and **c;** and ≥ 0.5% for **d** and **e** were reported. Bacteria with lower abundance were pooled and indicated as "Others"

Table 5 Mean of relative abundance (%) ± SD of the most prevalent phyla, classes, orders, families, and genera found in GMMC

Items	TM 0	TM 100	*P*-value
Phylum			
Firmicutes	19.51 ± 23.48	0.21 ± 0.19	0.747
Proteobacteria	29.00 ± 28.65	4.20 ± 5.20	**0.047**
Tenericutes	51.50 ± 38.26	95.56 ± 5.30	0.065
Class			
Clostridia	19.47 ± 23.43	0.18 ± 0.18	0.746
Betaproteobacteria	4.45 ± 5.15	0.05 ± 0.10	**0.012**
Gammaproteobacteria	24.55 ± 30.20	4.15 ± 5.12	0.336
Mollicutes	51.50 ± 38.26	95.56 ± 5.30	0.065
Order			
Clostridiales	19.47 ± 23.43	0.18 ± 0.18	0.746
Neisseriales	1.06 ± 1.12	0.05 ± 0.10	0.033
Aeromonadales	22.24 ± 30.74	3.98 ± 5.24	0.422
Enterobacteriales	2.27 ± 4.37	0.16 ± 0.37	0.144
Mycoplasmatales	51.50 ± 38.26	95.56 ± 5.30	0.065
Family			
Clostridiaceae	0.0 ± 0.0	0.2 ± 0.2	-
Ruminococcaceae	19.5 ± 23.4	0.0 ± 0.0	-
Neisseriaceae	1.1 ± 1.1	0.1 ± 0.1	**0.033**
Aeromonadaceae	22.2 ± 30.7	4.0 ± 5.2	0.422
Enterobacteriaceae	2.3 ± 4.4	0.2 ± 0.4	0.221
Mycoplasmataceae	51.5 ± 38.3	95.6 ± 5.3	0.065
Genus			
Deefgea	1.05±1.13	0.04±0.10	0.055
Citrobacter	2.20±4.38	0.00±0.00	-

P < 0.05 are in bold. "-" taxa detected only in one group

Proteobacteria, and especially Gammaproteobacteria, which constituted approximately half of the bacterial taxa found. This result is in agreement with previous studies on other fish species regardless of the technique used for bacterial identification [26–28, 30, 31, 50–52]. Gammaproteobacteria class includes several potentially pathogenic bacterial species for fish, such as *Vibrio anguillarum*, and *Photobacterium damselae*. Actually, there are several evidences supporting the role of fish skin microbiota as an important niche for mucosal pathogen evolution in nature [50]. For instance, potentially pathogenic *Vibrio*, such as *Vibrio anguillarum* and *Vibrio cholerae*, monopolize skin microbiome of wild eel (*Anguilla anguilla*) from estuary and wetland [50]. Other accidental pathogens identified in wild eel have been *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and *Aeromonas veronii*. Similarly, skin microbiome of coral reef fish showed a significant enrichment in Gammaproteobacteria, especially Vibrionaceae [31].

Although in the present study trout skin microbiome was dominated by the Gammaproteobacteria's family of Aeromonadaceae instead of Vibrionaceae, at genus level, *Pseudomonas*, *Stenotrophomonas* and *Citrobacter* were present in our samples likewise in wild and farmed eel skin microbiome [50]. This result is quite interesting, since previous studies have indicated that fish skin microbiome is species-specific, both in terms of bacterial diversity and bacterial community structure, showing significantly lower variability between individuals from the same species than between those of different species [26, 31].

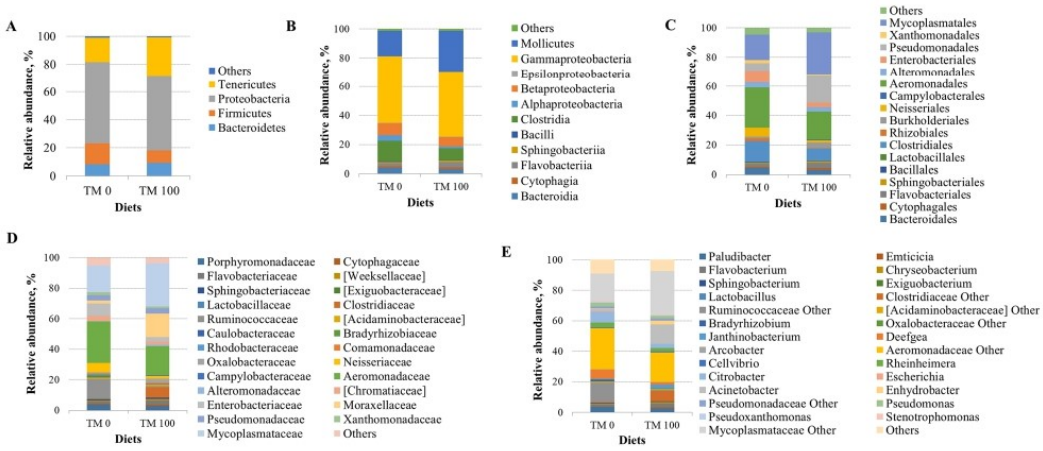

Fig. 4 Relative abundance (%) of the overall most prevalent bacterial phyla (**a**), classes (**b**), orders (**c**), families (**d**), and genera (**e**) in GMMC. All bacteria with an overall abundance of ≥1% for **a**, **b** and **c;** and ≥ 0.5% for **d** and **e** were reported. Bacteria with lower abundance were pooled and indicated as "Others"

The low frequency of *Vibrio* genera in trout skin microbial community could be explained by the fact that trout is a freshwater fish while *Vibrio* are mainly marine bacterial genera. It is widely accepted, indeed, that the skin of fish harbors a complex and diverse microbiota that closely interacts with the microbial communities of the surrounding water.

In line with our data, Lowrey et al. [27] reported that Proteobacteria and Bacteroidetes were the most abundant phyla of rainbow trout skin microbiota, however at genus level they found a skin bacterial community consistently composed by *Flectobacillus*. These apparently controversial evidences are inevitable since, up to date, few studies have investigated skin microbiome in freshwater fish, and it is not yet known if it fundamentally differs from that of marine fish [51].

With regard to skin microbial community composition, the two dietary groups did not display distinctive features, except for a decrease in the relative abundance of *Deefgea* genus (family Neisseriaceae) in skin microbiome of trout fed with insect meal. Changes in the skin microbiota of fish in response to stressors, such as hypoxia have been previously observed, in brook charr (*Salvelinus fontinalis*), in which probiotic-like bacteria decreased after stress exposure [53]. Studies in salmonids have also shown that parasitic infections or other microbial aetiological agents (e.g. viruses) may perturb skin microbiota [30].

In agreement with our recent study in rainbow trout [19], metagenomic analysis indicated that Tenericutes was the most abundant phylum in trout intestine, regardless of the diet. Specifically, within this phylum, the Mollicutes, mainly represented by Mycoplasmataceae family, were the dominant class. The Tenericutes are among the protagonists of gut symbionts of rainbow trout, indicating that they are possibly related to the metabolism of the host [27, 54, 55]. Although diet is the most important external factor affecting the gut microbiota composition, in this case we observed only a weak dietary modulation of intestinal bacterial communities. The only changes due to dietary FM substitution with TM meal were a decreased number of Proteobacteria and, at family level, a reduced number of taxa assigned to Ruminococcaceae and Neisseriaceae.

In line with our results, Antonopoulou et al. [20] reported that *T. molitor* meal replacement affected the dominant intestinal phyla less in rainbow trout than in sea bream and sea bass. In contrast, there are several evidences that FM replacement with insect meal from black soldier fly (*Hermetia illucens*) larvae positively modulates gut microbiota of rainbow trout by increasing the proportion of lactic acid bacteria (LAB), which are generally considered as beneficial microorganisms and frequently used as probiotics in fish and other vertebrates diet [18, 19, 56].

Actually, there is a study stating that the inclusion of 20% TM meal in the diet increased the intestinal population of *Lactobacillus* and *Enterococcus* genera in rainbow trout juveniles [23]. The increase of LAB by dietary insect meal could be related to the prebiotic properties of chitin. Chitin is an insoluble linear polysaccharide (a biopolymer of N-acetyl-β-*D*-glucosamine) that confers structural rigidity to insects' exoskeleton. Partial or full enzymatic deacetylation of chitin produces chitosan.

Table 6 Mean of relative abundance (%) ± SD of the most prevalent phyla, classes, orders, families, and genera found in SMMC

Items	TM 0	TM 100	*P*-value
Phylum			
Bacteroidetes	8.15 ± 6.71	9.28 ± 5.61	0.878
Firmicutes	14.82 ± 16.38	8.65 ± 7.18	0.810
Proteobacteria	58.77 ± 10.48	53.18 ± 20.76	0.617
Tenericutes	17.36 ± 9.49	28.19 ± 15.54	0.228
Class			
Bacteroidia	4.22 ± 4.88	3.17 ± 2.41	0.683
Cytophagia	0.80 ± 0.70	1.09 ± 0.82	0.802
Flavobacteriia	2.14 ± 1.21	3.74 ± 2.27	0.375
Sphingobacteriia	0.72 ± 0.49	0.93 ± 0.73	0.855
Bacilli	0.62 ± 0.63	0.29 ± 0.29	0.198
Clostridia	14.21 ± 16.56	8.35 ± 7.22	0.936
Alphaproteobacteria	3.80 ± 4.18	1.28 ± 0.90	0.153
Betaproteobacteria	8.17 ± 1.81	6.20 ± 4.24	0.247
Epsilonproteobacteria	0.18 ± 0.17	0.33 ± 0.25	0.397
Gammaproteobacteria	46.34 ± 7.74	45.12 ± 17.46	0.874
Mollicutes	17.36 ± 9.49	28.19 ± 15.54	0.228
Order			
Bacteroidales	4.22 ± 4.89	3.17 ± 2.41	0.683
Cytophagales	0.80 ± 0.70	1.09 ± 0.82	0.802
Flavobacteriales	2.14 ± 1.21	3.74 ± 2.27	0.375
Sphingobacteriales	0.72 ± 0.49	0.93 ± 0.73	0.855
Bacillales	0.39 ± 0.49	0.15 ± 0.18	0.211
Lactobacillales	0.23 ± 0.17	0.14 ± 0.11	0.275
Clostridiales	14.21 ± 16.56	8.35 ± 7.22	0.936
Rhizobiales	1.65 ± 3.49	0.30 ± 0.30	0.936
Burkholderiales	1.30 ± 1.51	3.60 ± 3.99	0.261
Neisseriales	5.94 ± 2.73	1.70 ± 2.45	**0.013**
Campylobacterales	0.18 ± 0.17	0.33 ± 0.25	0.397
Aeromonadales	27.21 ± 9.10	19.20 ± 9.11	0.154
Alteromonadales	3.69 ± 6.17	3.04 ± 2.49	0.969
Enterobacteriales	8.09 ± 5.16	2.96 ± 4.72	0.093
Pseudomonadales	5.43 ± 4.95	18.81 ± 23.79	0.471
Xanthomonadales	1.93 ± 1.68	1.12 ± 0.71	0.328
Mycoplasmatales	17.36 ± 9.49	28.20 ± 15.55	0.227
Family			
Porphyromonadaceae	3.64 ± 4.23	2.66 ± 2.17	0.636
Cytophagaceae	0.79 ± 0.71	1.08 ± 0.82	0.799
Flavobacteriaceae	1.50 ± 1.06	2.69 ± 1.87	0.368
[Weeksellaceae]	0.57 ± 0.29	0.99 ± 0.93	0.633
Sphingobacteriaceae	0.70 ± 0.48	0.90 ± 0.72	0.854
[Exiguobacteraceae]	0.13 ± 0.12	0.09 ± 0.11	0.367
Lactobacillaceae	0.02 ± 0.04	0.03 ± 0.06	0.549
Clostridiaceae	0.34 ± 0.21	6.68 ± 8.17	**0.013**

Table 6 Mean of relative abundance (%) ± SD of the most prevalent phyla, classes, orders, families, and genera found in SMMC *(Continued)*

Items	TM 0	TM 100	*P*-value
Ruminococcaceae	12.65 ± 17.33	0.63 ± 1.35	0.170
[Acidaminobacteraceae]	1.12 ± 1.48	0.95 ± 0.59	0.887
Caulobacteraceae	0.47 ± 0.53	0.30 ± 0.16	0.810
Bradyrhizobiaceae	1.15 ± 2.77	0.03 ± 0.03	0.683
Rhodobacteraceae	0.55 ± 0.62	0.16 ± 0.15	0.133
Comamonadaceae	0.48 ± 0.70	0.73 ± 0.42	0.433
Oxalobacteraceae	0.82 ± 0.81	2.87 ± 3.86	0.173
Neisseriaceae	5.94 ± 2.73	1.70 ± 2.45	**0.013**
Campylobacteraceae	0.18 ± 0.17	0.33 ± 0.25	0.397
Aeromonadaceae	27.21 ± 9.10	19.20 ± 9.11	0.154
Alteromonadaceae	0.31 ± 0.15	0.91 ± 1.55	0.936
[Chromatiaceae]	3.36 ± 6.09	2.11 ± 1.23	0.378
Enterobacteriaceae	8.09 ± 5.16	2.96 ± 4.72	0.092
Moraxellaceae	1.81 ± 1.60	15.47 ± 22.04	0.298
Pseudomonadaceae	3.62 ± 3.35	3.33 ± 2.08	0.818
Xanthomonadaceae	1.93 ± 1.68	1.12 ± 0.71	0.328
Mycoplasmataceae	17.36 ± 9.49	28.20 ± 15.55	0.228
Genus			
Paludibacter	3.60 ± 4.21	2.61 ± 2.15	0.629
Emticicia	0.21 ± 0.11	0.65 ± 0.53	0.197
Flavobacterium	1.49 ± 1.04	2.54 ± 1.92	0.440
Chryseobacterium	0.54 ± 0.31	0.92 ± 0.89	0.662
Sphingobacterium	0.44 ± 0.26	0.52 ± 0.47	0.964
Exiguobacterium	0.13 ± 0.12	0.09 ± 0.11	0.369
Lactobacillus	0.02 ± 0.04	0.02 ± 0.05	0.549
Janthinobacterium	0.54 ± 0.64	2.29 ± 3.78	0.378
Deefgea	5.85 ± 2.90	1.58 ± 2.48	**0.017**
Arcobacter	0.18 ± 0.17	0.33 ± 0.25	0.397
Cellvibrio	0.31 ± 0.15	0.91 ± 1.55	0.936
Rheinheimera	3.36 ± 6.09	2.11 ± 1.23	0.378
Citrobacter	7.11 ± 5.75	2.66 ± 4.54	0.471
Escherichia	0.53 ± 0.80	0.02 ± 0.05	0.253
Acinetobacter	1.46 ± 1.27	12.87 ± 18.51	0.185
Enhydrobacter	0.23 ± 0.24	2.32 ± 3.30	0.183
Pseudomonas	2.44 ± 2.13	1.92 ± 1.06	0.604
Pseudoxanthomonas	0.80 ± 1.13	0.41 ± 0.33	0.486
Stenotrophomonas	0.67 ± 0.50	0.43 ± 0.30	0.406

$P < 0.05$ are in bold

Both chitin and chitosan are hardly digested by the majority of fish [21]; therefore, once consumed, the fermentation of both polysaccharides is largely performed by gut microbiota. The lack of enrichment in intestinal LAB during the present study was an unexpected result, especially when compared to what has been previously observed in the intestine of trout fed with diets containing *H. illucens* larvae meal [18, 19]. The main effect of the dietary inclusion of this type of insect meal was a significant increase of Firmicutes at the expense of Proteobacteria phylum. The dietary administration of TM meal caused instead

only a decrease in relative amount of Proteobacteria without any increase in Firmicutes.

