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Use of alternative protein sources to fishmeal in aquafeed formulations and their effects on gut microbiota of cultured fish

Fonti proteiche alternative alla farina di pesce per l'alimentazione di specie ittiche di allevamento: studio del loro effetto sul microbiota intestinale

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ABSTRACT

Complex microbial ecosystems, collectively referred to as microbiomes, inhabit and interact with fish with ultimate beneficial outcomes for the host. Among these microbial ecosystems, the most explored to date is the gut microbiome, which refers to the large ensemble of microbes hosted in fish intestinal tract, including components from bacteria, fungi, and their viruses. Fish gut microbiome is involved in the bioconversion of dietary components, leading to the production of a wide and diverse range of bioactive small molecules, including short-chain fatty acids (SCFAs), and vitamins, with major impacts on host nutrition, and protection against pathogen colonization. Innovative technologies are providing us with more details about the fish microbiome, which is emerging as a very dynamic, changing, and adaptable system. However, the interplay between gut microbiome and nutrition is not fully clarified in fish. Therefore, it is important to define a strategy to predict and investigate the biological functions of fish gut microbiome when different dietary approaches are used, in line with the aquaculture strategy for replacing fishmeal (FM) and fish oil in aqua feeds with alternative terrestrial sources of protein and oil. On the other hand, modulation of fish gut microbiome using different diets, can open new perspectives for the fish farming by improving host's health and productivity, but until now only few studies have investigated this opportunity.

Accordingly, the aim of this PhD research is to provide the scientific basis for developing effective strategies to manipulate gut microbial communities through the diet, promoting fish health and improving productivity. To meet such aim three studies were conducted.

In the first study, we investigated the effects of partial replacement of dietary FM with a mix of animal by-product meals (ABP) and plant proteins on intestinal microbiota composition of rainbow trout (*Oncorhynchus mykiss*) in relation to growth and feeding efficiency parameters. We used 1540 trout fed for 12 weeks with 7 different feed formulations. The growth data showed that trout fed on diets rich in animal by-product meals grew as well as fish fed on control diet, which was rich in FM (37.3%) and ABP-free. High-throughput 16S rRNA gene amplicon sequencing (MiSeq platform, Illumina) was utilized to study the gut microbial community profile. Five thousand three hundred ninety-nine operational taxonomic units (OTUs) were identified, which predominantly mapped to the phyla of *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*. The ratio between vegetable and animal proteins proved to play a central role in determining microbiome profiles and *Firmicutes* and *Proteobacteria* phyla were particularly discriminatory for diet type in trout. Plant ingredients favored a higher *Firmicutes*:*Proteobacteria* ratio than animal proteins. In summary, animal by-product meals, as replacements to FM, improved fish growth performance and did not induce negative changes in gut microbial richness, thus proving to be a suitable protein source for rainbow trout.

In the second study, we investigated in gilthead sea bream (*Sparus aurata*), the effects of partial replacement of dietary FM with either fish hydrolysate or autolyzed yeast from *Saccharomyces cerevisiae* (HiCell produced by Biorigin Europe), on the gut microbiota richness and composition, and on fish growth and feeding efficiency parameters. Our data showed that dietary HiCell inclusion promoted specific gut bacterial taxa potentially beneficial for the host such as *Prevotella,*

Megasphaera and *Bacillus* genera. These effects are likely due to the yeast characteristics and not only to the bioavailability of the short peptides of which it is composed; in line with this finding, fish fed with the hydrolysate-based diet did not show the same changes in gut microbiota composition as fish fed the HiCell supplemented diet.

The third study focused on the effects of substitution of FM with insect meal (IM) from *Hermetia illucens* in the diet of rainbow trout, on fish growth performances, and intestinal microbiome. *H. illucens* larvae that were used to produce IM were cultivated on fruit and vegetables wastes from the wholesale market of Milano. Three diets, with increasing levels of replacement of FM with IM (10%, 20% and 30%) and a control diet without IM was tested in a 16-weeks feeding trial. Fish gut microbiome was analyzed by High-throughput 16S rRNA sequencing (MiSeq platform, Illumina). Although substituting up to 30% of FM with HI meal in trout diet did not affect fish growth performance, it increased gut microbiota richness and diversity. In particular, fish fed IM presented an increase in the relative abundance of lactic bacteria belonging to *Staphylococcaceae*, *Lactobacillaceae*, and *Leuconostocaceae* families, which are known to play an important role in degrading complex carbohydrates leading to the production of metabolic end products such as SCFAs. The abundance of other bacterial taxa known to promote fish health was increased, too.

The data obtained from the present research could contribute to improve the sustainability of aquaculture by developing new diets with positive effects on fish growth performances, metabolism, health, feed conversion ratio, and final product quality.

RIASSUNTO

Il microbiota è un complesso ecosistema costituito da una grande varietà di batteri, funghi e virus, che convive ed interagisce con l'organismo animale, apportando molteplici effetti benefici all'ospite. Tra le communita microbiche dei vari distretti corporei, il microbiota intestinale è quello ad oggi più studiato. Il microbiota intestinale svolge un ruolo molto importante nella produzione di molecole bioattive, come gli acidi grassi a corta catena (short-chain fatty acids - SCFAs), derivati dalla bioconversione dei carboidrati complessi assunti con la dieta, e che condizionano lo stato di salute dell'intestino. L'utilizzo di tecnologie innovative, ha permesso negli ultimi anni di descrivere il microbiota intestinale del pesce come un sistema dinamico e propenso agli adattamenti. Tuttavia, non è ancora chiara l'interazione tra la dieta dell'animale e la composizione delle comunità microbiche intestinali. Inoltre, la manipolazione della composizione microbica intestinale tramite la dieta, potrebbe aprire nuove prospettive nelle biotecnologie animali e in particolare in acquacoltura, contribuendo a migliorare la salute e la produttività dei pesci allevati.

In tale ambito, obiettivo di questo progetto di dottorato è stato quello di fornire conoscenze scientifiche utili per modificare le comunità microbiche intestinali attraverso la dieta, promuovendo la salute del pesce e al contempo aumentando la qualità del prodotto. Per raggiungere questo obiettivo sono stati condotti tre studi diversi.

Nel primo studio, è stato valutato l'effetto della parziale sostituzione di farina di pesce, con un mix di proteine vegetali e altre derivanti da scarti di lavorazione degli avicoli, sul microbiota intestinale di trota iridea (*Oncorhynchus mykiss*), monitorando i parametri di crescita degli

animali e l'efficienza di conversione dell'alimento. È stata allestita una prova sperimentale nella quale 7 differenti diete sono state testate su 1540 trote, per 12 settimane. Il microbioma intestinale degli animali è stato analizzato utilizzando la piattaforma MiSeq (Illumina) e attuando un sequenziamento "high-throughput" di alcune regioni variabili del gene rRNA 16S. Sono state identificate 5399 unità operative tassonomiche (Operational Taxonomic Units - OTUs), predominate dai phyla *Firmicutes*, *Proteobacteria*, *Bacteroidetes* e *Actinobacteria.* Il rapporto tra proteine vegetali e animali ha mostrato di svolgere un ruolo importante nel determinare il profilo microbico. I phyla dei *Firmicutes* e dei *Proteobacteria* sono risultati particolarmente influenzati dal tipo di dieta. Infatti, una dieta ricca in proteine vegetali ha favorito un rapporto *Firmicutes*:*Proteobacteria* più alto rispetto ad una dieta costituita principalmente da proteine di origine animale. Da questo studio è emerso che la sostituzione di farina di pesce con idrolizzati proteici da scarti di lavorazione degli avicoli, ha migliorato le performance di crescita del pesce senza indurre effetti negativi sul microbiota intestinale in termini di ricchezza delle specie, dimostrando di essere una fonte proteica ottimale per la trota.

Il secondo studio ha valutato gli effetti sul microbioma intestinale, nonché sui parametri di crescita e sull'efficienza di conversione alimentare, in orata (*Sparus aurata*). Le orate divise in tre gruppi sono state alimentate per 90 giorni con una dieta commerciale di controllo e altre due diete contenenti: la prima, idrolizzati proteici derivati da pesce in parziale sostituzione della farina di pesce e l'altra, il prodotto HiCell (Biorigin Europe), un autolisato di lievito *Saccharomyces cerevisiae*. I dati hanno mostrato che l'inclusione di HiCell nella dieta aumenta la presenza di taxa batterici benefici per l'ospite come i generi *Prevotella,*

Megasphaera e *Bacillus*. Questo effetto è presumibilmente dovuto alle caratteristiche del lievito e non solo alla maggiore biodisponibilità di piccoli peptidi liberi di cui è composto, dato che il microbioma di pesci alimentati con idrolizzati proteici, che sono ugualmente ricchi in oligopeptidi liberi, non ha portato allo stesso aumento.

Il terzo ed ultimo studio, si è focalizzato sugli effetti della sostituzione di farina di pesce con farina di insetto (*Hermetia illucens)* sulla crescita e sul microbioma intestinale di trota iridea. Le larve di *H. illucens* utilizzate per produrre la farina proteica sono state allevate su scarti di origine vegetale provenienti dall'ortomercato di Milano. Sono state testate, per 16 settimane di sperimentazione, tre diete differenti contenenti quantità crescenti di farina di insetto (10%, 20% e 30%) in sostituzione della farina di pesce e una dieta di controllo priva di farina di insetto. La sostituzione fino al 30% della farina di pesce con farina di insetto non ha influenzato le performances di crescita delle trote, ma ha apportato dei cambiamenti in termini di ricchezza e di diversità delle comunità microbiche intestinali. In particolare, le trote alimentate con farina di insetto hanno mostrato un aumento dell'abbondanza relativa di batteri lattici appartenenti alle famiglie *Staphylococcaceae*, *Lactobacillaceae* e *Leuconostocaceae*, noti per il loro ruolo nella degradazione dei carboidrati complessi con conseguente produzione di acidi grassi a catena corta. Infine, la sostituzione ha influenzato positivamente anche l'abbondanza di altri taxa batterici noti per essere benefici sullo stato di salute degli animali.