Conclusions

In summary, the data demonstrated that yellow mealworm (*T. molitor*) larvae meal is a valid alternative animal protein to replace FM in the aquafeeds. In summary, the data demonstrated that yellow mealworm (*T. molitor*) larvae meal is a valid alternative animal protein to replace FM in the aquafeeds. The totally replacement of FM with TM did not cause negative effects on rainbow trout gut and skin microbial communities. No evident sign of dysbiosis was detected, but only slight microbiota changes after total FM substitution with insect meal. Specifically we assisted to a reduction in relative abundance of Neisseriaceae bacterial family, in both gut and skin. Differences at genus level were identified only at the skin leveln with a two-fold decrease of Deefgea genus in trout fed with TM 100 diet. Last, but not least, the mapping of the trout skin microbiota represents a novel contribution of the present study since fish skin microbiota is still scarcely investigated, in particular in freshwater fish. Indeed, in contrast to the increasing knowledge on gut microbiota, the skin microbiota of major farmed fish species remains largely unmapped but it deserves thorough consideration.

Abbreviations

FM: Fishmeal; TM: *Tenebrio molitor*; GMMC: Gut mucosa microbial communities; SMMC: Skin mucosa microbial communities; LAB: Lactic acid bacteria

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Authors' contributions

Conceptualization, G.T.; methodology E.G.; L.G.; and S.R.; Data collection, curation and analysis, M.A.; F.M.; E.G; and L.G.; writing—original draft preparation, G.T.; S.R.; writing—review and editing, S.R; and G.T.; funding acquisition, G.T. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All DNA sequencing data were deposited as FASTQ files at the European Nucleotide Archive (EBI ENA) public database under the accession code: **PRJEB38845.**

Ethics approval and consent to participate

The experimental protocol was designed according to the guidelines of the current European and Italian laws on the care and use of experimental animals (European directive 86 609/EEC, put into law in Italy with D.L. 116/92). The Ethical Committee of DISAFA (protocol n°143811) approved the experimental protocol.

Consent for publication

All the authors consent to the publication of data in JASB.

Competing interests

The authors declare that they have no competing interests. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Chapter 4

The Effects of Nisin-Producing *Lactococcus lactis* Strain Used as Probiotic on Gilthead Sea Bream (*Sparus aurata*) Growth, Gut Microbiota, and Transcriptional Response

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ORIGINAL RESEARCH
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The Effects of Nisin-Producing *Lactococcus lactis* Strain Used as Probiotic on Gilthead Sea Bream (*Sparus aurata*) Growth, Gut Microbiota, and Transcriptional Response

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The present research tested the effects of dietary nisin-producing *Lactococcus lactis* on growth performance, feed utilization, intestinal morphology, transcriptional response, and microbiota in gilthead sea bream (*Sparus aurata*). A feeding trial was conducted with fish weighting 70–90 g. Fish were tagged with passive, integrated transponders and distributed in nine 500 L tanks with 40 fish each. Fish were fed for 12 weeks with either a control (diet A) or experimental diets (diets B and C) in triplicate (3 tanks/diet). Extruded pellets of diets B and C were supplemented with a low (2×10^9 CFU/kg) and a high (5×10^9 CFU/kg) dose of probiotic, respectively. No significant differences were found between groups for the feed conversion ratio or specific growth rates. However, the final body weight of fish fed diet C was significantly higher than the control group with intermediate values for fish fed diet B. Histological analysis conducted using a semi-quantitative scoring system showed that probiotic did not alter the morphology of the intestine and did not trigger inflammation. With regard to the transcriptomic response, a customized PCR array layout was designed to simultaneously profile a panel of 44 selected genes. Significant differences in the expression of key genes involved in innate and acquired immunity were detected between fish fed probiotic and control diets. To analyze the microbiota associated to the feeds and the gut autochthonous microbial communities, we used the Illumina MiSeq platform for sequencing the 16S rRNA gene and a metagenomics pipeline based on VSEARCH and RDP databases. The analysis of gut microbiota revealed a lack of colonization of the probiotic in the host's intestinal mucosa. However, probiotic did modulate the fish gut microbiota, confirming that colonization is not always necessary to induce host modification. In fact, diets B and C were enriched with Actinomycetales, as compared to diet A, which instead showed a

higher percentage of *Pseudomonas*, *Sphyngomonas*, and *Lactobacillus* genera. These results were confirmed by the clear separation of gut bacterial community of fish fed with the probiotic from the bacterial community of control fish group in the beta-diversity and PLS-DA (supervised partial least-squares discriminant analysis) analyses.

Keywords: aquaculture, gilthead sea bream, probiotic, *Lactococcus lactis*, gut microbiota, transcriptomic

INTRODUCTION

The definition of "Probiotics" has changed many times during this century. However, according to (Food and Agriculture Organization of the United [FAO] and World Health Organisation [WHO], 2001) probiotics are "live microorganisms that confer a health benefit on the host when administered in adequate amounts." The most commonly used probiotics are bacteria belonging to *Lactobacillus*, *Bifidobacterium*, *Bacillus,* and *Enterococcus* genera (European Medicines Agency [EMA], and European Food Safety Authority [EFSA], 2017; EFSA FEEDAP [EFSA Panel on Additives and Products or Substances used in Animal Feed] et al., 2018), but some fungal genera have also been reported as novel probiotics.

In the last 25–30 years, the use of probiotics in animal production has increased (Chaucheyras-Durand and Durand, 2010; Ezema, 2013). Indeed, several publications have reported numerous beneficial effects associated with the supplementation of live yeast or bacteria (mostly *Lactobacillus*) in the diet of terrestrial animals, including amelioration of resistance to pathogens, improvement in growth parameters (in swine and poultry), increase in productivity and quality of eggs in laying hens, and enhancement of milk production in cattle (Gallazzi et al., 2008; Shabani et al., 2012; Puphan et al., 2015; Uyeno et al., 2015; De Cesare et al., 2017; Wang et al., 2017; Dowarah et al., 2018; Forte et al., 2018).

In aquaculture, a great number of bacterial species are currently used as probiotics (for a review, please see Newaj-Fyzul et al., 2014). These microorganisms can be administered as multi-species (multi-strain) or single-species (single-strain) (Food and Agriculture Organization of the United [FAO], 2016) and provided either as a suspension in water, or added to the feed. However, use in feed is considered the best option; therefore, this approach is employed most frequently (Nayak, 2010; Jahangiri and Esteban, 2018). In the European Union (EU), probiotic strains, must obtain a market authorization by the EFSA (European Safety Food Authority)[1], which grants a QPS (Qualified Presumption of Safety) status. The QPS is based on reasonable evidence. No microorganism belonging to a QPS status group needs to undergo a full safety assessment, but microorganisms that pose a safety concern to humans, animals, or environment are not considered suitable for QPS status and must undergo a full safety assessment. The QPS assessment requires: (1) the identity of the strain to be conclusively established, and (2) absence of resistance to antibiotics (for bacteria) or antimycotics (for yeasts) used in human and veterinary medicine (EFSA Panel on Biological Hazards (BIOHAZ) et al., 2020).

The increase in the use of probiotics in aquaculture is mostly related to the need to decrease or even avoid the use of antibiotics, increasing at the same time the sustainability of the aquaculture industry. The negative effects of antibiotics overuse include the accumulation of residue in the aquatic environment, particularly in the marine sediments where antibiotics can persist for months, favoring the selection of multi-antibiotic-resistant bacterial strains. Indeed, there is an increasing risk that antibiotic-resistant bacteria, initially derived from food-producing animals, could render the latest generation of antibiotics virtually ineffective for humans (Cabello, 2006; World Health Organisation [WHO] et al., 2006). Another negative outcome of antibiotics being used as growth promoters in cultured fish is the reduction of biodiversity and quantity of indigenous gut microbiota, which can impair fish immune responses (Borch et al., 2015).

For these reasons, the use of antibiotics as growth promoters in animal production has been fully banned in the EU since 2006 (Casewell et al., 2003; European Parliament and the Council of the European Union, 2003, 2019; European Medicines Agency [EMA], and European Food Safety Authority [EFSA], 2017) and many research efforts have been undertaken to replace them with probiotics for animal health management (Ezema, 2013).

Several studies have demonstrated that probiotics can reduce pathogenic bacteria due to direct competition-colonizing dynamics, through which microorganisms can partition spatial niche habitats in the intestinal mucosa (Balcázar et al., 2007b; Sugimura et al., 2011). Probiotics can also produce inhibitory molecules, such as bacteriocins, siderophores, enzymes, and hydrogen peroxide, or inhibit pathogenic bacteria by decreasing the intestinal pH through the release of organic acids (Ringø, 2008; Zhou X. et al., 2010; Ustyugova et al., 2012; Perez et al., 2014; Dahiya et al., 2020).

In addition, probiotics enhance the host immune system by generating systemic and/or local responses (Balcázar et al., 2006b; Salinas et al., 2008) that include activation of various antioxidant pathways and an increase in several innate immune parameters, such as phagocytosis, lysozyme levels, respiratory burst peroxidase and antiprotease activity, cytokine production, and white blood cell count (Nayak, 2010; Lazado and Caipang, 2014; Newaj-Fyzul et al., 2014; Simó-Mirabet et al., 2017).

In cultured fish, probiotics improve fish growth and feed conversion rates, too, due to an increase in feed digestibility and absorption of nutrients (Dimitroglou et al., 2011; Martínez Cruz et al., 2012). These effects stem from the capacity of probiotics to secrete enzymes, such as proteases, amylases, and lipases that hydrolyze molecules, which the fish intestine cannot

[1]https://www.efsa.europa.eu/en

otherwise digest (Balcázar et al., 2006b; Abd El-Rhman et al., 2009). Furthermore, the use of probiotics can restore the eubiotic state of the intestinal microbiota after antibiotic treatment or a pathogenic insult or can help maintain gut microbiota homeostasis, even in larval stages, when vaccination is difficult (Abdelhamid et al., 2009; Borch et al., 2015).

Hence, positive effects of different probiotics have been reported in several fish species, such as Nile tilapia (*Oreochromis niloticus*) (Ridha and Azad, 2012), common carp (*Cyprinus carpio*) (Feng et al., 2019), African catfish (*Clarias gariepinus*) (Al-Dohail et al., 2009), olive flounder (*Paralichthys olivaceus*) (Heo et al., 2013), Asian sea bass (*Lates calcarifer*) (Ringø, 2008; Lin et al., 2017), red drum (*Sciaenops ocellatus*) (Zhou Q.C. et al., 2010), European sea bass (*Dicentrarchus labrax*) (Carnevali et al., 2006; Mahdhi, 2012), common dentex (*Dentex dentex*) (Hidalgo et al., 2006), gilthead sea bream (*Sparus aurata*) (Suzer et al., 2008; Varela et al., 2010), rainbow trout (*Oncorhynchus mykiss*) (Merrifield et al., 2010), and abalone (*Haliotis midae*) (Macey and Coyne, 2005), and in crustaceans, such as white shrimp (*Litopenaeus vannamei*) (Lin et al., 2004).

According to the above findings, the aim of the present research was to evaluate the effects of the lactic acid bacteria *Lactococcus lactis* subsp. *lactis* SL242, used as feed additive, on growth performance, feed utilization, intestinal morphology, transcriptional response, and microbiota in gilthead sea bream (*Sparus aurata*).

The probiotic strain *L. lactis* subsp. *lactis* SL242 was selected due to important characteristics of *Lc. lactis* in general and SL242 in particular. *Lc. lactis* are mesophilic lactic acid bacteria that are present in the intestinal microbiota of fish (Tarnecki et al., 2017; Ringø et al., 2020) and can adapt to the water temperature of many reared fish species. Lactococci are proteolytic bacteria (Samaržija et al., 2001) that are potentially useful for improving the digestion of proteins contained in fish feed. The proteolytic system of lactococci includes a cell wall-associated proteinase and an extracellular peptidase (Samaržija et al., 2001). Furthermore, SL242 produces the antibiotic nisin A (Malvisi et al., 2016), which can inhibit or kill vegetative cells and bacterial spores (European Safety Food Authority [EFSA], 2005). Due to its antibacterial activity, nisin is of great interest in aquaculture. Nisin-susceptible bacterial species are found among *Bacillus*, *Clostridium*, *Listeria*, *Staphylococcus*, *Streptococcus*, and *Vibrio* genera (European Safety Food Authority [EFSA], 2005; Malvisi et al., 2016; Hamid et al., 2020), including known aquatic pathogens, such as *V. parahaemolyticus*, and *V. alginotlyticus* (Hamid et al., 2020). *Lc. lactis* probiotics have also shown inhibitory action against *Yersinia rukeri* and *Aeromonas salmonicida*, which can affect fish growth (Balcázar et al., 2007a, 2006b). Furthermore, *Lc. lactis* probiotic has been effective against *Aeromonas hydrophila* in *Oreochromis niloticus* (Zhou X. et al., 2010).

MATERIALS AND METHODS

Ethics Statement

Procedures for fish manipulation and tissue collection were carried out according to the Spanish (Royal Decree RD53/2013) and the current EU legislation (2010/63/EU) for handling of experimental fish. All procedures were approved by the Ethics and Animal Welfare Committees of Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain) (Permit number 824/2019) and "Generalitat Valenciana" (permit number 2019/VSC/PEA/0197).