In conclusione, i risultati ottenuti dal presente progetto di ricerca potrebbero contribuire al miglioramento della sostenibilità dell'acquacoltura, mediante lo sviluppo di nuove diete che non gravano sulla risorsa oceanica ed hanno effetti positivi sulle performances di

crescita, sul metabolismo e sulla salute del pesce, nonché sulla qualità del prodotto finale.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 The fish microbiota

The term "microbiota" (or microflora) pertains to the collection of microbes colonizing a particular host niche, while "microbiome" refers to all of the genomes of the microbes constituting microbiota.

Microbiota communities were first correlated with fish in the 1920s (Merrifield and Rodiles, 2015). Since these studies were published, scientific interest in this relationship has increased.

Figure 1.1 shows different body district characterized by complex microbial communities that contribute to the development of the host immune system, angiogenesis, fat storage, and nutrition (Llewellyn et al., 2014). The composition of each microbial community is perfectly adapted to that part of the body, playing an essential role in its physiology. The quantity of bacterial population differs among the districts. According to the literature, there is a low bacterial abundance on the skin, 10^2 to 10^4 CFU (colony-forming unit) cm^{-2} and the amount results being influenced by the surrounding environment, in particular by water pollution. Conversely, gills are characterized by 10^6 CFU g^{-1} of tissue, and intestine by 10^6 – 10^8 CFU g^{-1}, a number that seems higher than that of the surrounding water (Austin, 2006).

Microbiota balance can be influenced by numerous biotic (fish lifestyle, genotype and physiology) and abiotic factors, such as stress, health status, environment and diet (Llewellyn et al., 2014). Undoubtedly, microorganisms present in the water and in sediment can influence both the microbiota on external surfaces (gills and skin) and

the microflora in the digestive tract, which receive water and feed abundant in microorganisms (Austin, 2006).

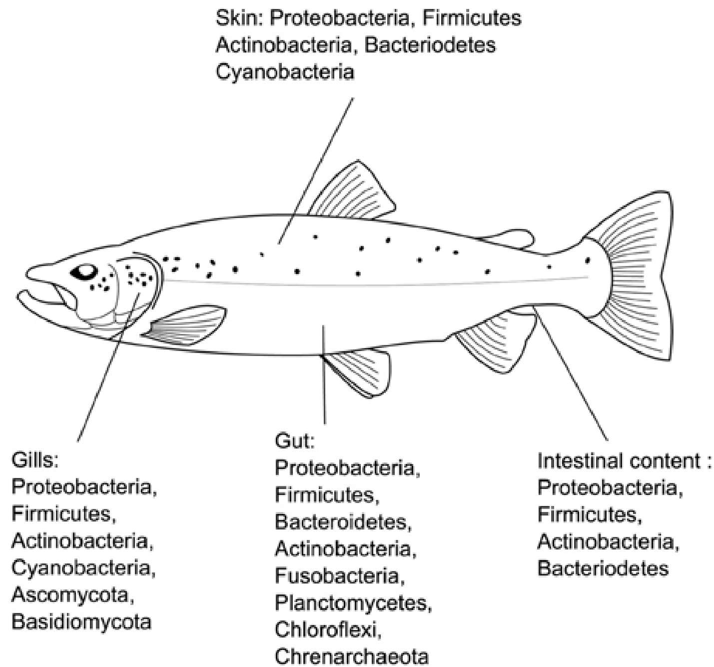

Figure 1.1 Different fish body districts with complex microbial communities. The bacterial phyla included are those that represent more than 80% of the sequences characterized by a specific tissue/organ (Llewellyn et al., 2014).

1.2 Fish gut development stages

Fish microbiota begins to form in the early stages of development and process is finished in the adult stage when the bacterial composition becomes stabilized (Fig 1.2). Microflora associated with the gastrointestinal tract of the early life stages of fish larvae and fry have been described in several investigations, but the establishment of a balanced gut flora in fish larvae is complex, and seems to be influenced by the microflora of the egg, the live feed and the bacteria present in the water. As known, in the first development stages, embryo is

protected by the chorion of the egg, which presents a microbial community, called epibiota, or epiflora. The microbes that compose the epibiota can be mutualistic or commensal species that function as a barrier against overgrowth of potential pathogens, and the dominating bacterial species are *Cytophaga*, *Flavobacterium* and *Pseudomonas* (Merrifield and Rodiles, 2015; Wang et al., 2017). The protective function of the egg-adherent microbiota was demonstrated by various studies that found bactericidal activity in fish eggs as well as the occurrence of immune-related molecules and glycoproteins, with agglutinative activity that immobilizes pathogens. Moreover, some studies dealing with eggs of rainbow trout (*Oncorhynchus mykiss*), Coho salmon (*O. kisutch*) and Chinook salmon (*O. tshawytscha*) reported high levels of lysozyme bactericidal activity against both Gram-positive and Gram-negative bacterial fish pathogens (Hansen and Olafsen, 1999, 1989). According to the authors of these studies, lysozyme activity may explain why only certain bacterial fish pathogens are transmitted vertically from mother to progeny. Thus, the presence of lysozyme in eggs of new fish species for aquaculture might reveal important information regarding disease resistance.

When the egg hatches, the microbe-free larva is immediately colonized by bacteria of the surrounding water and larvae's gastrointestinal (GI) tract acquires the first microbes with the ingestion of egg debris, allowing the epiflora to contribute to the first microbiota development (Merrifield and Rodiles, 2015). Generally, the GI tract of newly hatched larvae tend to contain few bacteria. Ringø and Birkbeck (1999), summarized in a review several studies that reported the principal bacterial genera found in the intestinal tract of freshwater and marine fish at the larval and fry stages. The bacterial families most

frequently reported in 11 marine species [European sea bass (*Dicentrarchus labrax*), red sea bream (*Pagrus major*), black sea bream (*Acanthopagrus schlegeli*), Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), Dover sole (*Solea solea*), turbot (*Scophthalmus maximus*), herring (*Clupea harengus*), rockfish (*Sebastes schlegeli*), milkfish (*Chanos chanos*), and wolfish (*Anarhichaslupus*)], and 3 freshwater species [Channel catfish (*Ictalurus punctatus*), Goldfish (*Carassius auratus*), and Masu salmon (Oncorhynchus masou)] were *Vibrio* (15 times), *Pseudomonas* (9), *Cytophaga* (8), *Flavobacterium* (7) and the family *Enterobacteriaceae* (7).

The bacterial flora of the surrounding water and, later, ingested food mainly influence the composition of the intestinal microbiota when it is established. The microflora of some nutrient rich and confined water environments is undoubtedly different from the oceanic microflora, and this could affect the commensal relationship between the normal indigenous microflora and adverse strains or opportunistic pathogens on mucosal surfaces of eggs and larvae (Hansen and Olafsen, 1989).

However, the same authors found also species-specific differences in bacterial colonization of fish eggs suggesting that genetic factors play a role, too (Hansen and Olafsen, 1989). Indeed, the establishment of a normal or protective microflora is regulated by factors such as specific receptors for adhesion and suitable conditions for proliferation of the bacteria. In addition, nonspecific defense factors and specific immunity of the host also play an important role (Hansen and Olafsen, 1999).

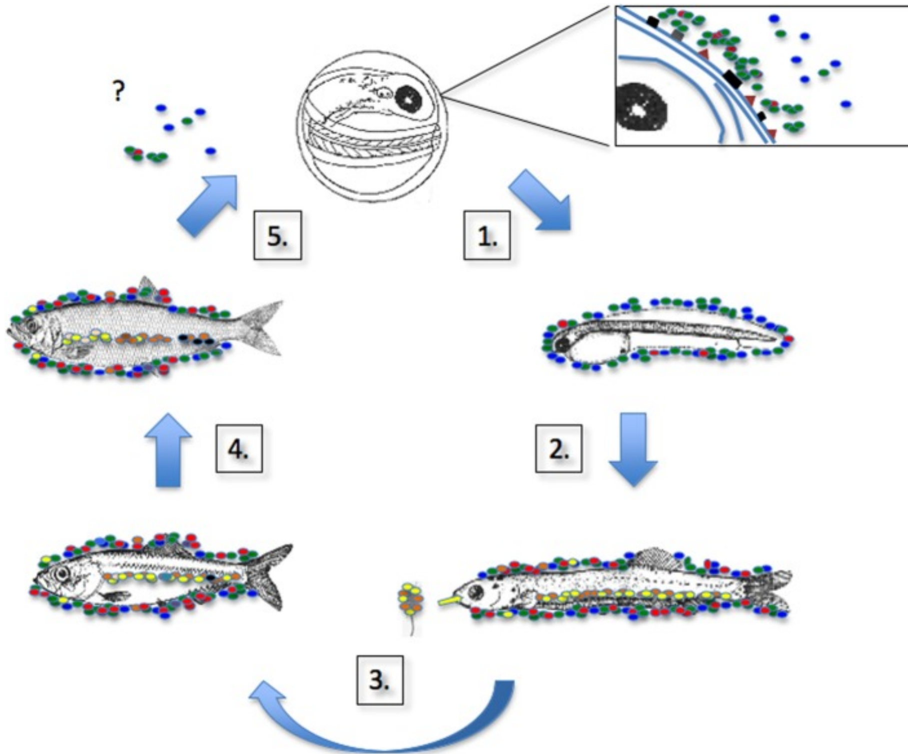

Figure 1.2 Microbiota formation during fish development. Figure shows the schematic of the generalized lifecycle of teleost and accessory indigenous bacteria (different taxa represented by colored ellipses). (1) Bacteria colonize the chorion of the egg. Taxonomic differences of bacteria between fish species suggest specific early interactions, perhaps through precursors of innate immunity (symbolized by squares and triangles on the chorion surface). (2) Egg hatches, in addition to bacteria originally present on the chorion, larva is colonized by environmental bacteria. (3) Early digestive tract colonization occurs when larva commence feeding. Bacterial taxa strongly resemble those associated with food source. (4) Microbiome develops, accumulates diversity and matures. (5) Adult microbiome is diverse assemblage of microbial taxa. Differences exist between surface mucosal and intestinal bacterial communities. Intestinal communities also be compartmentalized/specialized to niches within the alimentary tract. Question mark indicates possible vertical transmission of microbiota components to eggs during oviposition (Llewellyn et al., 2014).