Animals

On June 2019, juveniles of gilthead sea bream were purchased from a Mediterranean hatchery (Piscimar, Burriana, Spain) and adapted for more than 2 months to the indoor experimental facilities of IATS-CSIC, under natural photoperiod and temperature conditions (40°5′N; 0°10′E). Seawater was pumped ashore (open system); oxygen content of water effluents was always above 85% saturation, and unionized ammonia remained below 0.02 mg/L. During the acclimation and experimental period, water temperature increased from 20–22°C in June to 28°C in August, decreasing thereafter from 24–25°C in mid-September to 13–16°C in December.

Diets

Extruded pellets of a control (diet A) and two experimental diets (diets B and C) were manufactured by VRM Srl Naturalleva (Verona, Italy), mimicking commercial fish feed formulations with traditional vegetable proteins and oils as the main replacers of fishmeal and fish oil, respectively (**Table 1**). The mash of each diet was extruded using a single-screw extruder (X-165, Wenger United States). To ensure product stability, the probiotic was homogenized with the dietary oil and included by vacuum coating (La Meccanica vacuum coater, Italy) during the post-extrusion process. During the vacuum process, only dry basal extruded pellets of diets B and C were supplemented with 2.5 and 6.2 g/100 kg of *L. lactis* subsp. *lactis* SL242, corresponding to a probiotic dosage of 2×10^9 CFU/kg (low dose) and 5×10^9 CFU/Kg (high dose), respectively. Sacco S.r.l [Cadorago (Co), Italy] provided the probiotic strain.

The two doses were chosen on the basis of our experience and literature data (Villamil et al., 2002; Adel et al., 2017) in order to verify the most effective one. They are also in line with dosages that could be used commercially in a cost-effective manner.

The final feeds were stored in a refrigerated room (6–7°C) for the entire duration of the feeding trial. A preliminary stability study of SL242 in the feed supplemented with probiotic was conducted for 12 weeks (the duration of the experiment), at 6°C. At the end of this period, the average loss of viability determined by plate count resulted about 50%, consistent with our expectations. Although further improvement may be warranted for a commercial probiotic product, at this stage of the process, the observed stability is considered acceptable.

Feeding Trial

In September 2019, fish weighing 70–90 g were randomly distributed in nine 500 L tanks to establish triplicate groups of 40 fish each (initial rearing density, 6.6–6.7 kg/m^3). All fish were tagged with PIT (passive integrated transponders) (ID-100A 1.25 Nano Transponder, Trovan) in the dorsal skeletal muscle. Fish were individually weighed and measured at initial, intermediate,

TABLE 1 | Ingredients and chemical composition (%) of control diet (Diet A) used in the trial.

Ingredients	Diet A
Fishmeal	10.1
Corn gluten	24.3
Guar germ meal	10.0
Soybean meal	13.1
Soya protein concentrate	13.6
Wheat	10.8
Fish oil	7.5
Rapeseed oil	3.5
Camelina oil	3.5
Lactic bacteria	0.0
Lysine	0.9
DL-methionine	0.4
Monoammonium phosphate	1.2
Taurine	0.4
Vitamins[a] and Minerals[b]	0.7
Proximate composition (%)	
Gross energy (MJ/kg)	18.92
Digestible energy, DE (MJ/kg)	17.26
Crude fat	18.0
Crude protein	43.8
Digestible protein, DP	38.8
DP/DE (mg/kJ or g/MJ)	22.5
Fiber	2.6
Nitrogen free extract	24.6
Starch	8.7
Non-starch polysaccharides	18.5

Diet B and C were formulated with the addition of probiotic (5×10^6 CFU/g feed).
[a]Vitamin premix (IU or mg/kg diet): DL-α tocopherol acetate 60 IU; sodium menadione bisulfate 5 mg; retinyl acetate 15,000 IU; DL-cholecalciferol 3,000 IU; thiamine 15 mg; riboflavin 30 mg; pyridoxine 15 mg; vitamin B_{12} 0.05 mg; nicotinic acid 175 mg; folic acid 500 mg; inositol 1,000 mg; biotin 2.5 mg; calcium pantothenate 50 mg.
[b]Mineral premix (g or mg/kg of diet) bi-calcium phosphate 500 g, calcium carbonate 215 g, sodium salt 40 g, potassium chloride 90 g, magnesium chloride 124 g, magnesium carbonate 124 g, iron sulfate 20 g, zinc sulfate 4 g, copper sulfate 3 g, potassium iodide 4 mg, cobalt sulfate 20 mg, manganese sulfate 3 g, sodium fluoride 1 g.

and final sampling points (every 4 weeks), by using a FR-200 Fish Reader W (Trovan, Madrid, Spain) for data capture and pre-processing.

The trial lasted 12 weeks (October 2019–December 2019). Fish were hand-fed once daily (12 a.m.), 5–6 days per week to visual satiety with either control or experimental diets for the entire duration of the trial. Feed intake and mortalities (<1%) were recorded daily and normal fish behavior was assessed routinely by camera monitoring.

Sample Collection

At the end of the feeding trial, four fish per replicate (12 fish/diet) were anesthetized with 0.1 g/L of tricaine-methasulfonate (MS-222, Sigma-Aldrich) and then sacrificed by severing the spinal cord. The intestine (excluding the pyloric ceca) of each fish was dissected out, weighed, and measured aseptically to calculate the intestine weight index (IWI) and intestine length index (ILI). Then, anterior (AI) and posterior (PI) intestine tissue portions (~0.4 cm) were put either into RNAlater, or in 10% neutral buffered formalin for subsequent molecular (AI) and histological (AI, PI) analyses. The remaining part of AI was opened and washed with sterile Hank's balanced salt solution before collecting the autochthonous intestinal bacteria by scraping intestinal mucosa with the blunt end of a clean scalpel. Then, mucus samples were transferred to a sterile Eppendorf tube and stored in ice until subsequent (within 2 h) DNA extraction for microbiota analysis.

To characterize feed-associated bacterial communities, two samples of 200 mg each from each feed were taken at the end of the trial and used for bacterial DNA extraction and sequencing.

Histological Analysis

Fixed samples of AI and PI were dehydrated in ethanol solutions with gradually increasing concentrations and then, embedded in paraffin. Sections of 5 μm were obtained with a microtome (Leica RM2245) and stained with hematoxylin and eosin (H&E), following standard histological protocols. The sections were examined under a stereomicroscope Eurotek Tecno NB50T (Orma Srl, Milan, Italy) and photographed with a digital camera Eurotek CMOS MDH5 (Orma Srl, Milan, Italy). Based on previous studies (Knudsen et al., 2007; Uran et al., 2008; Urán et al., 2009; Khojasteh, 2012), the semi-quantitative scoring system focused on five different gut morphological parameters (mucosal folds, connective tissue, *lamina propria* of simple folds, and supranuclear vacuoles). Histological alterations of each morphological parameter were classified using a score value ranging from one (normal condition) to five (severe alteration). The final values, obtained by the sum of score values for each parameter, were then used to classify the severity of the morphological damage by using a class-based scoring system: Class I (values $\leq$ 10)—normal tissue structure with slight histological alterations; Class II (values 11–15)—moderate histological alterations; and Class III (values > 15)—severe histological alterations of the organ.

Gene Expression Analysis

Total RNA from AI was extracted using a MagMax-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, United States). The RNA yield was higher than 3.5 μg with absorbance measures (A260/280) of 1.9–2.1. cDNA was synthesized with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, United States), using random decamers and 500 ng of total RNA in a final volume of 100 μL. Reverse transcription (RT) reactions were incubated 10 min at 25°C and 2 h at 37°C. Negative control reactions were run without the enzyme. As reported previously (Estensoro et al., 2016), a customized PCR array layout was designed to simultaneously profile a panel of 44 selected genes, including markers of epithelial integrity (11), nutrient transport (4), mucins (3), cytokines (9), immunoglobulins (2), cell markers and chemokines (7), and pattern recognition receptors (8) (**Table 2**). qPCR reactions were performed using an iCycler IQ Real-Time Detection System (Bio-Rad, Hercules, CA, United States). Diluted RT

TABLE 2 | PCR-array layout for intestine gene expression profiling.

Function	Gene	Symbol	GenBank
Epithelial integrity	Proliferating cell nuclear antigen	*pcna*	KF857335
	Transcription factor HES-1-B	*hes1-b*	KF857344
	Krueppel-like factor 4	*klf4*	KF857346
	Claudin-12	*cldn12*	KF861992
	Claudin-15	*cldn15*	KF861993
	Cadherin-1	*cdh1*	KF861995
	Cadherin-17	*cdh17*	KF861996
	Tight junction protein ZO-1	*tjp1*	KF861994
	Desmoplakin	*dsp*	KF861999
	Gap junction Cx32.2 protein	*cx32.2*	KF862000
	Coxsackievirus and adenovirus receptor homolog	*cxadr*	KF861998
Nutrient transport	Intestinal-type alkaline phosphatase	*alpi*	KF857309
	Liver type fatty acid-binding protein	*fabp1*	KF857311
	Intestinal fatty acid-binding protein	*fabp2*	KF857310
	Ileal fatty acid-binding protein	*fabp6*	KF857312
Mucus production	Mucin 2	*muc2*	JQ277710
	Mucin 13	*muc13*	JQ277713
	Intestinal mucin	*i-muc*	JQ277712
Cytokines	Tumor necrosis factor-alpha	*tnfα*	AJ413189
	Interleukin 1 beta	*il1β*	AJ419178
	Interleukin 6	*il6*	EU244588
	Interleukin 7	*il7*	JX976618
	Interleukin 8	*il8*	JX976619
	Interleukin 10	*il10*	JX976621
	Interleukin 12 subunit beta	*il12*	JX976624
	Interleukin 15	*il15*	JX976625
	Interleukin 34	*il34*	JX976629
Immunoglobulins	Immunoglobulin M	*igm*	JQ811851
	Immunoglobulin T	*igt*	KX599201
Cell markers and chemokines	CD4	*cd4-1*	AM489485
	CD8 beta	*cd8b*	KX231275
	C-C chemokine receptor type 3	*ccr3*	KF857317
	C-C chemokine receptor type 9	*ccr9*	KF857318
	C-C chemokine receptor type 11	*ccr11*	KF857319
	C-C chemokine CK8/C-C motif chemokine 20	*ck8/cl20*	GU181393
	Macrophage colony-stimulating factor 1 receptor 1	*csf1r1*	AM050293
Pattern recognition receptors (PRR)	Galectin 1	*lgals1*	KF862003
	Galectin 8	*lgals8*	KF862004
	Toll-like receptor 2	*tlr2*	KF857323
	Toll-like receptor 5	*tlr5*	KF857324
	Toll-like receptor 9	*tlr9*	AY751797
	C-type lectin domain family 10 member A	*clec10a*	KF857329
	Macrophage mannose receptor 1	*mrc1*	KF857326
	Fucolectin	*fcl*	KF857331

reactions (×6) were used for qPCR assays in a 25 μL volume in combination with a SYBR Green Master Mix (Bio-Rad, Hercules, CA, United States) and specific primers at a final concentration of 0.9 μM (**Supplementary Table 1**). The program used for PCR amplification included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. All the pipetting operations were executed by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (>92%) was checked, and the specificity of reactions was verified by analyzing the melting curves (ramping rates of 0.5°C/10 s over a temperature range of 55–95°C), and linearity of serial dilutions of RT reactions ($r^2 > 0.98$). Fluorescence

data acquired during the extension phase were normalized by the delta-delta CT method (Livak and Schmittgen, 2001), using beta-actin as housekeeping gene due to its stability in different experimental conditions (average CT between experimental groups varied less than 0.2).

Bacterial DNA Extraction

The bacterial DNA was extracted from feeds (2 samples/feed) and from intestinal samples (7–10 fish/dietary group). Intestinal mucus samples (200 μl) were treated with 250 μg/ml of lysozyme (Sigma) for 15 min at 37°C. Then, DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's instructions. DNA concentration, quality, and purity were measured using a NanoDrop 2000c (Thermo Fisher Scientific) and agarose gel electrophoresis (1% w/v in Tris-EDTA buffer). Samples were stored at −20°C until sequencing. The same procedure was used to extract DNA from the control and experimental feeds (previously ground to a fine powder) to evaluate the concentration of the probiotic supplement.

Illumina MiSeq Sequencing and Bioinformatic Analysis

The V3-V4 region of the 16S rRNA gene (reference nucleotide interval 341–805 nt) was sequenced using the Illumina MiSeq system (2 × 300 paired-end run) at the Genomics Unit from the Madrid Science Park Foundation (FPCM, Spain). The details on the PCR and sequencing of amplicons have been described elsewhere (Piazzon et al., 2019). Raw sequence data were uploaded to the NCBI (National Center for Biotechnology Information) and Sequence Read Archive (SRA) under NCBI BIOPROJECT ID: PRJNA679278; NCBI BIOSAMPLE ID: SAMN16828235-61; and SRA ACCESSION: SRR13081673-99. Raw forward and reverse reads were quality filtered using FastQC[2], and pre-processed using Prinseq (Rahlwes et al., 2019). Terminal N bases were trimmed at both ends and sequences with >5% of total N bases were discarded. Reads that were <150 bp long with a Phred quality score <28 in both of the sequence ends and with a Phred average quality score <26 were excluded. Then, forward and reverse reads were merged using fastq-join (Aronesty, 2013).

Bacterial taxonomy was assigned using the Ribosomal Database Project (RDP) release 11 as a reference database (Cole et al., 2014). Reads were aligned with a custom-made pipeline using VSEARCH and BLAST (Altschul et al., 1990; Rognes et al., 2016). Alignment was performed establishing high stringency filters (≥90% sequence identity, ≥90% query coverage). Taxonomic assignment results were filtered and data were summarized in an Operational Taxonomic Units (OTUs) table. Sample depths were normalized by total sum scaling and then made proportional to the total sequencing depth, following previously described recommendations (McKnight et al., 2019). Species richness estimates and alpha diversity indexes were calculated using the R package Phyloseq (Mcmurdie and Holmes, 2013). Rarefaction curves were obtained by plotting the number of observed taxonomic assignations in an OTU table against the number of sequences in each sample using the R package phyloseq.

[2]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

Inferred Metagenome and Pathway Analysis

Piphillin was used to normalize the amplicon data by 16S rRNA gene copy number and to infer the metagenomics content (Iwai et al., 2016). This analysis was performed with the OTUs significantly driving the separation by probiotic in the PLS-DA analysis (described in the section "Statistics"). For the analysis, a sequence identity cut-off of 97% was implemented, and the inferred metagenomics functions were assigned using the Kyoto Encyclopedia of Genes and Genomes database (KEGG, Oct 2018 Release). Raw KEGG pathway output from Piphillin was analyzed with the R Bioconductor package DESeq2 using default parameters, after flooring fractional counts to the nearest integer (Love et al., 2014; Bledsoe et al., 2016; Piazzon et al., 2019). Comparisons were also performed between different diets to evaluate possible pathway differences across diets.

Statistics

Data on growth and gene expression were analyzed by one-way ANOVA using SigmaPlot v14 (Systat Software Inc., San Jose, CA, United States). Normality of the data was verified by Shapiro-Wilk test, and Dunn's *post hoc* test was used for multiple comparisons between groups. For analysis of qualitative histological data, we conducted the non-parametric Kruskall-Wallis test, followed by Dunn's test for the multiple comparisons. GraphPad Prism8 (GraphPad Software, Inc., La Jolla, CA, United States) was used for both analyses. Microbiota species richness, alpha diversity indexes, and phylum abundance between experimental groups were determined by Kruskal-Wallis test followed by Dunn's *post hoc* test. Beta diversity was tested with permutational multivariate analysis of variance (PERMANOVA), using the non-parametric method *adonis* from the R package Vegan with 10,000 random permutations. To further study microbiota differences between dietary groups, supervised partial least-squares discriminant analysis (PLS-DA) and hierarchical clustering of samples were sequentially applied using EZinfo v3.0 (Umetrics, Umea, Sweden) and *hclust* function (gplots R package), respectively. Hotelling's T^2 statistic was calculated by employing the multivariate software package, whereby points above the 95% confidence limit for T^2 were considered as outliers and discarded. Values of normalized counts of OTUs present in 3 or more samples were included in the analyses, and the significant contribution to the group separation was determined by the minimum variable importance in the projection (VIP) values (Wold et al., 2001; Li et al., 2012), which renders an accurate clustering using the average linkage method and Euclidean distance feasible. The quality of the PLS-DA model was evaluated by the parameters R2Y (cum) and Q2 (cum), which indicate the fit and prediction ability, respectively. To assess whether the supervised model was being overfitted, a validation test consisting

on 600 random permutations was performed using SIMCA-P + (v11.0, Umetrics).

RESULTS

Growth Performance

Data on growth performance, feed intake, and feed conversion ratio (FCR) are reported in **Table 3**. All fish grew efficiently during the first 30 days of the trial (FCR = 1.27–1.28), reaching an overall FCR of 1.55–1.60 at the end of trial. The decrease in the length of the day and temperature from October to December should be noted.

No statistically significant differences were found between groups for the condition factor and specific growth rates (SGR), although the highest SGR tended to be achieved in fish fed diet C (high dose of probiotic). Indeed, the final body weight of these animals was higher than in the control group (diet A) ($P < 0.05$) with intermediate values for fish fed diet B (low dose of probiotic). Thus, total weight gain varied from 97% in fish fed diet A to 106% in fish fed diet C.

Histological and Biometric Scoring

Histological analysis of gilthead sea bream intestine was performed according to the aforementioned morphological criteria. The intestinal scoring data are reported in **Table 4**. The AI (**Figures 1A–C**) and PI (**Figures 1D–F**) portions were not affected by probiotic administration. Although the mucosal folds of the PI were significantly different ($P < 0.05$) between groups fed diets A and B, the total scores, calculated for each group, fall within an evaluation of Class I. In particular, the simple and complex folds appeared thin and regularly branched, *lamina propria* and connective tissue appeared normally proportioned and supranuclear vacuoles were numerous and well-distributed. Regarding the index of intestine length (ILI) (**Table 4**), diet B showed a significantly lower ILI than the control group (diet A) ($P < 0.05$), but no differences were observed between the other groups. No differences in the intestine weight index (IWI) were observed between groups.

Gene Expression Profiling

All genes included in the PCR-array were found at detectable levels with the highest expression level for markers of nutrient transport (*alpi, fabp1, and fabp2*), epithelial integrity (*cx32.2*), mucus production (*muc2, muc13*) and pattern recognition receptors (*fcl*) (**Supplementary Table 2**). Regarding the probiotic effect, statistically significant changes were found in the expression patterns of 5 out of 44 genes ($P < 0.05$) (**Figure 2**). In particular, expression of interleukin 10 (*il10*), interleukin (*il12),* and toll-like receptor 2 (*tlr2*) was upregulated in fish fed diet C (high probiotic dose) with intermediate values (not statistically different from the control group) in fish fed diet B (low probiotic dose). In contrast, the highest values of toll-like receptor 5 (*tlr5)* and galectin-8 (*lgals8)* were seen in fish fed diet B, whereas intermediate values were found in fish fed diet C. The probiotic treatment altered other markers (desmoplakin, *dsp*; interleukin 34, *il34*; C-C chemokine receptor 3, *ccr3*; and macrophage mannose receptor 1, *mrc1*) to a lesser extent, with an overall enhancement of gene expression that was especially evident in fish fed diet C ($P < 0.1$).

TABLE 3 | Growth performance of gilthead sea bream (*Sparus aurata*).

Diet	Mean body weight (g)		WG[1] (%)	SGR[2] (%)	Feed intake	CF[3]		FCR[4]
	Initial	Final			(g dry feed/fish)			
Period T0-T1, 24/09/2019–24/10/2019								
A	82.67 ± 0.86	130.53 ± 1.35	57.9 ± 0.4	1.52 ± 0.01	61.82 ± 0.59^{ab}	2.89 ± 0.02	A	1.27 ± 0.01
B	83.45 ± 0.74	130.01 ± 1.20	55.8 ± 0.8	1.48 ± 0.02	59.58 ± 0.73^{a}	2.84 ± 0.02	B	1.28 ± 0.01
C	83.28 ± 0.83	132.08 ± 1.32	58.6 ± 0.8	1.54 ± 0.02	60.61 ± 0.75^{b}	2.86 ± 0.01	C	1.27 ± 0.01
Period T1-T2, 25/10/2019–15/11/2019								
A	130.53 ± 1.35	149.43 ± 1.56	14.5 ± 0.6	0.66 ± 0.03	38.78 ± 2.17	2.76 ± 0.02	A	1.80 ± 0.02
B	130.01 ± 1.20	150.08 ± 1.40	15.4 ± 0.4	0.68 ± 0.01	36.99 ± 1.19	2.74 ± 0.01	B	1.84 ± 0.04
C	132.08 ± 1.32	152.99 ± 1.66	15.8 ± 0.8	0.70 ± 0.02	33.93 ± 2.06	2.73 ± 0.01	C	1.86 ± 0.06
Period T2-T3, 15/11/2019–18/12/2019								
A	149.43 ± 1.56	163.04 ± 2.02^{a}	9.1 ± 0.6	0.28 ± 0.02	35.71 ± 0.69	2.78 ± 0.02	A	2.40 ± 0.05
B	150.08 ± 1.40	166.30 ± 1.90^{ab}	10.8 ± 0.3	0.31 ± 0.03	33.74 ± 1.37	2.73 ± 0.03	B	2.31 ± 0.03
C	152.99 ± 1.66	171.24 ± 2.07^{b}	11.9 ± 1.8	0.36 ± 0.02	32.69 ± 1.46	2.81 ± 0.01	C	2.02 ± 0.25
Overall, 24/09/2019–18/12/2019								
A	82.67 ± 0.86	163.04 ± 2.02^{a}	97.2 ± 1.4	0.80 ± 0.01	135.85 ± 3.29	2.78 ± 0.02	A	1.57 ± 0.05
B	83.45 ± 0.74	166.30 ± 1.90^{ab}	99.3 ± 0.7	0.81 ± 0.01	129.62 ± 2.71	2.73 ± 0.03	B	1.60 ± 0.03
C	83.28 ± 0.83	171.24 ± 2.07^{b}	105.6 ± 2.5	0.85 ± 0.02	126.44 ± 6.86	2.81 ± 0.01	C	1.55 ± 0.02

Data are reported as mean ± SEM, different superscript letters indicate significant differences ($P < 0.05$) between diet groups in the same sub-column.
[1]Weight gain, WG = (100 × body weigh increase)/initial body weight.
[2]Specific growth rate, SGR = 100 × (ln final body weight–ln initial body weight)/days.
[3]Condition factor, CF = 100 × (body weight/standard length).
[4]Feed conversion ratio, FCR = dry feed intake/wet weight gain [total feed supplied (g DM, dry matter)/WG (g)].

TABLE 4 | Histological scoring (for anterior and posterior intestine) and biometric measurement [intestinal length index (ILI) and intestinal weight index (IWI)] of gilthead sea bream (*Sparus aurata*) juveniles fed the control (A) and experimental (B and C) diets.

Diet	Mucosal folds	Connective tissue	Lamina propria of simple folds	Supranuclear vacuoles	Total score	ILI[1] (cm)	IWI[2] (g)
			Anterior intestine			Biometric measurement	
A	1.1 ± 0.1	1.7 ± 0.06	1.7 ± 0.06	1.5 ± 0.04	6.1 ± 0.2	97.21 ± 7.62^{a}	2.43 ± 0.06
B	1.0 ± 0.04	1.5 ± 0.2	1.5 ± 0.1	2.2 ± 0.5	6.3 ± 0.9	75.73 ± 6.74^{b}	2.38 ± 0.12
C	1.1 ± 0.04	1.7 ± 0.1	1.5 ± 0.2	2.0 ± 0.4	6.2 ± 0.7	86.43 ± 8.02^{ab}	2.40 ± 0.17
			Posterior intestine				
A	1.2 ± 0.2^{a}	1.6 ± 0.09	1.9 ± 0.2	2.2 ± 0.3	6.9 ± 0.8		
B	1.8 ± 0.3^{b}	2.0 ± 0.2	1.7 ± 0.1	2.1 ± 0.2	7.6 ± 0.8		
C	1.3 ± 0.07^{ab}	1.8 ± 0.04	1.7 ± 0.08	2.0 ± 0.07	6.8 ± 0.07		

Data are reported as mean ± SEM of 12 fish per diet. Different superscript letters indicate significant differences (Dunn's pot-hoc test, $P < 0.05$) between dietary groups in the same sub-column.
[1] Intestinal length index, ILI = 100 × (intestine length/standard length).
[2] Intestinal weigth index, IWI = 100 × (intestine weight/fish weight).

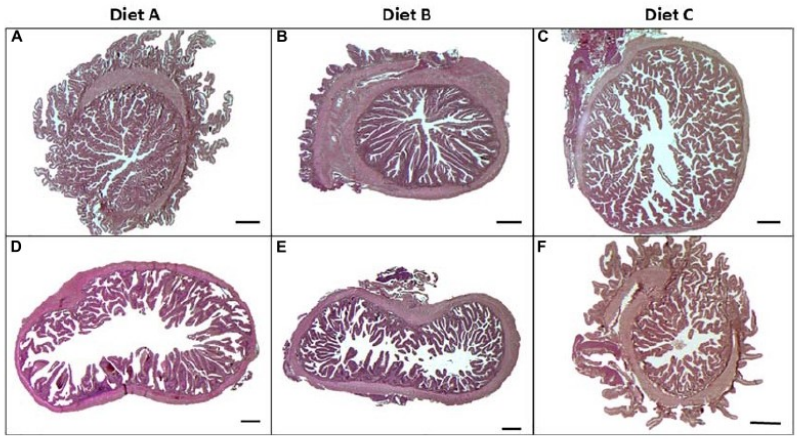

FIGURE 1 | Light microscope images obtained from anterior **(A–C)** and posterior **(D–F)** intestine of gilthead sea bream juveniles (*Sparus aurata*) fed with diets A, B, and C, stained with hematoxylin and eosin (H&E). Scale bar = 500 μm.

Characterization of Feed-Associated Bacterial Communities

At the end of the trial, the normalized counts of *L. lactis* subsp. *lactis* resulted 8–11 in diet A (<0.0001% total bacterial counts); 30,204 in diet B (2.5% total counts); and 61,828 (5.4% total counts) in diet C (**Figure 3**). By excluding Cyanobacteria/Chloroplast (>90% total counts), Firmicutes and Proteobacteria proved to be the most highly represented bacterial phyla in the three feeds, whereas the rest of the bacterial population consisted of Bacteriodetes and Fusobacteria phyla (**Supplementary Figure 1A**). However, the percentage of Firmicutes varied considerably between feeds, with higher values in feed B (4.2%) and C (7.8%) than in the control feed, in which Firmicutes represented only 2% of the total counts. Thus, by recalculating the relative bacterial abundances after excluding Cyanobacteria/Chloroplast, the percentage of Firmicutes rose from 34% in the control diet A to 70% in diet B and 79% in diet C (**Supplementary Figure 1B**). Then, by specifically analyzing the relative abundance of the probiotic *L. lactis subsp. lactis* in comparison to the most representative genera within the phylum Firmicutes, the percentage of *L. lactis* subsp. *lactis* was close to 0% in the control diet, whereas in B and C diets, it was significantly higher, reaching values of 64 and 71%, respectively (**Supplementary Figure 1C**).

Alpha Diversity and Gut Microbiota Composition

Illumina sequencing of AI-adherent bacteria yielded 3,677,860 high-quality and merged reads, with an average value of 136,217 reads per sample (**Supplementary Table 3**). When annotated, the

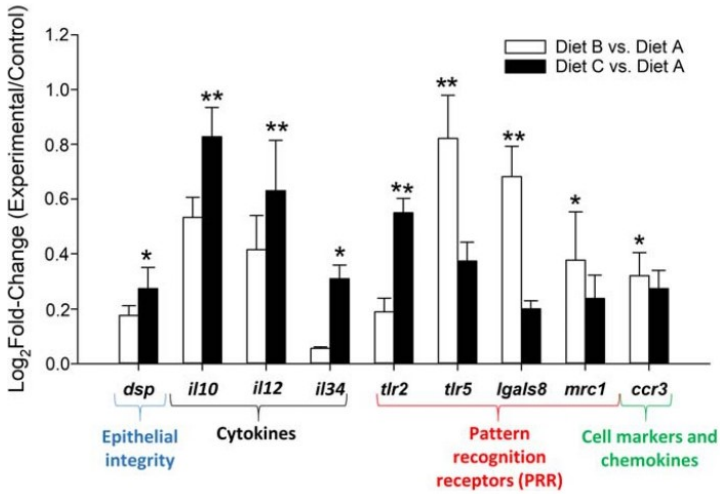

FIGURE 2 | Fold change of differentially expressed genes (Dunn's *post hoc* test; $^{**}P < 0.05$; $^{*}P < 0.1$) in the anterior intestine of fish fed experimental diets (diets B and C) relative to the control diet (A). Data are the mean + SEM of 9–12 fish per diet. White columns (fish fed diet B). Black columns fish fed diet C.