1.3 Fish gut microbiota

Knowledge of the principle composition of fishes gut microbiota and understanding the role they play in digestion and whole body function is critical. Research on GI bacteria did not attain much popularity until the 1970s (Ringø et al., 2003); however during the past few decades, numerous studies have been carried out to characterize the GI microbiota in a wide range of fish species (Ray et al., 2012). Bacteria adherent to the alimentary tract mucosa live in intimate contact with the host and play essential roles in host metabolism, immunity, and development. The bacterial community in the fish gut is very dense compared to surrounding water, which suggests that GI tract provides a favourable ecological niche for survival. Each region of the intestine (anterior gut from pyloric caeca to first diffused sphincter and posterior gut from the first diffused sphincter to rectum sphincter) is characterized by a specific microbiotic profile made up of aerobic and anaerobic bacteria and the bacterial density generally increased along the intestinal tract.

Gut microbial community can be divided in allochthonous or transient microbiota consisting of free living microbial species that have been ingested and passes through the lumen associated with the digesta, and autochthonous microbiota that comprise residential species which are able to colonise the mucosal surface of the host gut and make up the core community (Egerton et al., 2018; Romero et al., 2014).

Mainly bacteria compose the fish GI microbiota, but it also harbors yeasts, viruses, fungi, and protoctista. In fish gut the yeast identified have been typically *Ascomycota* and *Basidiomycota*, which are producers of bioactive immunostimulant that modulate the mucosal leucocyte populations, cytokine gene expression, and goblet cell

abundance in host mucosal tissue. However, bacteria are the dominant microbes of the fish intestine (Egerton et al., 2018) and have been almost the sole focus of research in this field thus far. The predominant bacterial phyla of fish gut are *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, and which comprise together the 90% of gut microbiota composition of fish species studied so far (Egerton et al., 2018; Merrifield and Rodiles, 2015; Romero et al., 2014).
The composition of transient microbiota differs among teleost which live in a different environments and consume different diets. In particular, marine fish mainly harbor facultative anaerobic bacteria, such as *Pseudomonas*, *Vibrio*, *Alteromonas*, *Corynebacterium*, *Acinetobacter*, *Flavobacterium* and *Micrococcus*, whereas freshwater fish host *Bacterides*, *Aeromonas*, *Pseudomonas*, *Enterobacteriaceae*, *Micrococcus*, and *Clostridium* (Austin, 2006; Wang et al., 2017).
Commensal GI bacteria play a crucial role in various aspects of host physiology, being involved in nutrition, development, immune responses, and resistance against pathogens. In particular, GI microbiota synthesises enzymes, such as amylase, lipase, chitinase, cellulase, phytase, and protease. These enzymes are responsible for the anaerobic fermentation of undigested dietary complex carbohydrates. The end products of such fermentation are short-chain fatty acids than are readily absorbed and utilized by the host affecting positively intestinal epithelial cell differentiation and proliferation. Moreover, gut microbiota can prevent overgrowth of pathogens, creating an integrated defence system with the host immune system (Hooper et al., 2002; Kitano and Oda, 2006; O'Hara and Shanahan, 2006; Wang et al., 2017). Finally, GI microbiota stimulates fatty acid uptake and controls fat storage. Semova et al (2012) proposed in zebrafish four mechanisms

whereby microbes stimulate fatty acids (FAs) absorption and lipid droplets accumulation in enterocytes. Microbiota could promote the availability of FAs acting directly on luminal lipolytic activity, acting on bile salts production, reducing FAs oxidation, and promoting increased storage of them in lipid droplets; otherwise microbes might have an indirect effect on physiologic response (Semova et al., 2012; Wang et al., 2017).

The GI bacterial flora is affected by several factors, among them host (gender, genetic, and immunity) environmental (water, diet and additives), and microbial factors (adhesion capacity and metabolic capacity). Among the environmental factors, GI microbiota sense water temperature and salinity, and the seasonal intestinal lactic bacteria composition. In addition, diet type heavily affects the GI bacteria composition; we can observe this by comparing herbivorous and carnivorous fish (Egerton et al., 2018; Wang et al., 2017). Actually, some researchers have demonstrated the existence of a stable gut microbiota, called core gut microbiota. Roeselers and colleagues, for the first time, showed that there was a similar gut bacterial community in wild (recently caught) and in domesticated zebrafish (*Danio rerio*) independent of environmental influences and diet. In particular, these core components were dominated by *Proteobacteria* and *Fusobacteria* phyla (Roeselers et al., 2011). More recently, in another study, authors found in trout (*Oncorhynchus mykiss*) a large core intestinal microbiota composed by of 52 bacterial species that remains unaltered in spite of the diet and rearing density, which otherwise significantly changes fish growth performance, fillet quality and welfare. Specifically, the dominant phyla identified were *Firmicutes* and *Proteobacteria*, and

mainly *Bacilli*, *Alphaproteobacteria* and *Gammaproteobacteria* classes (Wong et al., 2013).

1.4 Fish diet and gut microbiota

In the last few years, fish nutrition has become an important topic for researchers. In aquaculture, feed is a factor that above all influences fish growth, health, the quality of the final product, and manufacturing costs.

To produce aquafeeds, better sources of proteins and lipids are fishmeal (FM) and fish oil (FO) that come from small pelagic species (sardine, herring, anchovies and mackerel) and fish processing by-products for human consumption. To avert ecological harm from overexploitation of oceans and defray rising costs due to increased competition for FM or reduced FM production, fish farmers and commercial feed producers have made substantial efforts to reduce the proportion of FM and FO in aquaculture feed, by replacing ground-up forage fish with terrestrial plants (mainly soybean, sunflowers and rape) (Tacon and Metian, 2009). However, in carnivorous fish species (such as rainbow trout, Atlantic salmon, and cod) fed with plant-based diet characterized by low level of indispensable amino acids (in particular lysine, and methionine) and the presence of a wide variety of anti-nutritional factors, damage to the intestinal tract, were observed, thus reducing nutrient absorption and fish growth (Francis et al., 2001; Uran et al., 2008). As a result, fish tended to produce excessive amounts of waste, which attracts bacteria and diseases and in the case of fish farmed in open ocean pens, disrupts the normal ecology of the marine environment in the immediate vicinity and for some distance beyond. For example, phytate that is considered an anti-nutritional factor is a

common constituent of plant- derived fish feed, but phytate- bound phosphorus is not available to gastric or agastric fish. Hence, phytate contributes to an increase of phosphorus load to aquatic environment, causing eutrophication that has several negative effects on the aquatic environment (Kumar et al., 2011).

Recently, interest has been increasing in alternative protein sources that are more economically sustainable and without side effects on the animal health. Moreover, a few studies conducted using high-throughput sequencing methods showed the effects of different diets on gut microbiota composition. For instance, microbiome analysis in Atlantic salmon (*Salmo salar*) showed an increased number and diversity of gut bacterial taxa, but characterized by a decreased number of lactic bacteria, in fish fed with soybean meal that presented enteritis in the distal intestine as compared to fish fed with a fishmeal-based diet. This study showed a correlation between the inflammation state and changes in gut microbiota according to diet (Bakke-McKellep et al., 2007).

In this context, gut microbiota is involved in numerous effects on host metabolism mucosal development and maturation, nutrition, immunity and disease resistance, raising the question of whether it is possible to change the microbiota composition through diet, increasing bacterial taxa with beneficial actions on intestinal epithelial tissue, or with an important role in the defence against pathogens. In this regard, some alternative protein sources have been proposed, focusing in particular on animal by-product meals, autolyzed yeast, and insect meal.

1.4.1 Animal by – product meals

Animal by product meals (ABP), from the rendering industry, are considered as suitable, alternative feedstuffs in aquaculture practice

since they are rich in most essential amino acids and contain important amounts of water-soluble proteins, which are highly digestible and improve the feed palatability (Burr et al., 2012). The use of ABP in animal feed industries was restricted by European Union with the Regulation 999/2001, after the outbreak of the transmissible spongiform encephalopathies (TSE). The re-authorization of the use of non-ruminant ABP in aquafeed has been issued since June 2013 by a Regulation 56/2013, which defined them safe (Jedrejek et al., 2016).

Among ABP, poultry by product meal (PBM) is one of the most promising alternatives for fish feed formulations and consists of rendered clean parts of the poultry carcass such as necks, feet, intestines, and undeveloped eggs (Yu, 2007).

This part is described extensively in chapter 2 of this thesis (Rimoldi et al., 2018).