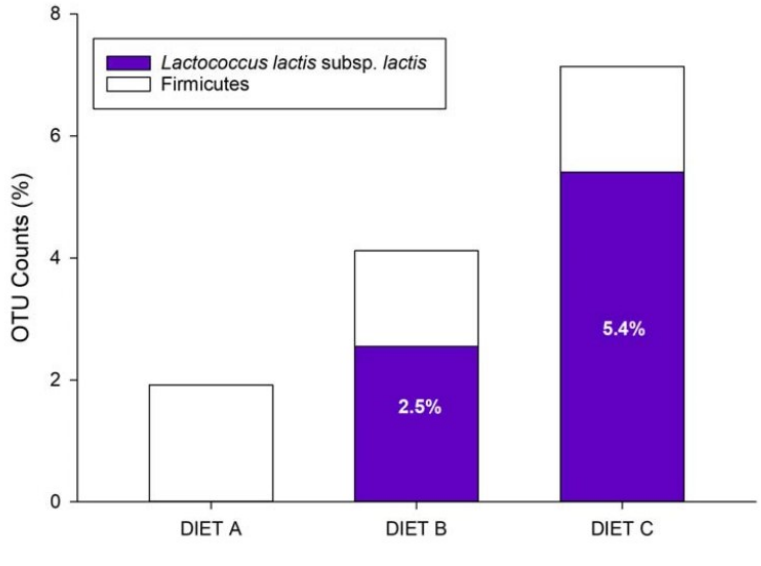

FIGURE 3 | Relative abundance of Firmicutes phylum and *Lactococcus lactis* subsp. *lactis* in control (Diet A) and experimental diets (Diets B and C).

reads were assigned to 1,313 OTUs at 97% identity threshold. Rarefaction analysis showed curves that approximated saturation (horizontal asymptote); thus, a good coverage of the bacterial community was achieved and the number of sequences for analysis was considered appropriate (**Supplementary Figure 2**). Indeed, up to 85% of the OTUs were classified at the level of species and more than 90% at the level of genus (94.1%), family (96%), order (97%), class (97.2%), and phylum (99%).

As shown in **Table 5**, the richness estimator (ACE) indicated a higher OTU richness in fish fed diet B than in fish fed diet A or diet C. At the same time, alpha diversity estimators (Shannon and Simpson) disclosed a reduced evenness in fish fed diet C, which

TABLE 5 | Species richness estimate (ACE) and diversity indexes (Shannon and Simpson) of the adherent microbial communities in the anterior intestine of fish fed diet A (10), diet B (10), and diet C (7).

	Diet			K-W test
	A	B	C	*P*-value
ACE	205.17 ± 16.76^{b}	294.98 ± 32.04^{a}	162.08 ± 23.39^{b}	0.006
Shannon	2.14 ± 0.12^{a}	2.4 ± 0.13^{a}	1.58 ± 0.2^{b}	0.006
Simpson	0.82 ± 0.02^{a}	0.85 ± 0.02^{a}	0.65 ± 0.08^{b}	0.02

Different superscript letters indicate significant differences be dietary groups [Kruskal-Wallis (K-W) test, Dunn's pot-hoc test, P < 0.05].

indicates that abundant OTUs predominated over the others in this group of fish.

Changes in bacterial composition were also found at the phylum level (**Figure 4**). Proteobacteria was the most abundant phylum in the three groups, ranging from 55.9% in fish fed diet C to 55.7% in fish fed diet A, and 50.1% in the diet B fed group. The second-most abundant phylum was Firmicutes, representing the 26.6% of the OTU counts in fish fed diet A, decreasing progressively with the probiotic supplementation in fish fed diet B (26.2%) and diet C (5.6%). The same trend was shown by the phylum Bacteroidetes, ranging from 2.7% in fish fed diet A to 1.3% in fish fed diet B and 0.1% in fish fed diet C. The phylum Actinobacteria increased from 9.3% in fish fed diet A to 16.3% in fish fed diet B but decreased to its minimum level in group C (3.2%). Finally, Spirochetes appeared in a significant proportion (32%) only in fish fed with diet C, being practically absent in the other groups (<3%).

Beta Diversity, Discriminant Analysis, and Inferred Pathways

No significant differences in beta diversity were found when experimental groups were computed independently (PERMANOVA, $P = 0.34$, $F = 1.031$, $R^2 = 0.04$). In contrast, when B and C groups were computed together, beta diversity became statistically significant (PERMANOVA, $P = 0.032$, $F = 1.8789$, $R^2 = 0.099$). Taking this analysis further, a PLS-DA model was constructed with a 99% to the total variance explained (**Figure 5**). During the statistical processing to construct the model, two fish from the Diet A group and one fish from the Diet C group appeared as outliers and were excluded from the model. This approach displayed a clear separation of control fish and fish fed probiotic diets (B + C group) along component 1 (84.52%) with a higher individual variability within fish fed diet B than in those fed diet C. This PLS-DA model was successfully validated with a permutation test (pCV ANOVA = 0.015) discarding the possibility of over-fitting of the supervised model (**Supplementary Figure 3**).

Differences between control fish and the probiotic-fed merged groups were driven by 81 OTUs (VIP > 1), mainly belonging to the phyla Proteobacteria, Spirochetes, and Firmicutes. A detailed list of the VIPs can be found in **Supplementary Table 3**. The inferred metagenomic analysis using DESeq2 disclosed nine differentially abundant pathways across groups (**Figure 6**). Pathways related to protein digestion and absorption, as well as renin secretion were over-represented in the probiotic fed fish groups, whereas the control group showed a relative preponderance of pathways related to shigellosis, proteasome and autophagy.

DISCUSSION

In aquaculture the use of probiotics is significantly increasing and a growing number of studies are demonstrating their positive effects in the most economically important fish species (Merrifield et al., 2010; Varela et al., 2010; Mahdhi, 2012; Ridha and Azad, 2012; Chauhan and Singh, 2019).

As mentioned previously, one of the most interesting effects of probiotics is the increase in the animals' growth performance (Sun et al., 2012; Nguyen et al., 2017; Won et al., 2020). In the present study, gilthead sea bream fed diets C and B, supplemented with high and low doses of *L. lactis* subsp. *lactis*, respectively, reached a higher final biomass than control fish fed with diet A, and differences in biomass gain were statistically significant between groups C and A. Although differences between fish groups arose at the December sampling, most of the weight gain was attained during September–October, as this period still corresponds to the active fish feeding behavior at IATS-CSIC latitude. This result highlights, albeit slightly, the beneficial action of the probiotic, suggesting a more efficient digestion and utilization of nutrients in gilthead sea bream fed probiotics. Indeed, although no significant differences were detected in FCR and SGR between dietary groups, the lowest FCR (1.60 ± 0.03) and the highest SGR (0.85 ± 0.02) were registered in fish fed diet C. Similar results were obtained in gilthead sea bream by Suzer et al. (2008) and Varela et al. (2010), using *Lactobacillus* spp. and *Shewanella putrefaciens* Pdp11, respectively. Positive results in fish growth performance, using *L. lactis* as probiotic, were also obtained in other cultured fish species, such as common carp, European sea bass, tilapia, and olive flounder (Balcázar et al., 2006a; Carnevali et al., 2006; Heo et al., 2013; Xia et al., 2018; Feng et al., 2019).

Histological analysis was conducted using a semi-quantitative scoring system. The parameters taken into account for the AI and PI morphological evaluation were related to the mucosal folds that represent the intestinal absorptive surface area, and to the associated connective tissue (Dimitroglou et al., 2011; Khojasteh, 2012; Puphan et al., 2015). Our results confirmed that probiotic did not alter the morphology of the gut and did not trigger intestinal inflammation. Indeed, no structural modifications were detected in fish fed with diets supplemented with probiotic (diets B and C), in comparison to the control group fed diet A. In line with our results, other studies have shown that probiotics improve gut morphology, leading to an increase in intestinal absorption capacity (Batista et al., 2016; Won et al., 2020). In contrast, Cerezuela et al. (2012; 2013) reported several negative effects related to the administration of probiotics in gilthead sea bream. In particular, those authors showed that both *Tetraselmis chuii* and *Bacillus subtilis* induced intestinal inflammation with numerous signs of edema in the

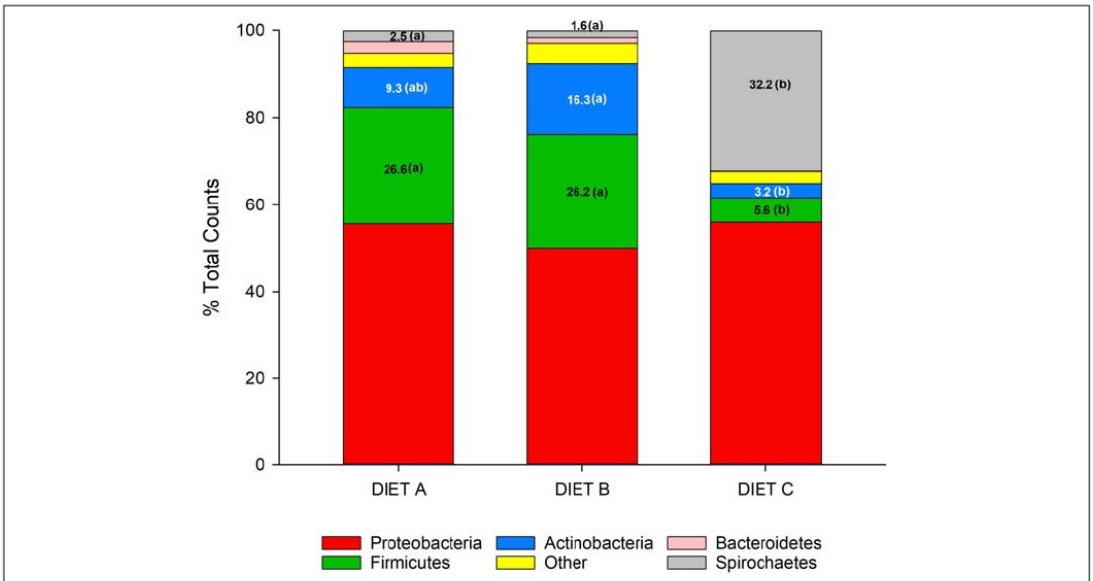

FIGURE 4 | Stacked bar chart representing the relative abundance (%) of bacterial phyla in fish fed control (A) and experimental (B and C) diets. The Kruskal–Wallis test (Dunn's *post hoc* test, $P < 0.05$) showed significant differences between groups for the phyla Firmicutes, Actinobacteria and Spirochetes. The differences are indicated by different letters in parenthesis that correspond to pairwise comparisons within each phylum between groups.

mucosal folds. Therefore, more in-depth histological analyses are needed to better understand the effects of different probiotic strains on the adsorptive surface area in fish intestine and, in particular, on the villi length and density.

Numerous studies that have investigated the effects of probiotics on the piscine immune system have reported an enhanced immune response, thus improving survival rates and resistance to a pathogenic attack (Nayak, 2010; Lazado and Caipang, 2014). Different probiotic strains stimulate the immune system in fish, but the effect appears to be species-specific. *L. lactis* supplementation increased the concentration of several pro- and anti-inflammatory cytokines (Tnfα, Il1β, Il6, Il12, Il10 and Tgfβ) in common carp serum (Feng et al., 2019) and upregulated the expression of *tnfα, ifnγ, hsp70,* and *il1β* genes in the intestine of tilapia (Xia et al., 2018; Won et al., 2020). Conversely, *L. lactis* did not induce any differences in the abundance of cytokines and pattern recognition receptors (PRRs) transcripts in intestine or head kidney of trout (Pérez-Sánchez et al., 2011). In gilthead sea bream, the anti-inflammatory action of a *Bacillus*-based probiotic induced decreased expression of *lgals8* and *cd4* transcripts in anterior intestine, lower amounts of circulating IgM and cortisol, a lower respiratory burst activity of blood leukocytes, and lower numbers of eosinophilic granulocytes (in particular, mast cells) in the intestinal submucosa (Simó-Mirabet et al., 2017). Herein, significant differences in the expression of key genes involved in innate and acquired immunity (interleukins and PRRs) were detected between fish fed probiotic and control diets. Among the mechanisms induced by probiotics, it has been postulated that the activation of immunity derives from the interaction of the host with the probiotic microbial associated molecular patterns (MAMPs) (Yang et al., 2014). The direct effect of MAMPs was recently demonstrated by feeding grouper (*Epinephelus coioides*) with MAMPs isolated from the probiotic *Bacillus pumilus* SE5. Indeed, an activation of intestinal immunity via up-regulation of TLR signaling pathways was observed (Yang et al., 2019). Thus, the observed activation of the immune system in the present study is likely taking place by direct induction of gilthead sea bream PRRs by components on the cell wall of the probiotic, such as peptidoglycan or lipoteichoic acid, which are in fact TLR2 agonists (Dammermann et al., 2013).

The density, composition and function of intestinal microbiota of fish, including gilthead sea bream, are shaped by numerous factors, such as diet, sex, developmental stage, and rearing conditions (Piazzon et al., 2017, 2019; Rimoldi et al., 2020), as well as multiple endogenous host-microbe interactions, such as the host's genetic background (Piazzon et al., 2020), and possible intestinal disorders or intestinal diseases (Bakke-Mckellep et al., 2007; Green et al., 2013). Furthermore, microbiota vary taxonomically and functionally in different sections of the GIT of fish (Kokou et al., 2020). There is also a distinction between the allochthonous, i.e., free-living, transient microbiota associated with the digesta (feces), and autochthonous communities that colonize the mucosal surface of the digestive tract and make up the core community (Merrifield et al., 2010; Ringø et al., 2016; Nguyen et al., 2017; Egerton et al., 2018).