1.4.2 Yeast – based meals

Yeasts are a natural component of the fish microbiota. In 1963, the presence of yeasts was demonstrated for the first time in fish. Typically, fish GI tract harbors two phyla: *Ascomycota*, of which *Saccharomycetaceae* is the more representative family, and *Basidiomycota*, characterized principally by *Rhodotorula* genus (Merrifield and Rodiles, 2015; Romero et al., 2014).

In recent years, yeasts have been suggested as an alternative protein source for aquafeed. Yeast has a high nutritional value for its high crude protein, B-vitamins, peptides, free nucleotides, that promote fish growth and feed efficiency, and mannan oligosaccharide (MOS), which enhances fish immune response. Indeed, some studies showed a natural commensalism relationship between fish intestinal mucus and yeasts that produce killer toxins that can protect the host against fungal and

bacterial pathogens (Gatesoupe, 2007; Merrifield and Rodiles, 2015). In order to promote that ability and functions, yeasts are mainly used as prebiotics and probiotics. In particular, in some studies, the outer cell wall of yeast (MOS) is used to enhance the fish immune response (Terova et al., 2009), modifying gut morphology (Salze et al., 2008) and gut microbiota composition (Dimitroglou et al., 2010).

In particular, *Saccharomyces cerevisiae* that contains numerous immunostimulant components such as MOS, β-glucan, chitin (as a minor component), and nucleic acids, was used in many studies (Oliva-Teles and Goncalves, 2001; Ozório et al., 2012; Zerai et al., 2008). Indeed, among the effects of this yeast are the enhancement of animal growth, feed efficiency, survival rate, and blood biochemistry (Romero et al., 2014). Moreover, in a study on juvenile beluga (*Huso huso*), adding a low percentage (2%) of inactive brewer's yeast to the diet increased intestinal lactic acid bacteria (LAB) compared to the control group, in addition to increasing fish final weight and specific growth rate and decreasing the feed conversion ratio (Hoseinifar et al., 2011).

1.4.3 Insect – based meals

Insects are part of the natural diet of both marine and freshwater fish and could become a sustainable and commercially viable alternative to FM in aquaculture and a key player in the fish feed market. Insects have high nutritional value and are rich in proteins (30-65% of dry matter), fats (7-32%), vitamins and minerals based on the life stage. Insects are considered highly sustainable; indeed, they can be reared on agricultural, agro-industrial, or human waste, creating a recycling process of nutrients (Barroso et al., 2014; Makkar et al., 2014). In 2011, FAO research estimated that 1.3 billion tons of food waste is produced per year, equivalent to one third of all food produced globally, and that

the inadequate waste treatment in developing countries represents a great source of pollution. In this regard, food waste could instead become a sustainable resource for the production of insects (FAO, 2011). On a global scale, insect meal based on organic waste has great potential as it could provide three times as much protein as the entire amount of soya produced today. For these reasons, insects have been suggested as an environmentally more friendly alternative to conventional feed sources, also due to their low greenhouse gas emission (Oonincx et al., 2010; Oonincx and de Boer, 2012) and high feed conversion efficiency (FAO, 2013; van Huis, 2013). Some scientific trials have already been carried out to investigate the use of insects as a feed for tilapia (Ogunji et al., 2008), rainbow trout (St-Hilaire et al., 2007), and Atlantic salmon (Lock et al., 2016) among other species, demonstrating that fish growing on insect meals performed equally well as their counterpart fish growing on traditional commercial fishmeal. During the production of fish feed, insects must be degreased to make feeds with only 10% of lipid content and dechitinized to avoid digestibility problems in the animals. Chitin is a polysaccharide present in the exoskeleton of insects, composed of N-acetylglucosamine units linked to each other with a β-1,4 bond. In fish some chitinolytic enzymes were found, produced by intestinal bacteria, and some studies have reported on the antimicrobial activity of low levels of chitin, stimulating the fish innate immune system (Gopalakannan and Arul, 2006; Lin et al., 2012; Rumpold and Schlüter, 2013).

A 2013 FAO publication concluded that insect meal could replace up to 70 percent of soymeal or FM in the diet of different fish species with no adverse effects for the growth and that such a diet does not affect the

taste or quality of the final product to market. Currently, the insect species that can be reared in the European Union and which satisfy the safety conditions in the feed industry, are: black soldier fly (*Hermetia illucens*), housefly (*Musca domestica*), mealworm (*Tenebrio molitor*), house cricket (*Acheta domesticus*), tropical house cricket (*Gryllodes sigillatus*) and Jamaican field cricket (*Gryllus assimilis*). Among them, one of the most promising species for large-scale production is *Hermetia illucens* (Hi) (van Huis, 2015). The larvae of Hi grow on different organic substrates consuming twice their weight each day and the prepupa contain a very high percentage of protein (36-48%; 420 g/kg) and fat (31-33%; 350 g/kg) on a dry matter basis with no slaughter waste, meaning that the entire larvae can be used (van Huis, 2013).

1.5 Microbiota: an active part in the host immune response

Teleost fish have an adaptive immune system associated with each of their mucosal surfaces. The main mucosa-associated lymphoid tissues (MALTs) are the nasopharyx-associated lymphoid tissue (NALT), the skin-associated lymphoid tissue (SALT), the gill-associated lymphoid tissue (GIALT), and the gut-associated lymphoid tissue (GALT). These districts harbor microbial populations that produce molecules locally or systematically active, with immunostimulatory and immunosuppressive effects on innate and adaptive host immune cells. Microbiota present in these fish mucosal body sites vary greatly and differ mainly between the GALT and the external mucosal surfaces (NALT, SALT, and GIALT), suggesting the presence of a unique and specialized symbiotic relationships between microbiota and each particular mucosal site (Kelly and Salinas, 2017).

Numerous studies were performed using germ-free zebrafish (*Danio rerio*) colonized with a single microbial species or a group of microrganisms. These challenges showed three different responses at the transcriptional level in epithelial cells proliferation, nutrient metabolism, and innate immune response (Kelly and Salinas, 2017). Given the ability of microbiota to manipulate host gene expression determining mutually advantageous cooperation, it is defined as a "metabolically active organ" (Hooper et al., 2002). The host benefits from the enhanced robustness against environmental perturbations and protection from pathogens by microbiota bacteria, while the microbiota lives in a protected and nutrient-rich environment where it can reproduce (Hooper et al., 2002; Kitano and Oda, 2006). In this context, a commensal microbiome is characterized by a colonization resistance, a term defined for the first time in 1971 (Van Der Waaij et al., 1971), according to which autochthonous microorganisms perform a passive (via competitive exclusion) and an active (via toxic secondary metabolites) form of inhibition (Llewellyn et al., 2014; Stecher and Hardt, 2008). Hypothesizing a co-evolution of the fish immune system and microbiota, a balance is created between microbes, their products, and host-produced metabolites mainly after a stressed perturbation or disease, and this balance has a crucial role in maintaining the healthy of the animals. In this regard, microorganisms will be able to evade the host immune system, while the latter will calibrate to hinder the pathogens action and at the same time tolerate all different types of microbes that inhabit various districts of fish mucosa (Kelly and Salinas, 2017).

1.6 Microbiota manipulation

Knowing the GI bacteria content of the most widely cultivated fish can help in manipulating their intestinal microbiota by adding probiotics to the feed, which are live microorganisms that can colonize the fish intestine and balance the positive and negative bacterial strain and ameliorate the health status or enhance the intestinal defense barrier producing antibacterial compounds (Austin, 2006; Burr et al., 2005). The major probiotics used by fish farmer are *Bacillus*, *Lactococcus*, *Aeromonas*, and *Shewanella* genera (Egerton et al., 2018).

Moreover, some prebiotics (non-digestible feed ingredients) are given in aquaculture owing to their positive effects on the tissue, stimulating growth and cells differentiation or activity of advantageous bacteria (Austin, 2006; Burr et al., 2005).

The prebiotics mainly utilized are fructo-oligosaccharides (FOS), short-chain fructo-oligosaccharides (scFOS), xylooligosaccharides (XOS), inulin, oligofructose, mannanoligosaccharides (MOS), trans-galactooligosaccharides (TOS), isomaltooligosaccharides (IMO), arabinoxylooligosaccharides (AXOS) and galactooligosaccharides (GOS) (Egerton et al., 2018). Numerous studies have demonstrated how the gut microbiota ferments the prebiotics fermentation in short-chain fatty acids, the principal source of energy for intestinal tissue (Llewellyn et al., 2014).

Probiotics and prebiotics can be valid alternatives to the use of antibiotics in aquaculture, where their use is highly regulated by Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009. Indeed, the continued use of antibiotics may increase the antibiotic-resistant bacteria and decrease the fish immune system.

1.7 Aim of work

Due to the overfishing of wild marine resources and the increasing share of aquaculture products in fish consumption, there is need to find alternative protein sources to replace fishmeal (FM) in aquafeeds. This prompted our interest in the study of the effects of different aquafeeds formulations on growth performance and gut microbiome of both, marine and freshwater fish species.

In aquaculture research, one important aim of gut microbiota studies is to provide the scientific basis for developing effective strategies to manipulate gut microbial communities through the diet, promoting fish health and improving productivity.

Accordingly, the aim of this PhD research was to investigate the effects of partial replacement of dietary FM with different alternative protein sources in fish diet on intestinal microbiota composition, in relation to fish growth and feeding efficiency parameters.

Three dietary trials have been completed in the present PhD Thesis, using three different alternative sources to fishmeal (animal by-products, insect meal from *Hermetia illucens* larvae and yeast, *Saccharomyces cerevisiae*) in two teleost species: rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*).

High-throughput 16S rRNA gene amplicon sequencing (MiSeq platform, Illumina) was utilised to study the gut microbial community profile.