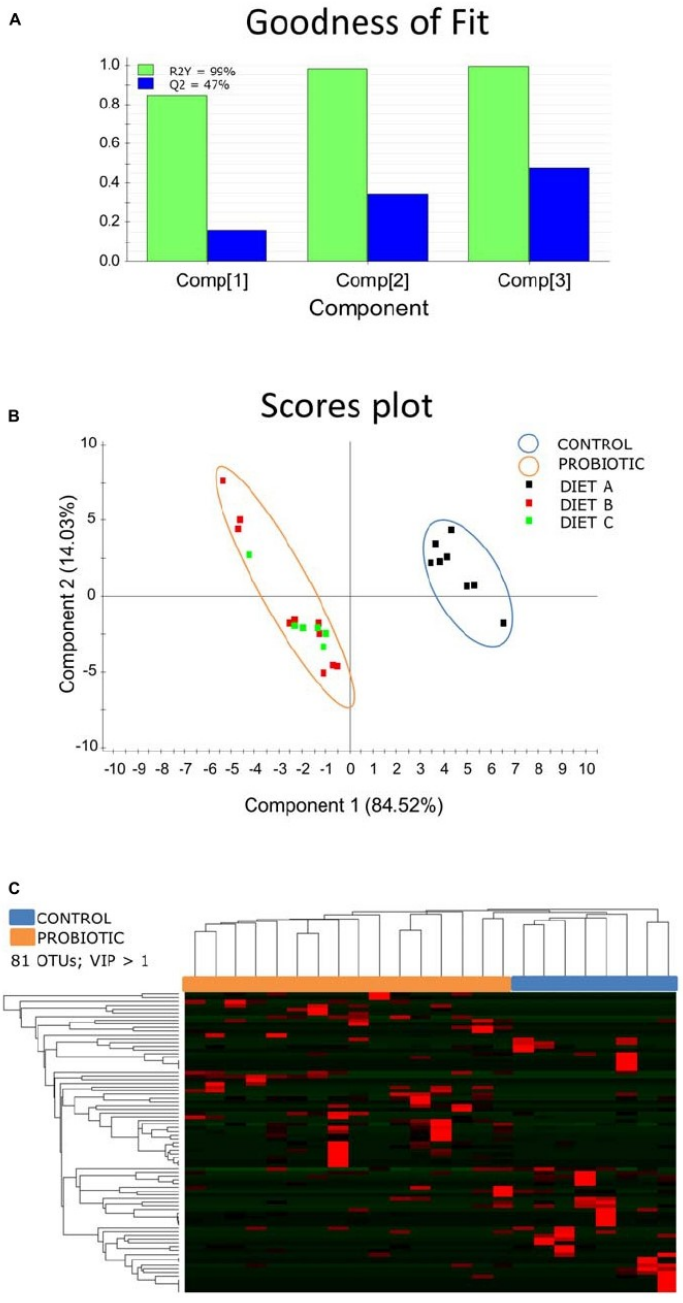

FIGURE 5 | (A) Graphical representation of the goodness-of-fit of the PLS-DA model. **(B)** Two-dimensional PLS-DA score plot representing the distribution of the samples between the first two components in the model. **(C)** Heatmap showing the abundance distribution (*z*-score) of the OTUs identified to be driving the separation between fish fed probiotic diets (B + C; orange) and fish fed diet A (blue).

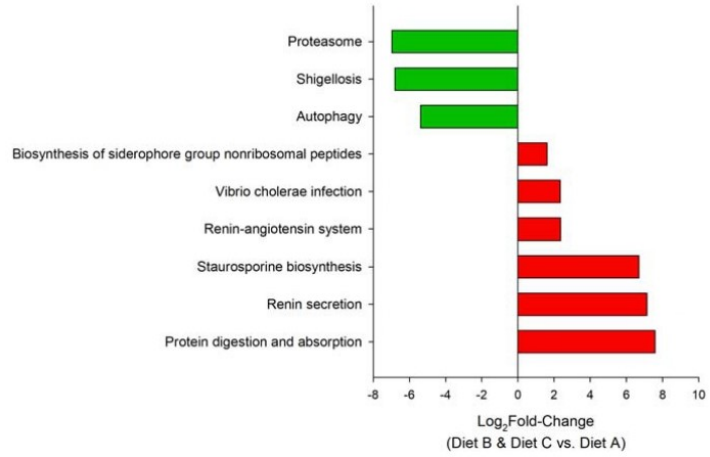

FIGURE 6 | Bar plots depicting the changes in metabolic capacities in the comparison between fish fed probiotic diets (B + C group) and fish fed the control diet A. Bars show the $\log_2$-fold change in the metabolic pathway.

Taxonomically, gut bacteria are classified according to phyla, classes, orders, families, genera, and species. The "core" intestinal microbiota, which can often persist in spite of changing factors is constituted by Proteobacteria, Firmicutes, and Actinobacteria phyla in both freshwater and marine fish species (Silva et al., 2011; Kormas et al., 2014; Ghanbari et al., 2015; Piazzon et al., 2019). These taxa are largely considered important players in nutritional provisioning, immune defense, and metabolic homeostasis (Estruch et al., 2015; Givens et al., 2015; Rimoldi et al., 2019; Terova et al., 2019).

Accordingly, in the present experiment, gilthead sea bream were fed with three different feeds and at the end of the experiment, the microbiota of these feeds was analyzed. Data revealed that Firmicutes and Proteobacteria were the bacterial phyla represented most, followed in descending order by Bacteriodetes and Fusobacteria. Then, by analyzing specifically the relative abundance of the probiotic *L. lactis* subsp. *lactis* compared to the most representative genera of Firmicutes phylum, we found that the percentage of *L. lactis* subsp. *lactis* was close to 0% in diet A (control), whereas in diets B and C, it was definitely high, reaching values of 64 and 71%, respectively. This results is in agreement with the supplementation of a low and a high dose of probiotic to diets B and C, respectively.

With regard to the gut microbiota, gilthead sea bream fed diet C showed a significant increase in bacteria belonging to the Spirochetes phylum, which were practically absent in the gut of fish fed diets B and A (<3%). In the same fish group, a decrease in Actinobacteria, Bacteroidetes, and Firmicutes phyla was recorded. The Firmicutes phylum is composed of more than 200 different genera, such as *Lactobacillus*, *Bacillus*, *Enterococcus*, *Ruminococcus, and Clostridium.* Lactic acid bacteria (LAB) include, among others, *Streptococcus* sp., *Lactobacillus* sp., *Leuconostoc* sp. and *Carnobacterium* sp., which are considered as beneficial microorganisms that contribute to an healthy status of the fish intestine (Kim et al., 2012; Terova et al., 2019). It is known that commensal Firmicutes and Bacteroidetes are the major producers of short chain fatty acids, such as butyrate, acetate, and propionate that are the end products of fiber fermentations.

While is difficult to assess from genomic data alone the physiological effect on the host of the microbiota changes we found, it is worth noting that Firmicutes/Bacteroidetes ratio in the gut has been directly related to lean body mass in both human and animals (Magne et al., 2020). Indeed, the ratio of Firmicutes vs. Bacteroidetes was increased in obese individuals as compared to lean ones. Actually, gilthead sea bream fed with diets containing probiotic showed a higher Firmicutes/Bacteroidetes ratio than control fish and this could be correlated to their better growth performances. Likewise in mice, the reduced amount of Bacteroidetes was a direct consequence of probiotic supplementation (Grazul et al., 2016). In addition, gilthead sea bream fed diet C, showing the best FCR and SGR values, had the highest percentage of Spirochetes. In swine, the Spirochaetaceae bacterial family was shown to correlate positively with the host weight (Unno et al., 2015). The gut microbiome of the feeding group C was also characterized by a Proteobacteria/Firmicutes ratio five times higher than in the other groups. This result is not surprising because *Lc. lactis* subsp. *lactis* SL242 produces the antibiotic nisin, displaying strong activity against Gram-positive bacteria (Li et al., 2018), and a vast majority of Firmicutes are Gram-positive.

The analysis of gut-adherent (autochthonous) microbiota did not reveal significant differences between fish groups in relation to *L. lactis*, suggesting a lack of colonization of the probiotic in the host's intestinal mucosa. This was not a surprising result since it is known that probiotics generally do not colonize the digestive tract i.e., they do not become established permanently or for a long-term (weeks, months, or years) in the intestinal tract (Marco, 2019). Thus, the ingested bacteria can be beneficial

while they are in the gut, but they do not have a lasting effect and continued probiotic consumption is needed for sustained impact. Thus, instead of colonizing, the new bacteria may temporarily complement resident microbial communities, forming part of a transient (allochthonous) microbiome in fish without displacing the native gut microbiota, but instead altering digestive tract function by producing active metabolites that modulate the activity of the gut microbiota, or by stimulating the intestinal epithelium directly (Marco, 2019). Hence, in the present trial, although the probiotic did not colonize the host's intestinal mucosa, it did modulate the fish gut microbiota, confirming that colonization is not always necessary to induce host modification. Indeed, diets B and C were enriched with Actinomycetales, as compared to diet A, which instead showed a higher percentage of *Pseudomonas*, *Sphyngomonas*, and *Lactobacillus* genera. These results were confirmed by the clear separation of bacterial community of fish fed with the probiotic from the bacterial community of control fish group (diet A) in the beta-diversity and PLS-DA analyses. Furthermore, the KEGG pathway analysis underlined such differences, highlighting several pathways potentially affected by the diet. Particularly interesting were those related to protein absorption and digestion.

In the present study, the analysis of gut microbial communities revealed significant differences between fish groups in term of species richness and diversity. Among alpha diversity indices, fish fed with diet B showed the highest level of richness estimator ACE and biodiversity, in comparison to the other two fish groups. In contrast, dietary group C, although achieving the best growth performances, showed the lowest gut bacterial diversity.

A reduction in bacterial diversity is usually considered an adverse outcome, since this could lead to less competition for opportunistic or invading pathogens due to a functionally unbalanced ecosystem (Cerezuela et al., 2013; Li et al., 2014; Rimoldi et al., 2020). However, while an increase in intestinal microbial biodiversity following prebiotics (dietary compounds that induce the growth or activity of gut microbiota) administration has been frequently described, the data currently available on the effects of probiotics in fish are more controversial. For instance, in line with our results, the species richness and diversity indexes decreased in gilthead sea bream in response to dietary administration of the probiotic *Bacillus subtilis*, either alone or in combination with prebiotics or microalgae (Cerezuela et al., 2012, 2013). In contrast, in line with what we found in fish fed diet B, lactic acid bacteria supplementation was associated with an increase in bacterial diversity in the intestinal mucus of Atlantic salmon (Gupta et al., 2019). In addition, probiotics, such as lactic acid bacteria, are known to produce several antimicrobial compounds capable of suppressing the growth of other microorganisms, which can alter the gut microbiota in terms of both composition and biodiversity (Collado et al., 2007).

CONCLUSION

According to analysis of gut-adherent (autochthonous) microbiota, the probiotic *L. lactis* subsp. *lactis* did not colonize in the host's intestinal mucosa. However, the probiotic did modulate the fish gut microbiota, confirming that colonization is not always necessary to induce host modification. Indeed, gut microbiota of fish fed diets B (low dose of probiotic) and C (high dose) were clearly separated from the bacterial community of control fish in the beta-diversity and PLS-DA analyses. Furthermore, the KEGG pathway analysis underlined such differences, highlighting several pathways potentially affected by the diet. Particularly interesting were those related to protein absorption and digestion.

With regard to fish growth performance, there were no significant differences between groups for the FCR and SGR. The only difference was the final body weight of fish fed diet C (high dose of probiotics) that resulted higher than the control group.

Dietary probiotic administration did not alter the morphology of the intestine and did not trigger inflammation.

Researches such as these highlight the interaction between fish diet and their microbiota and suggest that manipulating diet to tune the gut microbiome may be a promising intervention, together with well-designed probiotics.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (accession: SAMN16828235–SAMN16828261 and PRJNA679278).

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics and Animal Welfare Committees of Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain) (Permit number 824/2019) and "Generalitat Valenciana" (Permit number 2019/VSC/PEA/0197).

AUTHOR CONTRIBUTIONS

FM, FN-C, and MP: experimental investigation, methodology, data curation, formal analysis, and writing—review and editing. SR: methodology, formal analysis, and writing—review and editing. JC-G: methodology, data curation, formal analysis, and writing—review and editing. AG: conceptualization and writing—review and editing. IM: writing—review and editing. FB: experimental investigation and methodology. JP-S: conceptualization, experimental investigation, data curation, and writing—review and editing. GT: conceptualization, data curation, and writing—review and editing. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.659519/full#supplementary-material

Supplementary Figure 1 | (A) Relative abundance of phyla in control (Diet A) and experimental diets (Diets B and C). **(B)** Relative abundance of phyla excluding Cyanobacteria/Chloroplast in control and experimental diets. **(C)** Relative abundance of Firmicutes genera, including *Lactococcus lactis* subsp. *lactis* in control and experimental diets.

Supplementary Figure 2 | Rarefaction curves obtained from the sequencing data of the different groups included in this study (Diets A, B, C).

Supplementary Figure 3 | Validation plot of the PLS-DA model between control (Diet A) and experimental (Diets B and C) groups, consisting of 600 random permutations.

Supplementary Table 1 | Primers for qPCR gene expression. Colors in letters of column Gene show different gene functions included in the array as follows: red: Epithelial integrity; dark blue: Nutrient transport; green: Mucus production; Orange: Cytokines; purple: Immunoglobulins; black: Cell markers and chemokines; light blue: Pattern recognition receptors.

Supplementary Table 2 | Relative gene expression of anterior intestine in fish fed control **(A)** and experimental **(B,C)** diets. Data are the mean ± SEM of 9–12 fish. All data values were referred to the expression level of *hes1-b* of control fish with an arbitrary assigned value of 1. [1]*P*-value results from ANOVA. Bold font indicates statistically significant differences between groups ($P < 0.05$).

Supplementary Table 3 | Sequencing and taxonomy assignment results.

Supplementary Table 4 | OTUs driving the separation between fish fed probiotic diets (B + C) and fish fed control diet A in the PLS-DA model (VIP > 1).