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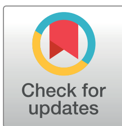
CHAPTER 2

NEXT GENERATION SEQUENCING FOR GUT MICROBIOME CHARACTERIZATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) FED ANIMAL BY-PRODUCT MEALS AS AN ALTERNATIVE TO FISHMEAL PROTEIN SOURCE



RESEARCH ARTICLE

Next generation sequencing for gut microbiome characterization in rainbow trout (*Oncorhynchus mykiss*) fed animal by-product meals as an alternative to fishmeal protein sources

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Abstract

Animal by-product meals from the rendering industry could provide a sustainable and commercially viable alternative to fishmeal (FM) in aquaculture, as they are rich in most essential amino acids and contain important amounts of water-soluble proteins that improve feed digestibility and palatability. Among them, poultry by-product meal (PBM) have given encouraging results in rainbow trout (*Oncorhynchus mykiss*). However, the introduction of new ingredients in the diet needs to be carefully evaluated since diet is one of the main factors affecting the gut microbiota, which is a complex community that contributes to host metabolism, nutrition, growth, and disease resistance. Accordingly, we investigated the effects of partial replacement of dietary FM with a mix of animal by-product meals and plant proteins on intestinal microbiota composition of rainbow trout in relation to growth and feeding efficiency parameters. We used 1540 trout with an initial mean body weight of 94.6 ± 14.2 g. Fish were fed for 12 weeks with 7 different feed formulations. The growth data showed that trout fed on diets rich in animal by-product meals grew as well as fish fed on control diet, which was rich in FM (37.3%) and PBM-free. High-throughput 16S rRNA gene amplicon sequencing (MiSeq platform, Illumina) was utilised to study the gut microbial community profile. After discarding *Cyanobacteria* (class *Chloroplast*) and mitochondria reads a total of 2,701,274 of reads taxonomically classified, corresponding to a mean of 96,474 ± 68,056 reads per sample, were obtained. Five thousand three hundred ninety-nine operational taxonomic units (OTUs) were identified, which predominantly mapped to the phyla of *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria*. The ratio between vegetable and animal proteins proved to play a central role in determining microbiome profiles and *Firmicutes* and *Proteobacteria* phyla were particularly discriminatory for diet type in trout. Plant ingredients favoured a higher *Firmicutes*:*Proteobacteria* ratio than animal proteins. Acceptable abundance of *Firmicutes* was guaranteed by including at least 25% of vegetable

specific role of Fabio Brambilla is articulated in the 'author contributions' section.

Competing interests: Naturalleva provided support by manufacturing the fish diets and conducting fish feeding trial. This does not alter our adherence to all PLOS ONE policies on sharing data and materials.

proteins in the diet regardless of animal protein source and percentage. In summary animal by-product meals, as replacements to FM, gave good results in terms of growth performances and did not induce significant changes in gut microbial richness, thus proving to be a suitable protein source for use in rainbow trout aqua feed.

Introduction

In aquaculture, feed accounts for over 50 percent of the production cost. This high cost is in large part due to the use of expensive ingredients such as fishmeal (FM) and fish oil (FO). Shepherd and Jackson (2013) [1] gave a detailed picture on the global volumes of production and consumption of FM and FO for the period 2001–2011, based on IFFO and Oil World data. The nutritive value of fish feed depends on the quality of the proteins used. For this, FM is a preferred ingredient, in particular for carnivorous species, such as salmonids. However, limited availability and high price of FM have driven the aquafeed industry to look for alternative protein sources in order to satisfy the demand of the continuously growing aquaculture sector [2,3].

Rational use of limited marine protein sources and development of nutritionally adequate feed formulations based on more readily available and economical alternative protein ingredients, are thus required [4–6]. In the last few years, significant advances have been made in this direction; currently, some commercial fish feeds can contain even less than 10% of FM. The most commonly used alternatives to expensive FM are of plant origin, such as oilseed meals (soybean, canola, and sunflower), grains (wheat and corn), and legumes (lupine, bean, and peas) [7]. Nevertheless, several nutritional issues are associated with the utilization of plant ingredients, due to their unbalanced amino acid profile and to the presence of anti-nutritional factors (ANFs) [8–11]. In plant feedstuffs, ANFs include indigestible components such as fibers, phosphorous-rich phytic acid, saponins, and protease inhibitors [10] that may reduce fish feed intake, growth, nutrient digestibility and utilization, and alter disease resistance, thus leading to poor fish growth [8, 12–14]. Therefore, it is crucial to find appropriate protein sources alternative to FM for aquafeed production. In this regard, some recent studies have shown that animal by-product meals, arising from the rendering industry, could be suitable for use as dietary FM replacers [15–18]. Unlike plant proteins, animal proteins are rich in most essential amino acids and contain important amounts of water-soluble proteins, which, besides being highly digestible, also improve feed palatability [16,17,19,20]. In recent years, after previous bans following the outbreak of the transmissible spongiform encephalopathy [21], the European Union has re-authorized the use of non-ruminant animal by-product meal (meat meal, blood meal, poultry by-product meals, and hydrolyzed feather meal) in aquafeeds. Since June 2013, it is thus possible to partially replace FM with different blends of non-ruminant animal proteins. Of these, one of the most promising and attractive options for fish feed formulations is poultry by-product meal (PBM), which consists of rendered clean parts of the poultry carcass such as neck, head, feet, undeveloped eggs, gizzard, and intestine [22]. PBM is generally a palatable, high-quality protein source due to its proper balance of essential amino acids, fatty acids, vitamins, and minerals [17,23].

The use of PBM in several fish species such as grass carp (*Ctenopharyngodon idellus*), turbot (*Scophthalmus maximus*), Nile tilapia (*Oreochromis niloticus*), and rainbow trout (*Onchorynchus mykiss*) has had positive effects on feed palatability, fish survival rate, growth performances, and protein retention [16,17,20,24–27].

However, the introduction of new ingredients in the diet needs to be carefully evaluated since diet is one of the main factors putting selective pressure on the gastrointestinal microbial composition in vertebrates, including fish [28]. Several studies in humans and mammals have undoubtedly correlated gut microbial communities with host physiology, nutrition, and growth [29–31]. Like in mammals, the intestinal microbiota of fish has important functions in host metabolism, mucosal development and maturation, nutrition, immunity, and disease resistance [32–35]. Fish gut microbiota is responsible for the synthesis of vitamins, digestive enzymes, and metabolites such as short-chain (volatile) fatty acids that represent the main energy source for intestinal epithelial cells [28,35–38]. Furthermore, fish intestine harbors a wide range of bacteria, mainly lactic acid bacteria, that can inhibit bacterial pathogens by secreting antimicrobial compounds such as lactic and acetic acids [28,39]. On the other hand, an imbalanced microbiota could negatively affect fish nutrition and growth and lead to an alteration of gut immune functions contributing thus to the development of diseases. Therefore, a better understanding of gut/microbe interactions and gut microbial diversity in fish could be highly relevant for aquaculture practice.

Fish microbiota has traditionally been studied by culture methods and subsequent identification based on biochemical and phenotypic characteristics of bacteria. However, culture-dependent techniques give a limited picture of intestinal microbiota because only a low fraction, down to about 1% of the bacteria from fish intestine can be cultivated. Therefore, in the last few years, several culture-independent molecular techniques have been developed and applied to studies of fish gut microbiota [35]. The most powerful approach to study the composition of complex intestinal microbial communities is represented by Next-generation Sequencing (NGS) technology [37,40]. Metagenomic profiling by high-throughput sequencing of 16S rRNA or cpn60 gene, was applied in some recent studies to investigate the impact of dietary plant ingredients on fish gut microbiota composition [38,41–44]. In rainbow trout, for example, a diet containing proteins from terrestrial plants such as pea and soy generally led to a higher *Firmicutes*:*Proteobacteria* ratio than a FM-based diet [44]. Conversely, replacing FM with a mixture of plant meals in the diet of sea bream (*Sparus aurata*) had a negative effect on the relative abundance of *Firmicutes* phylum throughout the gut, in particular, on lactic acid bacteria belonging to genera *Streptococcus* and *Lactobacillus* [42]. Whereas several studies have thoroughly investigated the effects of plant-based diets on fish gut microbiota composition, only a limited number of researches have been focused on the effects of FM replacement with animal by-product meals [45].

Accordingly, the present study aimed to investigate, for the first time, the effect of replacement of FM with seven different blends of terrestrial animal and plant proteins on intestinal microbiota of rainbow trout, trying to correlate any changes in microbial communities' profile to the performance outcomes of fish. The Illumina MiSeq platform for high-throughput sequencing of 16S rRNA gene was utilized to analyze and characterize the whole gut microbiome of trout fed with five different experimental formulations and two commercial feeds. Our assumption was that animal by-product meals could not negatively affect intestinal microbial profile of rainbow trout being thus a valid alternative to FM in feed formulation.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Edmund Mach Foundation (F.E.M) (San Michele all'Adige, Trento, Italy), and in accordance with EU Directive 2010/63/EU for animal experiments. The Committee on the Ethics of Animal Experiments of the same Foundation

approved all of the protocols performed [approval n. 120/2008-A of 03/09/2008 (Art.12 of D. Lgs.116/92). Fish handling was performed under tricaine methanesulfonate (MS222) anesthesia, and all efforts were made to minimize discomfort, stress, and pain to the fish.

Fish, rearing conditions, and diets

All procedures involving rainbow trout (*O. mykiss*) were conducted at the indoor experimental facility of Edmund Mach Foundation (F.E.M) (San Michele all'Adige, Trento, Italy).

We used 1540 trout (13 months old, all female) with an initial mean body weight of 94.6 ± 14.2 g and a total length of 21.4 ± 1.2 cm. Fish were randomly distributed into 14 fiberglass tanks of 3600 litres (110 fish/tank, at a rearing density of 2,89 kg/m^3) connected to a flow-through fish rearing system. Experimental tanks were supplied with degassed ground water with an approximately constant temperature of 12.5 ± 0.3˚C and dissolved oxygen concentration at 9.1 ±0.6 mg/l (DO saturation over 85%). Fish were acclimatized for six days under natural photoperiod and fed to visual satiety with a standard commercial diet (VRM S.r.l, Naturalleva, Italy). After the acclimation period, fish were fed twice daily for 12 weeks with seven different extruded diets (4.5 mm diameter pellets) in duplicate (2 tanks/diet). Five diets (A-E) were formulated specifically for this study by Naturalleva (VRM S.r.l Italy), whereas diets F and G were commercial feeds manufactured by competitors. We have reported the proximate composition of all the diets in Table 1 and the formulation of the experimental diets (A-E) in Table 2. In the first four experimental diets (A-D) (Tables 1 and 2), FM was partially replaced by different mixtures of plant and animal by-product proteins, i.e. poultry by-product meal (PBM) and porcine blood meal. The latter ones derived from animals, which passed as fit for human consumption under veterinary supervision, before their slaughter. In particular, diets A and B had a discrete content of FM and high levels of animal by-product and plant proteins. Diets C and D had a higher percentage of animal by-product meals and a lower percentage of FM than the two previous diets, but the highest content of plant proteins. Diet E (control) contained only FM, porcine blood meal, and vegetable meal as protein sources (no PBM) (Tables 1 and 2). Diets F and G (commercial feeds manufactured by competitors) were instead

Table 1. Proximate composition (g · kg^{-1} diet) and amount (%) of different protein sources used for the formulation of the experimental diets.

	DIET						
	A	B	C	D	E	F	G
Moisture	70.0	70.0	70.0	70.0	70.0	70.0	70.0
Crude protein	410.0	420.0	410.0	420.0	430.0	420.0	430.0
Crude lipids	260.0	240.0	240.0	180.0	260.0	280.0	220.0
Crude fiber	20.0	21.0	28.0	28.0	13.0	18.0	30.0
NFE	175.0	184.0	187.0	237.0	162.0	128.0	180.0
Ash	65.0	65.0	65.0	65.0	65.0	84.0	70.0
Phosphorus	5.5	5.4	5.1	5.4	4.7	4.6	3.8
GE (MJ kg^{-1})	23.0	22.5	22.5	21.1	23.2	23.3	22.0
Relative amount of different protein sources (%):							
FP/TP	20.6	20.9	10.6	11.2	37.3	20.0	11.0
TAP/TP	68.0	68.0	64.0	56.0	62.0	75.0	80.0
AP (TAP-FP)	47.4	47.4	53.4	44.8	24.7	55.0	69.0
VP/TP	32.0	32.0	36.0	44.0	38.0	25.0	20.0

NFE: Nitrogen-free extract; **GE**: gross energy (calculated using combustion values for protein, lipid and carbohydrate of 23.6; 39.5; and 17.2 MJ/kg, respectively); **FP**: fish proteins; **TP**: total proteins; **TAP**: total animal proteins; **AP**: animal proteins from alternative sources; **VP**: vegetable proteins.

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Table 2. Formulations of the experimental diets (in percentage).

	A	B	C	D	E
Fish meal	13.49	13.84	6.92	7.21	26.06
Dried swine hemoglobin	0.00	0.00	0.00	0.00	4.25
Dried swine plasma	12.01	12.32	12.32	11.12	8.16
Poultry by-products meal	12.71	13.04	15.54	12.60	0.00
Fish oil	16.39	14.90	3.61	2.42	16.02
Rapeseed meal	6.86	7.04	12.32	8.95	0.00
Soybean meal	6.65	6.82	15.72	10.95	7.30
Guar germ meal	2.57	2.64	0.00	10.68	4.79
Wheat flour	6.73	6.90	5.07	10.71	7.38
Corn gluten	0.00	0.00	0.00	0.00	3.36
Vital wheat gluten	3.35	3.44	0.00	0.00	0.00
Peas	11.93	12.24	10.98	12.95	9.60
Soy protein concentrate	0.00	0.00	0.00	0.00	6.59
Soybean oil	5.51	5.04	15.84	10.74	5.47
DL- methionine	0.55	0.56	0.40	0.44	0.31
Lisin	0.33	0.30	0.29	0.20	0.10
Taurin	0.30	0.32	0.40	0.43	0.00
Antioxidants premix[a]	0.06	0.04	0.03	0.04	0.05
Vitamin and mineral premix[b]	0.50	0.50	0.50	0.50	0.50
Stay C 35%	0.06	0.06	0.06	0.06	0.06
	100.0	100.0	100.0	100.0	100.0

[a] Propyl Gallate: 9.9%; B.H.A.: 5.0%, Ethoxyquin: 9.9%; Citric acid: 11.0%; Carrier (= SiO_2) ad 100%.

[b] Vitamin and mineral premix (quantities in 1 kg of mix): Vitamin A, 4,000,000 IU; Vitamin D3, 800,000 IU; Vitamin C, 25,000 mg; Vitamin E, 15,000 mg; Inositol, 15,000 mg; Niacin, 12,000 mg; Choline chloride, 6,000 mg; Calcium Pantothenate, 3,000 mg; Vitamin B1, 2,000mg; Vitamin B3, 2,000mg; Vitamin B6, 1,800 mg; Biotin, 100 mg; Manganese, 9,000 mg; Zinc, 8,000 mg; Iron, 7,000 mg; Copper, 1,400 mg; Cobalt, 160 mg; Iodine 120 mg; Anticaking & Antioxidant + carrier, making up to 1000 g.

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characterized by the highest percentage of animal by-product meals, most of them deriving from PBM (Table 1). The information provided in the labels of these two feeds are the followings. Diet F: PBM, FM, wheat, fish oil, vegetable oils (soybean, rapeseed), porcine blood meal, vegetable meal (dehulled soybean and sunflower), wheat meal, volatile blood meal, vitamin, mineral, and antioxidant premixes. Diet G: PBM, vegetable meal (wheat, dehulled soybean), hydrolysed feather meal, FM, rapeseed oil, fish oil, porcine blood meal, sunflower seed meal, soybean oil, guar germ meal, vital wheat gluten. vitamin, mineral, and antioxidant premixes.

Fish feeding rates were restricted to 1.5% of biomass during the feeding trial. To calculate feed ratio, individual weight of 30 randomly chosen fish per tank (60 fish/diet) was assessed at 14, 42, and 70 days from the beginning of the trial, whereas all fish in the tank (220 fish/diet) were measured for their weight and body length at the beginning and the end of the experiment. Fish growth performance data were used as basis for the calculation of feed conversion ratio (FCR = dry feed intake/wet weight gain), condition factor [K = 100 (wet weight (g)/total length (cm)^3], and specific growth rate [SGR (%/day) = 100 x [ln (final body weight)–ln (initial body weight)]/days], for each dietary fish group.

Amino acid profile of diets

Total amino acid composition of each diet was determined by a Jasco HPLC system (Jasco-Europe S.r.l) consisting of a quaternary pump (Model PU-2089, Jasco) connected to a

Table 3. Amino acid composition (g · kg^{-1} diet) of the experimental diets.

	DIET						
	A	B	C	D	E	F	G
Alanine	27.4	27.1	25.1	24.7	27.2	26.4	27.7
Arginine	25.1	26.1	23.7	25.3	27.3	26.4	25.4
Aspartate	36.4	37.2	34.0	34.9	39.7	38.4	37.1
Glutamic Acid	50.5	52.9	50.9	54.3	57.9	56.1	54.7
Glycine	34.6	34.2	31.0	31.9	28.1	27.2	34.1
Histidine	13.0	12.7	12.3	11.5	13.5	13.1	13.6
Isoleucine	13.2	14.3	13.5	14.8	13.0	12.6	14.1
Leucine	33.7	34.4	32.7	32.0	36.3	35.1	35.5
Lysine	27.7	27.2	22.9	22.5	28.0	27.1	27.8
Methionine	9.9	10.2	8.4	9.6	10.1	9.8	10.4
Phenylalanine	19.9	20.4	19.2	19.0	21.7	21.1	20.9
Proline	28.0	29.0	27.1	28.2	21.6	20.9	29.0
Serine	24.7	26.2	24.3	25.1	20.0	19.3	25.7
Threonine	17.1	17.6	16.6	16.7	18.0	17.4	17.8
Tyrosine	10.9	11.4	10.6	11.0	14.0	13.5	11.5
Tryptophan	4.3	4.3	4.0	3.9	4.8	4.6	4.4
Valine	23.6	24.2	23.1	22.5	22.8	22.1	24.8

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degasser, a programmable fluorescence detector (Model FP-4025, Jasco) (excitation 250 nm, emission 395 nm) and a temperature control module. The amount of sample used was 100 mg, which contained approximately 5 mg of crude protein that were hydrolyzed with 6 M HCl at 110–120˚C for 22–24 h.

L-α-amino-*n*-butyric acid (Sigma Aldrich, Italy) was added as an internal standard before hydrolysis. Methionine (Met) and tryptophan (Trp) were determined separately. For Met quantification, performic acid oxidation followed by acid hydrolysis was used, whereas for Trp quantification, the procedure consisted of hydrolysis in 4.2 M NaOH at 100˚C for 4 h, followed by neutralization of hydrolysate, and dilution in ultrapure water. After borate buffer addition and filtration, amino acids were derivatized with AccQ-Fluor Reagent Kit (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, Waters S.p.A., Italy) at 55˚C for 10 min and injected in HPLC. Amino acids separation was performed by using a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm) (Waters, Italy) and a Phenomenex pre-column filter according to Liu et al. [46]. Briefly, the column was heated at 37˚C for total amino acids and at 31˚C for sulphur containing amino acids (Met), and Trp. The flow rate was fixed at 0.8 ml/min, mobile phase A consisted of acetate-phosphate aqueous buffer, mobile phase B of acetonitrile 100% and phase C was ultrapure water. The amino acid composition of each experimental diet is reported in Table 3.