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 5

Chapter 5

5.1 Discussion

Fisheries and aquaculture, considered as a great single industry, represent the most productive sector among all the animal-productive system, reaching the incredible value of 178 million tons of only aquatic animals produced and offered to the market (FAO, 2022). However, this value is the only final result of a continuously growing rate since 1950, which led to the development of a sector greater than the one registered in the agriculture production during the Green Revolution (Béné et al., 2015). Despite this, fish consumption, although it is still growing and with a current global average level around 20.2 kg $year^{-1}$, remains a protein source that is not equally distributed and used all around the world. The reasons behind this distribution are numerous, being both cultural and economic in nature; in fact, urbanization and growing income in several countries are leading to an increase in demand for animal products where production is unsustainable and with little possibility of further development (Thornton, 2010). Therefore, in the light of this growing demand for animal products and animal protein, reflecting the strong pressure being exerted by the great world population growth recorded in recent decades and which will bring the world population to about 10 billion before 2050, agriculture and animal production must find ways to increase production (United Nations Department of Economic and Social Affairs, 2022). One of the main constraints to the development of numerous sectors is environmental impact, considering the occupation of ice-free terrestrial arable area, loss of biodiversity and greenhouse gases emission. Accordingly, the aquaculture industry, which, contrary to fishing, is the main driver of the sector's growth with a great potential for further development, can represent a promising field to meet the future food supply demand. Nevertheless, although aquaculture sector still records low values of emissions and environmental impact compared to the others, it remains highly dependent on marine-derived materials, which are fished and could aggravate the environmental status of numerous endangered stocks (Hilborn et al., 2020). The

main products that historically have been used in aquaculture are FM and FO. They originally were inexpensive and abundant; in addition, they provide the perfect amount of dietary protein, essential amino acids, and essential FAs, together with numerous other beneficial compounds such as minerals and vitamins that meet the nutritional requirements of most farmed aquatic species (Tacon and Metian, 2015; Turchini et al., 2019). Today, although these ingredients continue to be essential for feed production and are still widely used, the worldwide level of inclusion has seen a slow decline in the last few decades (1.7 per year) due to their high market value and for the environmental issues associated with them (Naylor et al., 2009; Bandara, 2018). The path to developing a more sustainable sector converges necessarily with reducing the exploitation of marine resources. Hence, over the past 20 years, fish nutritionists have endeavoured to develop new aquafeed formulations, drastically reducing FM and FO inclusion rates and replacing them with numerous, promising alternative ingredients and strategies.

For the purpose of this project, two different insect species were used. In fact, including insect meal in fish feed is the perfect way to respond to the problems of the aquaculture industry related to the stability and reduction of feeding costs and to promote sustainable aquatic environment management, with relatively low impact. So far, several studies have shown that insect meal can partially replace fishmeal and completely replace soybean meal without affecting fish growth performance, feed utilization, digestibility, and fillet quality (Renna et al., 2017; Bruni et al., 2018, 2020; Terova et al., 2019). As freshwater fishes are natural predators of insects, it is reasonable to assume that they are evolutionarily adapted for consuming them. Nevertheless, fish growth performance is not the only outcome that defines successful aquaculture practice; fish welfare has to be taken into account, too. In this view, the intestinal microbiota, which directly affects digestive functions, and the immune response of the host should be considered a key indicator of a healthy fish (Ghanbari et al., 2015b).

The first of the two studies here presented regarding the use of insect, demonstrated that the inclusion of 15% HI larvae meal in the diet, to replace 50% of the FM content, can modify fish gut microbiota, thus improving the health status of trout. In two recent studies in trout, we have reported that the partial substitution of dietary FM with 10%, 20%, or 30% of a defatted HI meal had an important effect in modulating both the intestinal transient and resident bacterial communities (Rimoldi et al., 2019; Terova et al., 2019). So, as expected, the present metabarcoding analysis revealed that Firmicutes, Proteobacteria, and Tenericutes phyla were dominant in the gut of rainbow trout, regardless of the diet. The phylum Tenericutes is considered specifically adapted to the gastrointestinal environment of farmed rainbow trout. Several studies have reported that this phylum, with *Mycoplasma* being the dominant genus, is prominent in the distal intestine of rainbow trout as well as in other farmed salmonids (Lyons et al., 2017; Huyben et al., 2018). Therefore, our data provide further evidence of the importance of this genus in trout, thus corroborating the idea that this fish species could be a specific host for *Mycoplasma*. Although gut bacterial communities were dominated by the same phyla irrespective of the diet, species richness (Chao 1 index, observed OTUs) was significantly increased by dietary supply of 15% of insect meal in our study. Accordingly, Bruni et al. (2018) found a higher species richness in autochthonous intestinal microbiota of trout fed a diet containing 20% of Hi meal. A higher microbial richness should be considered a positive effect, since it may potentially provide further metabolic capabilities to the host thus improving its health status (Borrelli et al., 2017). Insect meals are rich in chitin, a form of insoluble fibre, which may act as prebiotic by selectively stimulating the growth of beneficial gut bacteria and promoting their colonization (Guerreiro et al., 2018). Furthermore, chitin and its deacetylate derivate chitosan have antimicrobial properties and a bacteriostatic effect against several harmful Gram-negative bacteria (Nawaz et al., 2018). Multivariate analysis of bacterial

community's diversity, based on unweighted UniFrac dissimilarity data, displayed a strong clustering of fish groups fed with HI meal and with the control diet that were cleanly separated into uniformly distant regions. Our data confirm previous research showing that the HI meal inclusion in the diet causes a significant reduction of gut Proteobacteria, predominantly belonging to the Gammaproteobacteria class, in comparison to the control diet without insect meal (Huyben et al., 2018; Rimoldi et al., 2019; Terova et al., 2019). In particular, in line with those studies, our metagenomic analysis highlighted the dramatic shift from a high Proteobacteria to Firmicutes ratio in the gut of fish fed with the Ctrl diet to a low ratio in fish fed with the insect meal diet. The most dominant genus in the control fish gut was *Aeromonas*, which includes several Gram-negative bacteria commonly present in fresh water and potentially pathogenic for fish, as they can cause skin ulcerations. In the current study, intestinal abundance of *Aeromonas* in trout fed Hi15 was significantly reduced, and this is in line with our findings on autochthonous intestinal microbiota of trout fed with Hi meal. In another study of our group, microbiota of trout fed with Hi meal showed a reduction of Gammaproteobacteria, mainly represented by genera *Shewanella, Aeromonas, Citrobacter*, and *Kluyera* (Rimoldi et al., 2019). Similarly, Bruni et al. (2018) found a high abundance of OTUs related to the *Aeromonas* genus only in the control fish group, but not in the intestine of the insect-fed groups. We also recorded an increase in the number of *Bacillus* and *Lactobacillus* genera in response to dietary insect meal. Proliferation of lactic acid bacteria (LAB) may be due to the prebiotic effect of chitin, and, as proposed by Bruni et al. (2018), it may indicate that chitin was a preferential growth substrate for LAB. Indeed, LAB play an important role in degrading fibers. Furthermore, they have an active role in host defense against pathogens, by producing bactericidal compounds, such as lactic acid, hydrogen peroxide, bacteriocins, and biosurfactants, which prevent pathogen colonization of the intestinal epithelial surface (Gudiña et al., 2015; Ringø et al.,

2018). Even the increased amount of *Bacillus* represents a positive effect of dietary chitin deriving from insect meal. Chitin, indeed, may have increased the proliferation of chitinolytic bacteria since several *Bacillus* species have been shown to secrete chitinase. Together with LAB, the *Bacillus* genus is one of the most common probiotics used in aquaculture to enhance host immune response and disease resistance. Up to date, several studies have demonstrated the immunomodulatory effects of *Bacillus subtilis* in fish (Cerezuela et al., 2013; Newaj-Fyzul et al., 2014b) and there are several evidences documenting that the use of insect meals from *H. illucens* may positively modulate trout gut microbiota, increasing LAB and Bacilli amount in both mucosa- and digesta-associated microbiota (Bruni et al., 2018; Huyben et al., 2018; Józefiak et al., 2019a; Terova et al., 2019). In addition to taxonomic characterization of gut microbiota in response to dietary insect meal, this study investigated the functional potential of the intestinal microbiome of rainbow trout using the computational approach PICRUSt. Indeed, the use of dietary insect meal clearly affected the structure of trout intestine–associated microbial community. Gut microbes carry out a multitude of biochemical reactions, which play a critical role in host nutrition by contributing to the digestion of several dietary ingredients. In agreement with Lyons et al. (2017a), we found that the principal functional pathways associated with bacterial communities of trout intestine, regardless of the diet, were metabolism, cellular processes, membrane transport, and genetic information processing. However, based on metagenome prediction, trout fed with insect meal showed an enhancement of pathways involved in sugar and starch metabolism. Members of the phylum Firmicutes are known to play a pivotal role in the fermentation of dietary carbohydrates (Corrigan et al., 2015). In our case, the increase of sugar metabolism observed in the Hi group of trout could be reasonably correlated to the higher presence of Bacilli that typify the intestinal microbiota of these fish. The fermentation of dietary carbohydrates and resistant starches by the

intestinal microbiota leads to the formation of a variety of beneficial substances, including short-chain fatty acids (SCFAs). It is well established that SCFAs (mainly acetate, propionate, and butyrate), in addition to being energy sources for colonocytes, promote fish intestinal health (Balcázar et al., 2006). Furthermore, the increased ability of gut microbiome to utilize dietary carbohydrates could be an interesting approach to improve feed digestibility in trout that is known as a poor user of dietary carbohydrates and fibres. In fact, *Bacillus* genera are widely used as probiotics in aquaculture to increase feed absorption and digestion. On the contrary, intestinal microbiome of trout fed with the Ctrl diet showed an increased capacity for peptidoglycan synthesis. Peptidoglycan is the major structural component of the cell wall of both Gram-positive and Gram-negative bacteria. It is the major wall structural component of the most pathogenic bacteria, and it is considered a proinflammatory molecule that stimulates host innate immune response (Mogensen, 2009). The hypothesis that control fish in the present study were affected by an inflammatory status seems to be supported by the increase of gene pathways of chaperones and protein-folding catalysts found in their intestinal microbiota. Indeed, secretion of chaperones and protein folding catalysts (foldase) from prokaryote cells acts as intercellular signal, principally for leukocytes. Effectively, Proteobacteria dominated intestinal microbiome of control trout, whereas Firmicutes were scarcely represented. This phylum was mainly represented by Gammaproteobacteria class, which includes important disease-causing pathogens of fish. Among these, *Aeromonas* resulted particularly abundant in the intestine of fish fed with Ctrl diet, possibly as a sign of intestinal dysbiosis or disease.

The second trial conducted during this PhD project, on the use of insect as innovative ingredients, was designed using another species, also widely used in aquaculture, for the total replacement of FM, the coleopterous TM. Numerous researches in the recent past have confirmed that the complete or partial

substitution of dietary FM with TM does not affect rainbow trout growth performance and fillet quality (Belforti et al., 2015; Rema et al., 2019; Chemello et al., 2020). Similarly, TM was successfully utilized and well accepted by several marine fish species (Gasco et al., 2016; Piccolo et al., 2017). While the effects of dietary FM/TM replacement on fish growth performances have been widely investigated, less evidence is available on the effects on host commensal bacterial communities. In particular, skin microbiome is underexplored in fish as well as in most farmed animals. The data showed no major effects of FM substitution with TM meal on species richness and diversity of both gut mucosa- and skin mucus-associated bacteria. In line with our results, the inclusion of hydrolyzed TM meal did not affect the total number of digesta-associated bacteria in sea trout (*Salmo trutta*) (Mikołajczak et al., 2020). In contrast, in the study of (Józefiak et al., 2019b), the total number of intestinal bacteria increased in rainbow trout fed a diet in which FM was partially replaced by TM in comparison to control fish that were fed a FM-based diet. Interestingly, (Antonopoulou et al., 2019) reported that the dietary inclusion of *T. molitor* larvae meal led to a five-fold increase of Simpson dominance index, and to a two-fold decrease of the Shannon index in rainbow trout gut microbiota, but not in sea bream and sea bass microbiota in which the same diversity indices remained practically unchanged. This evidence suggests a species-specific impact of insect meal on gut bacterial communities. Equally, in our previous studies, we found an increase of bacteria species richness and diversity in intestinal microbiome of trout fed diets with partial replacement of FM with *Hermetia illucens* meal (Rimoldi et al., 2019; Terova et al., 2019). Regardless of the diet type, marked differences in terms of alpha diversity were found between gut and skin microbiota, being the latter characterized by higher microbial diversity and richness. Although these divergences could be partly due to the different rarefaction depth applied to compute alpha diversity, it is also true that previous studies on trout and other freshwater species displayed a similar trend

with a lower alpha diversity in the gut than in the skin mucosal surface (Lowrey et al., 2015; Reinhart et al., 2019). Unfortunately, in contrast to high number of studies focused on fish gut microbiome, the skin mucus microbiome remains largely underexplored. Initially, fish skin is colonized by bacteria present in the water, but over time, the superficial mucus harbors an increasingly divergent microbial community. Like in intestine, the balance between members of skin microbial community, i.e., commensals, symbionts or pathogenic bacterial strains, collectively forming skin microbiome, is important to preserve fish health. It is well known that factors such as diet, water quality, seasonality, host physiology, infections, and stress can shape the composition of fish microbiomes and influence the balance of the microbic ecosystems (Rosado et al., 2019). Our metabarcoding analysis showed that rainbow trout skin microbiome was largely dominated by Proteobacteria, and especially Gammaproteobacteria, which constituted approximately half of the bacterial taxa found. This result agrees with previous studies on other fish species regardless of the technique used for bacterial identification (Lowrey et al., 2015; Krotman et al., 2020; Legrand et al., 2020). Gammaproteobacteria class includes several potentially pathogenic bacterial species for fish, such as *Vibrio anguillarum*, and *Photobacterium damselae*. Actually, there are several evidence supporting the role of fish skin microbiota as an important niche for mucosal pathogen evolution in nature. For instance, potentially pathogenic Vibrio, such as *Vibrio anguillarum* and *Vibrio cholerae*, monopolize skin microbiome of wild eel (*Anguilla anguilla*) from estuary and wetland (Carda-Diéguez et al., 2017). Other accidental pathogens identified in wild eel have been *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, and *Aeromonas veronii*. Although in the present study trout skin microbiome was dominated by the Gammaproteobacteria's family of Aeromonadaceae instead of Vibrionaceae, at genus level, *Pseudomonas, Stenotrophomonas* and *Citrobacter* were present in our samples likewise in wild