Fatty acid profile of diets

The fatty acid composition of each diet is listed in Table 4. Total lipids were extracted according to Folch et al. [47] by using dichloromethane instead of chloroform. Following lipid extraction, fatty acid methyl esters (FAME) were prepared by acid-catalyzed transmethylation of total lipids using boron trifluoride (BF_3) in methanol according to Santha and Ackman [48] and then analyzed by gas chromatography. The individual fatty acids were identified by comparing their retention times to that of standard FAME mixture (Supelco 37 Component FAME mix, Sigma Aldrich, Italy) and their relative proportions determined

Table 4. Fatty acid composition (% total fatty acids) of the experimental diets.

	DIET						
	A	B	C	D	E	F	G
12:0	0.02	0.02	0.01	0.02	0.02	0.02	0.02
14:0	1.86	1.79	0.72	0.76	1.99	1.97	1.76
15:0	0.15	0.15	0.06	0.07	0.16	0.16	0.14
16:0	10.37	9.97	10.62	10.69	9.90	9.89	10.20
17:0	0.18	0.17	0.13	0.14	0.18	0.18	0.17
18:0	3.19	3.07	3.95	3.94	2.95	2.95	3.16
20:0	0.48	0.46	0.36	0.35	0.47	0.48	0.46
22:0	0.34	0.33	0.37	0.38	0.28	0.28	0.35
24:0	0.07	0.07	0.07	0.06	0.07	0.07	0.07
25:0	0.02	0.02	0.04	0.04	0.02	0.02	0.02
14:1	0.02	0.02	0.01	0.02	0.01	0.01	0.02
16:1	2.27	2.19	1.00	1.13	2.29	2.26	2.21
17:1	0.14	0.13	0.08	0.09	0.14	0.14	0.13
18:1	0.01	0.01	0.01	0.01	0.01	0.01	0.01
18:1n-7	0.64	0.62	0.70	0.70	0.63	0.64	0.67
18:1n-9	28.82	27.61	24.28	24.01	27.26	27.53	28.14
20:1n-9	3.66	3.51	0.95	0.97	3.61	3.65	3.44
22:1n-9	0.53	0.50	0.11	0.11	0.54	0.55	0.49
22:1n-11	1.49	1.42	0.44	0.45	1.49	1.50	1.36
24:1n-9	0.09	0.09	0.03	0.03	0.09	0.09	0.09
18:2n-6	23.43	22.44	40.79	38.86	21.64	21.99	22.99
18:3n-6	0.20	0.19	0.08	0.08	0.20	0.20	0.19
20:2n-6	0.67	0.64	0.16	0.17	0.65	0.66	0.63
20:3n-6	0.26	0.25	0.06	0.06	0.25	0.26	0.25
20:4n-6	0.25	0.25	0.14	0.17	0.24	0.23	0.26
22:2n-6	2.3	2.2	0.6	0.6	2.3	2.3	2.1
18:3n-3	9.42	8.99	6.12	5.83	9.12	9.28	9.00
18:4n-3	0.64	0.61	0.22	0.24	0.68	0.68	0.60
20:3n-3	0.05	0.05	0.02	0.01	0.05	0.05	0.05
20:4n-3	0.32	0.30	0.11	0.11	0.33	0.33	0.29
20:5n-3	2.20	2.12	0.80	0.86	2.51	2.47	2.07
22:5n-3	0.68	0.65	0.23	0.24	0.71	0.71	0.63
22:6n-3	3.02	2.91	1.12	1.24	3.33	3.27	2.82
Σn-3PUFA	16.59	16.26	9.11	9.10	17.42	17.42	16.19
Σn-6PUFA	25.00	24.77	42.30	40.52	24.09	24.33	25.57
n-3/n-6	0.66	0.66	0.22	0.22	0.72	0.72	0.63
DHA/EPA	1.37	1.37	1.40	1.44	1.32	1.32	1.36

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Sampling

At the end of the feeding trial, two fish from each tank (4 fish/diet) were caught and euthanized with an overdose (320 mg/L at 22 °C) of anesthetic (tricaine-methasulfonate MS-222, Sigma-Aldrich, Italy). External surface of each fish was wiped with 70% ethanol to avoid the contamination of gut content by the external body surface microflora during dissection. With the aid of sterile scissors and forceps, the entire intestine (excluding pyloric caeca) was exposed from the ventral side, and then aseptically removed from each individual fish. The faecal content

was obtained by squeezing out and scrapping the intestinal mucosa with a sterile spatula, in order to collect the luminal and the mucosa-associated microbiota. The faecal samples were collected in sterile tubes, immediately frozen in dry ice and then stored at—80°C until analysis.

DNA extraction

We extracted total bacterial genomic DNA from all the collected faecal samples and used it as template in the 16S rRNA gene PCR amplification. Briefly, 1g of faeces from each fish was shaken with 5 ml of ASL buffer provided in the QIAamp DNA Stool Mini Kit (Qiagen, Italy). Then, 2 ml of homogenate were transferred into a microcentrifuge tube with two 5-mm stainless steel beads and then shaken on a TissueLyser II (Qiagen, Italy) for 5 min at 20 Hz. A sample of 2 ml of ASL buffer was processed in parallel as a negative control to check that no external DNA contamination was introduced during the extraction procedure. Bacterial DNA was then extracted according to the manufacturer's instructions. DNA concentration was measured by both, NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Italy) and Tecan Microplate Reader using Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Scientific, Italy). The extracted DNA samples were then diluted to a concentration of 5 ng/μl.

Intestinal microbiome analysis

16S rRNA gene amplicon sequencing library preparation. The Illumina protocol "16S Metagenomic Sequencing Library Preparation" (#15044223 rev.B) was applied to prepare 16S ribosomal RNA gene amplicons for Illumina MiSeq system. The variable V3 and V4 regions of the 16S rRNA gene were amplified from bacterial DNA obtained from fish faecal samples. The PCR reactions were performed using the 16S amplicon PCR forward primer (`5' CCTACGGGNGGCWGCAG 3'`) and reverse primer (`5' GACTACHVGGTATCTAATCC 3'`), which were selected by Klindworth et al. [49] as the most promising bacterial primer pair. Illumina adapter overhang nucleotide sequences were added at the 5' end of both primers. PCRs were carried out in 25-μl reactions containing 2.5 μl of microbial DNA (12.5 ng), 5 μl of each primer (1μM), and 12.5 μl of 2X KAPA Hifi HotStart Ready Mix (Kapa Biosystems Ltd, UK). A no template control, in which nuclease free water was added instead of bacterial DNA, and a negative control, with the extraction from the sample containing ASL buffer only, were included in this PCR. Reaction times and cycling conditions were 95°C for 3 min, 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min. The resulting PCR products were run on an Agilent 2200 TapeStation (Agilent Technologies, Italy) to verify the size. The expected size of amplicons was about 550 bp. The PCR products were then purified from primers and primer dimers using Agencourt AMPure XP Kit (Beckman Coulter Genomics, Italy). Dual indices and Illumina sequencing adapters (P5 and P7) were then attached to the amplicons using Nextera XT Index Kit (Illumina, San Diego, CA) to produce the final libraries. The index PCRs were carried out in 50-μl reactions containing 5 μl of DNA, 5 μl of Nextera XT Index Primer 1, 5 μl of Nextera XT Index Primer 2, 25 μl of 2x KAPA Hifi HotStart Ready Mix (Kapa Biosystems Ltd, UK), and 10 μl of nuclease-free water. The PCR reaction conditions were the followings: 95°C for 3 min, 8 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 5 min. Before quantification, the libraries were cleaned up using AMPure XP beads (Beckman Coulter Genomics, Italy) and the size of amplicons was verified on Agilent 2200 TapeStation (Agilent Technologies, Italy). The expected size of the final library was ~630 bp. Final libraries were quantified by absolute, quantitative PCR (qPCR) using KAPA Library Quantification Kits for Illumina® platforms (Kapa Biosystems Ltd, UK). In particular, library quantification was performed by amplifying the set of six diluted DNA standards and diluted

library samples via qPCR, using the KAPA SYBR® FAST qPCR Master Mix and primers targeting the Illumina® P5 and P7 flow cell oligo sequences. The qPCR was performed with the following cycling protocol: 95˚C for 5 min, 35 cycles of 95˚C for 30 s, 55˚C for 30 s, and 60˚C for 45 s. The average Cq score for each DNA standard was plotted against $\log_{10}$ of concentration (pM) to generate a standard curve. The concentrations of diluted library samples were then calculated against the standard curve, using absolute quantification. Final libraries were pooled in equimolar amounts, denatured and diluted to 4 pM before loading onto the MiSeq flow cell and sequenced on Illumina MiSeq platform (Illumina, San Diego, CA). According to Illumina protocol, 15% of PhiX Control library was combined with the amplicon library. MiSeq reagent Kit v3 (600 cycles) (Illumina, San Diego, CA) was used for library denaturating and for MiSeq sample loading. Sequencing was performed on Illumina MiSeq platform using a 2×300 bp paired end protocol.