and farmed eel skin microbiome (Carda-Diéguez et al., 2017). This result is quite interesting, since previous studies have indicated that fish skin microbiome is species-specific, both in terms of bacterial diversity and bacterial community structure, showing significantly lower variability between individuals from the same species than between those of different species (Larsen et al., 2013). The low frequency of *Vibrio* genera in trout skin microbial community could be explained by the fact that trout is a freshwater fish while *Vibrio* are mainly marine bacterial genera. It is widely accepted, indeed, that the skin of fish harbors a complex and diverse microbiota that closely interacts with the microbial communities of the surrounding water. In line with our data, (Lowrey et al., 2015) reported that Proteobacteria and Bacteroidetes were the most abundant phyla of rainbow trout skin microbiota, however at genus level they found a skin bacterial community consistently composed by *Flectobacillus*. These apparently controversial evidence is inevitable since, up to date, few studies have investigated skin microbiome in freshwater fish, and it is not yet known if it fundamentally differs from that of marine fish (Krotman et al., 2020). With regard to skin microbial community composition, the two dietary groups did not display distinctive features, except for a decrease in the relative abundance of *Deefgea* genus (family Neisseriaceae) in skin microbiome of trout fed with insect meal. In agreement with our recent study in rainbow trout (Rimoldi et al., 2019), metagenomic analysis indicated that Tenericutes was the most abundant phylum in trout intestine, regardless of the diet. Specifically, within this phylum, the Mollicutes, mainly represented by Mycoplasmataceae family, were the dominant class. The Tenericutes are among the protagonists of gut symbionts of rainbow trout, indicating that they are possibly related to the metabolism of the host (Lowrey et al., 2015; Lyons et al., 2017). Although diet is the most important external factor affecting the gut microbiota composition, in this case we observed only a weak dietary modulation of intestinal bacterial communities. The only changes due to dietary FM

substitution with TM meal were a decreased number of Proteobacteria and, at family level, a reduced number of taxa assigned to Ruminococcaceae and Neisseriaceae. In line with our results, (Antonopoulou et al., 2019) reported that *T. molitor* meal replacement affected the dominant intestinal phyla less in rainbow trout than in sea bream and sea bass. In contrast, there are several evidence that FM replacement with insect meal from black soldier fly (*Hermetia illucens*) larvae positively modulates gut microbiota of rainbow trout by increasing the proportion of lactic acid bacteria (LAB), which are generally considered as beneficial microorganisms and frequently used as probiotics in fish and other vertebrates' diet (Bruni et al., 2018; Rimoldi et al., 2019; Terova et al., 2019). The increase of LAB by dietary insect meal could be related to the prebiotic properties of chitin and chitosan. Both are hardly digested by the majority of fish (Ringø et al., 2012). Therefore, once consumed, the fermentation of both polysaccharides is largely performed by gut microbiota. The lack of enrichment in intestinal LAB during the present study was an unexpected result, as the main effect of the dietary inclusion of this type of insect meal is generally a significant increase of Firmicutes at the expense of Proteobacteria phylum. The dietary administration of TM meal caused instead only a decrease in relative amount of Proteobacteria without any increase in Firmicutes.

For the third and last part of the PhD program, it was used a different approach. Instead of testing a specific ingredient as FM replacement, it was tested the effects, including growth performance, histological alterations, gene expression and microbiota analyses, of the administration of two doses of probiotic, added to an experimental base diet that mimic a commercial fish feed formulation with traditional vegetable proteins and oils as the main replacers of FM and FO. In aquaculture, indeed, the use of probiotics is significantly increasing, and a growing number of studies are demonstrating their positive effects in the most economically important fish species (Merrifield et al., 2010; Ridha and Azad,

2012; Chauhan and Singh, 2019). In the present study, gilthead sea bream fed with high and low doses of *L. lactis* subsp. *lactis*, respectively, reached a higher final biomass than control fish, and differences in biomass gain were statistically significant between groups C and A. This result highlights, albeit slightly, the beneficial action of the probiotic, suggesting a more efficient digestion and utilization of nutrients in gilthead sea bream fed probiotics. Similar results were obtained in gilthead sea bream by (Suzer et al., 2008) and (Varela et al., 2010), using *Lactobacillus spp*. And *Shewanella putrefaciens* Pdp11, respectively. Positive results in fish growth performance, using *L. lactis* as probiotic, were also obtained in other cultured fish species (Heo et al., 2013; Xia et al., 2018; Feng et al., 2019). The histological evaluation, conducted using a semi-quantitative scoring system, and focused the mucosal folds, that represent the intestinal absorptive surface area, and to the associated connective tissue, confirmed that probiotic did not alter the morphology of the gut and did not trigger intestinal inflammation. Indeed, no structural modifications were detected in fish fed with diets supplemented with probiotic (diets B and C), in comparison to the control group fed diet A. In line with our results, other studies have shown that probiotics improve gut morphology, leading to an increase in intestinal absorption capacity (Batista et al., 2016; Won et al., 2020). In contrast, (Cerezuela et al., 2012, 2013) reported several negative effects related to the administration of probiotics in gilthead sea bream. Therefore, more in-depth histological analyses are needed to better understand the effects of different probiotic strains on the adsorptive surface area in fish intestine. Numerous studies that investigated the effects of probiotics on the piscine immune system have reported an enhanced immune response, thus improving survival rates and resistance to a pathogenic attack (Nayak, 2010; Lazado and Caipang, 2014). Different probiotic strains stimulate the immune system in fish, but the effect appears to be species-specific. *L. lactis* supplementation increased the concentration of several pro- and anti-inflammatory

cytokines (Tnfa, Il1b, Il6, Il12, Il10 and Tgfb) in common carp serum(Feng et al., 2019) and upregulated the expression of tnf a, ifng, hsp70, and il1b genes in the intestine of tilapia (Xia et al., 2018). Herein, significant differences in the expression of key genes involved in innate and acquired immunity (interleukins and PRRs) were detected between fish fed probiotic and control diets. Among the mechanisms induced by probiotics, it has been postulated that the activation of immunity derives from the interaction of the host with the probiotic microbial associated molecular patterns (MAMPs) (Sun et al., 2012). The direct effect of MAMPs was recently demonstrated by feeding grouper (*Epinephelus coioides*) with MAMPs isolated from the probiotic *Bacillus pumilus* SE5. Indeed, an activation of intestinal immunity via up-regulation of TLR signaling pathways was observed (Yang et al., 2019). Thus, the observed activation of the immune system in the present study is likely taking place by direct induction of gilthead sea bream PRRs by components on the cell wall of the probiotic, such as peptidoglycan or lipoteichoic acid, which are in fact TLR2 agonists. Regarding the microbiota analysis, to assess the stability of the probiotic inclusion in fish diets, at the end of the experiment, the microbiota populations associated with feeds was analyzed. Data revealed that Firmicutes and Proteobacteria were the bacterial phyla represented most, followed in descending order by Bacteriodetes and Fusobacteria. Then, by analyzing specifically the relative abundance of the probiotic *L. lactis* subsp. *lactis* compared to the most representative genera of Firmicutes phylum, we found that the percentage of *L. lactis* was close to 0% in diet A (control), whereas in diets B and C, it was definitely high, reaching values of 64 and 71%, respectively, in agreement with the supplementation of a low and a high dose of probiotic. Instead, with regard to the gut microbiota, gilthead sea bream fed diet C showed a significant increase in bacteria belonging to the Spirochetes phylum, which were practically absent in the gut of fish fed diets B and A (<3%). In the same fish group, a decrease in Actinobacteria, Bacteroidetes,

and Firmicutes phyla was recorded. The Firmicutes phylum is composed of more than 200 different genera, such as *Lactobacillus, Bacillus, Enterococcus, Ruminococcus*, and *Clostridium*. Lactic acid bacteria (LAB) include, among others, *Streptococcus sp*., *Lactobacillus sp*., *Leuconostoc* sp. and *Carnobacterium sp*., which are considered as beneficial microorganisms that contribute to a healthy status of the fish intestine (Kim et al., 2012; Terova et al., 2019). It is known that commensal Firmicutes and Bacteroidetes are the major producers of short chain fatty acids, such as butyrate, acetate, and propionate that are the end products of fiber fermentations. While is difficult to assess from genomic data alone the physiological effects on the host microbiota we found, it is worth noting that Firmicutes/Bacteroidetes ratio in the gut has been directly related to lean body mass in both human and animals (Magne et al., 2020). Indeed, the ratio of Firmicutes vs. Bacteroidetes was increased in obese individuals as compared to lean ones. Actually, gilthead sea bream fed with diets containing probiotic showed a higher Firmicutes/Bacteroidetes ratio than control fish and this could be correlated to their better growth performances. In addition, gilthead sea bream fed diet C, showing the best FCR and SGR values, had the highest percentage of Spirochetes. Even without a direct proved correlation in fish, in swine, the Spirochaetaceae bacterial family was shown to correlate positively with the host weight (Unno et al., 2015). The gut microbiome of the feeding group C was also characterized by a Proteobacteria/Firmicutes ratio five times higher than in the other groups. This result is not surprising because *L. lactis* subsp. *lactis* SL242 produces the antibiotic nisin, displaying strong activity against Gram-positive bacteria (Li et al., 2018), and a vast majority of Firmicutes are Gram-positive. The analysis of gut-adherent (autochthonous) microbiota did not reveal significant differences between fish groups in relation to *L. lactis*, suggesting a lack of colonization of the probiotic in the host's intestinal mucosa. This was not a surprising result since it is known that the mechanisms behind the permanently or

a long-term establishment of a probiotics in the host intestinal mucosa is difficult and mediated by complex molecular interactions, so generally it does not occur. Therefore, in order for ingested bacteria to be useful, continuous consumption of probiotics is necessary for a lasting impact. Thus, instead of colonizing, the new bacteria may temporarily complement resident microbial communities, forming part of a transient (allochthonous) microbiome without displacing the native gut microbiota, but nevertheless they can contribute and improve the digestive tract function by producing active metabolites that modulate the activity of the gut microbiota, or by stimulating the intestinal epithelium directly. Hence, in the present trial, although the probiotic did not colonize the host's intestinal mucosa, it did modulate the fish gut microbiota, confirming that colonization is not always necessary to induce host modification. Indeed, diets B and C were enriched with Actinomycetales, as compared to diet A, which instead showed a higher percentage of *Pseudomonas, Sphyngomonas*, and *Lactobacillus* genera. These results were confirmed by the clear separation of bacterial community of fish fed with the probiotic from the bacterial community of control fish group (diet A) in the beta-diversity and PLS-DA analyses. Furthermore, the KEGG pathway analysis underlined such differences, highlighting several pathways potentially affected by the diet. Particularly interesting were those related to protein absorption and digestion. The gut microbial analyses also revealed significant and controversial differences between fish groups in term of ecological indices. Among alpha diversity parameters, fish fed with diet B showed the highest level of richness estimator ACE and biodiversity, in comparison to the other two fish groups. And in fact, in line with what we found in fish fed diet B, lactic acid bacteria supplementation was associated with an increase in bacterial diversity in the intestinal mucus of Atlantic salmon (Gupta et al., 2019). In contrast, dietary group C, although achieving the best growth performances, showed the lowest gut bacterial diversity. A reduction in bacterial diversity is usually considered an

adverse outcome, since this could lead to less competition for opportunistic or invading pathogens due to a functionally unbalanced ecosystem (Cerezuela et al., 2013; Li et al., 2014; Rimoldi et al., 2020). However, while an increase in intestinal microbial biodiversity following prebiotics (dietary compounds that induce the growth or activity of gut microbiota) administration has been frequently described, the data currently available on the effects of probiotics in fish are more controversial. For instance, in line with our results, the species richness and diversity indexes decreased in gilthead sea bream in response to dietary administration of the probiotic *Bacillus subtilis*, either alone or in combination with prebiotics or microalgae (Cerezuela et al., 2012, 2013). In addition, it is worth to mention that probiotics, such as lactic acid bacteria, are known to produce several antimicrobial compounds capable of suppressing the growth of other microorganisms, which can alter the gut microbiota in terms of both composition and biodiversity (Collado et al., 2007).

5.2 Conclusion

In summary, the surge that aquaculture is experiencing, dictated by the ever-increasing global demand for food and the need for transformation towards a more sustainable horizon for the entire sector, has led to several developments in fish feed technology and applications in recent decades. The great need to reduce or even replace the historically marine-based ingredients FM and FO has prompted the research world to identify of different possible innovative alternatives and strategies. In the present PhD project, the effects of two different insect meals (*Tenebrio molitor, Hermetia illucens*), as partial or total replacement of FM, and the influence of administering a lactic acid bacterium (*Lactococcus lactis* subs *lactis* SL242) as probiotic were evaluated. The analyses conducted, during these three years were different, but the guiding thread of the project was evaluating of the intestinal microbiota communities and determining how they have been

modified as a result of the innovative approaches used. Including 15% of *H. illucens* increased the abundance of beneficial genera, such as Lactobacillus and Bacillus, while the number of bacteria assigned to the pathogenic Aeromonas genus was drastically reduced in the same fish group. The metagenomic functional data provided evidence that dietary IM inclusion can shape the metabolic activity of trout gut microbiota by complementing the endogenous digestive enzymes and improving dietary carbohydrate utilization. Therefore, *H. illucens* meal represents a promising alternative protein source for trout nutrition, able to modulate the gut microbial community. The same conclusions can be drawn for including *T. molitor* in aquafeeds. In fact, even with only slight microbiota changes, the total replacement of FM with TM did not cause negative effects or dysbiosis on rainbow trout gut and skin microbial communities. Specifically, we were able to reduce the relative abundance of Neisseriaceae bacterial family in both gut and skin, whereas differences at the genus level were identified only at the skin level with a two-fold decrease in *Deefgea* genus in trout fed with the insect meal diet. In conclusion, administering probiotics did modulate the fish gut microbiota, modifying the abundance of the taxa and potentially affecting several metabolic pathways related to protein absorption and digestion, even without a clear colonization of the host's intestinal mucosa. This confirms that probiotics' establishment is not always necessary to induce host modification. Research such as this highlights the interaction between diet and the intestinal microbiota, suggesting that manipulating the diet to regulate the gut microbiome may be a promising intervention to promote an economic and sustainable transition in the aquaculture sector for the future.

5.3 List of Publications of Federico Moroni (last three years)

- Bosi, A., Banfi, D., Moroni, F., Ceccotti, C., Giron, M. C., Antonini, M., et al. (2021). Effect of partial substitution of fishmeal with insect meal (*Hermetia illucens*) on gut neuromuscular function in Gilthead Sea bream (*Sparus aurata*). Sci Rep 11. doi: 10.1038/s41598-021-01242-1.
- Montero, D., Rimoldi, S., Torrecillas, S., Rapp, J., Moroni, F., Herrera, A., et al. (2022). Impact of polypropylene microplastics and chemical pollutants on European sea bass (*Dicentrarchus labrax*) gut microbiota and health. Science of the Total Environment 805. doi: 10.1016/j.scitotenv.2021.150402.
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