Sequencing data analysis. The sequencing raw data were processed by the QIIME pipeline [50] using the "closed reference" out picking strategy. Raw reads quality has been checked using FastQC v0.11.2 [51], and R1 and R2 paired reads were joined using QIIME with the "SeqPrep" join method. The quality control was performed by QIIME, setting the phred_quality_threshold to 19 (Phred $\geq$ Q20). Reads were collected into OTUs (with identity $\geq$ 97%) using QIIME closed reference otu picking strategy against reference QIIME formatted Greengenes v.13.8 database (http://greengenes.lbl.gov). The taxonomical classification was performed down to genus level. OTUs assigned to the phylum *Cyanobacteria* (class *Chloroplast*), were considered potential plant contaminants and removed from the downstream analysis. Reads of mitochondrial or eukaryotic origin were also excluded. Singletons (OTUs with only one read associated) were excluded using the "filter_otus_from_otu_table.py" QIIME script.

Alpha and beta diversity statistics have been performed using QIIME scripts 'alpha_rarefaction.py' and 'jackknifed_beta_diversity_.py', respectively. In the calculation of alpha diversity metrics, the normalization was performed using the "rarefaction" QIIME process with standard parameters setting the "max_rare_depth" (upper limit of rarefaction depths) to lowest sample size. Alpha diversity metrics were calculated using 'observed species', 'Chao1 index' (species richness estimator), 'Shannon's diversity index' and 'Good's coverage'. An alpha-rarefaction plot was created for each metric. The alpha diversity values at the same rarefaction level (at the lowest sample size) were calculated.

Beta diversity metrics is an estimation of between-sample diversity of microbial profile and it was calculated by QIIME 'jackknifed_beta_diversity_.py' script. This script performed a jackknife iterative resampling method to normalize data, using a subsampling at 75% of the lowest sample size. We used both weighted (presence/absence/abundance matrix) and unweighted (presence/absence matrix) UniFrac distances [52,53]. The distance matrices were graphically visualized by three-dimensional PCoA representations.

Definition of the overall core community. Core microbiome analysis was performed in QIIME using the 'compute_core_microbiome.py' script. For this study the core microbiome was defined as the OTUs present in 80% of the samples regardless of diet.

Statistical analysis

Normality and homoscedasticity of all data were checked by Shapiro–Wilk's and Levene's test, respectively, using STATISTICA v.7 (StatSoft, Inc). One-way analysis of variance (ANOVA) was performed on growth performance, feed conversion and α-diversity data. Statistical significance was set at *P*-value < 0.05, and Fisher's Least Significant Difference (LSD) test was applied for multiple comparisons, when the overall ANOVA resulted significant.

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 family level) and 0.5% at genus level were considered for statistical analysis.

Statistical analysis of intestinal microbial profiles was performed using the Statistical Analysis of Metagenomics Profiles (STAMP) program (http://kiwi.cs.dal.ca/Software/STAMP), retaining unclassified reads [54]. *P*-values were calculated by ANOVA followed by Tukey-Kramer post-hoc test and correction of multiple testing was done using Benjamini–Hochberg False Discovery Rate (FDR) method [55].

Differences in the beta diversity of bacterial communities were verified using the non-parametric Permutational Multivariate Analysis of Variance (PERMANOVA) and adonis tests with 999 permutations. Both tests were available with QIIME script 'compare_categories.py'. A "by diet" pairwise significance test was also performed. For each pairwise contrast a filtered distance matrix containing only the samples to be compared was created using the "filter_distance_matrix.py" QIIME script, then a PERMANOVA significance test on each pairwise filtered matrix was performed using the "compare_categories.py" QIIME script.

Results

Growth and feed efficiency parameters of fish fed different diets

For the entire duration of the trial, mortality was negligible (< 1 percentage) and not correlated with a specific diet whereas final body weight data showed a diet effect ($P < 0.05$), revealing significant differences between experimental groups (Table 5). Indeed, at the end of the 12-week feeding trial, mean body weight of fish fed with diets E (293.78 ± 51.30 g) and G (298.28 ± 48.24 g) was significantly higher than the weight of other groups ($P < 0.05$), whereas fish fed diet F reached a mean body weight similar to fish fed diet E (control), but significantly lower than that of the group G ($P < 0.05$). Among all feeding groups, fish fed diet A showed the lowest mean mass value (251.77 ± 41.90). In line with weight data, the best SGR were observed in fish fed diets E, F, and G. Fish receiving diet A, B and C presented, in contrast, the lowest values ($P < 0.05$) (Table 5), whereas fish fed diet D showed an intermediate SGR value. Fish fed diets E, F, and G were better able to utilize energy for growth, too, as indicated by their lower FCR values ($P < 0.05$), which were 0.89, 0.91, and 0.89, respectively (Table 5). Trout fed diets A, B, and C showed, instead, the highest FCR values whereas fish fed diet D were positioned in between. Conversely, condition factor (K), that was calculated considering the entire experimental period (12 weeks), did not resulted significantly affected by diet (Table 5).

Table 5. Final mean body weight, specific growth rate (SGR), feed conversion ratio (FCR), and condition factor (K) values of trout fed with different diets. The final weight data represent mean value ± SD (n = 220 fish per diet). Different letters indicate statistically significant difference between groups ($P<0.05$).

Diet	Final weight (g)	SGR	FCR	K
A	251.77 ± 42.83^{e}	1.19 ± 0.03^{d}	1.06 ± 0.034^{a}	1.14 ± 0.11
B	264.66 ± 46.92^{d}	1.23 ± 0.01^{cd}	1.03 ± 0.007^{ab}	1.13 ± 0.13
C	264.80 ± 41.78^{d}	1.25 ± 0.02^{c}	1.01 ± 0.012^{b}	1.14 ± 0.12
D	276.01 ± 44.30^{c}	1.29 ± 0.01^{b}	0.96 ± 0.003^{c}	1.12 ± 0.11
E	293.57 ± 51.82^{ab}	1.39 ± 0.02^{a}	0.89 ± 0.007^{d}	1.13 ± 0.13
F	286.12 ± 51.57^{b}	1.35 ± 0.00^{a}	0.91 ± 0.007^{d}	1.12 ± 0.11
G	298.21 ± 48.67^{a}	1.38 ± 0.02^{a}	0.89 ± 0.013^{d}	1.14 ± 0.14

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QIIME analysis of sequencing data

Sequencing data were exported as individual fastq files and has been deposited in European Nucleotide Archive (EBI ENA) under the accession code: PRJEB23230.

The sequence fastq files from the Illumina MiSeq were analysed using QIIME software. After filtering for quality, trimming length, and assigning taxonomies, the number of reads taxonomically classified according to the Greengenes database, discarding cyanobacteria and mitochondria reads, was 2,701,274. This value corresponded to an average number of 96,474 ± 68,056 reads per sample (range 5,573–283,511). We identified 5398 OTUs at 97% identity in trout faecal samples, of which 3304 were assigned to the genus level (S1 Dataset). After rarefaction, normalizing to the sample with the lowest number of sequences (5570 reads), the observed species number per sample was comprised between 113 and 682, corresponding to average counts per group comprised between 270 and 496 (Table 6). Good's coverage values for all dietary groups were $\geq$ 0.96, indicating that sequencing coverage was attained and that the OTUs found in the samples were representative of the sampled population (Table 6). All the rarefaction curves, tended to plateau (S1 Fig). The number of observed species as well as the species richness index (Chao1) resulted not affected by diet type (Table 6). Similarly, Shannon's diversity index, which accounts for both abundance and evenness of the species present, did not show significant differences between the tested feeding regimens. It reached, instead, a stable value in all samples, indicating that bacterial diversity in these communities was mostly covered (S1 Fig; Table 6).

Faecal microbiome profiling of trout fed different diets

We successfully outlined the microbial community structures for each experimental group of fish at the phylum, class, order, family, and genus level. By considering only taxa with a relative abundance of more than 1% (up to family level), and more than 0.5% at genus level, the overall gut microbial community was mainly comprised of 7 phyla, 13 classes, 21 orders, 33 families and 41 genera. We have presented the profiles of intestinal microbial communities for each dietary group and individual fish at the phylum (Fig 1A and 1B), family (Fig 2A and 2B), and genus (Fig 3A and 3B) taxonomic level. In Table 7, is reported the relative abundance of all taxa that resulted significantly affected by diet. The result of post hoc multiple comparisons is shown in S1 Table. Irrespective of diet, the dominant phyla in our samples were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* (Fig 1A and 1B). A total of 211 OTUs constituted the core gut microbiota, i.e. OTUs that were shared by 80% of the samples irrespective of

Table 6. Number of reads per sample assigned to OTUs, and alpha diversity metrics values (normalized at the lowest sample size: 5570 sequences) of gut microbial community of trout fed with different diets for 12 weeks. Reported data are expressed as means ± SD (n = 4).

Diet	Reads	Observed species	Good's coverage	Chao1	Shannon
A	92,418 ± 93,722	420 ± 67	0.97 ± 0.00	673 ± 95	5.65 ± 1.03
B	76,432 ± 70,693	270 ± 162	0.98 ± 0.01	540 ± 279	3.75 ± 2.00
C	104,521 ± 52,416	402 ± 102	0.97 ± 0.00	653 ± 129	5.28 ± 2.34
D	97,321 ± 68,096	494 ± 17	0.96 ± 0.01	803 ± 101	6.63 ± 0.12
E	155,045 ± 91,981	486 ± 91	0.97 ± 0.01	748 ± 188	6.67 ± 0.33
F	46,482 ± 15,417	496 ± 86	0.96 ± 0.01	767 ± 197	6.83 ± 0.14
G	103,097 ± 68,953	415 ± 250	0.96 ± 0.02	708 ± 348	4.49 ± 2.67
Total number of reads taxonomically classified			2,701,274		
Mean number of reads/sample			96,474 ± 68,056		
Total number of OTUs			5398		

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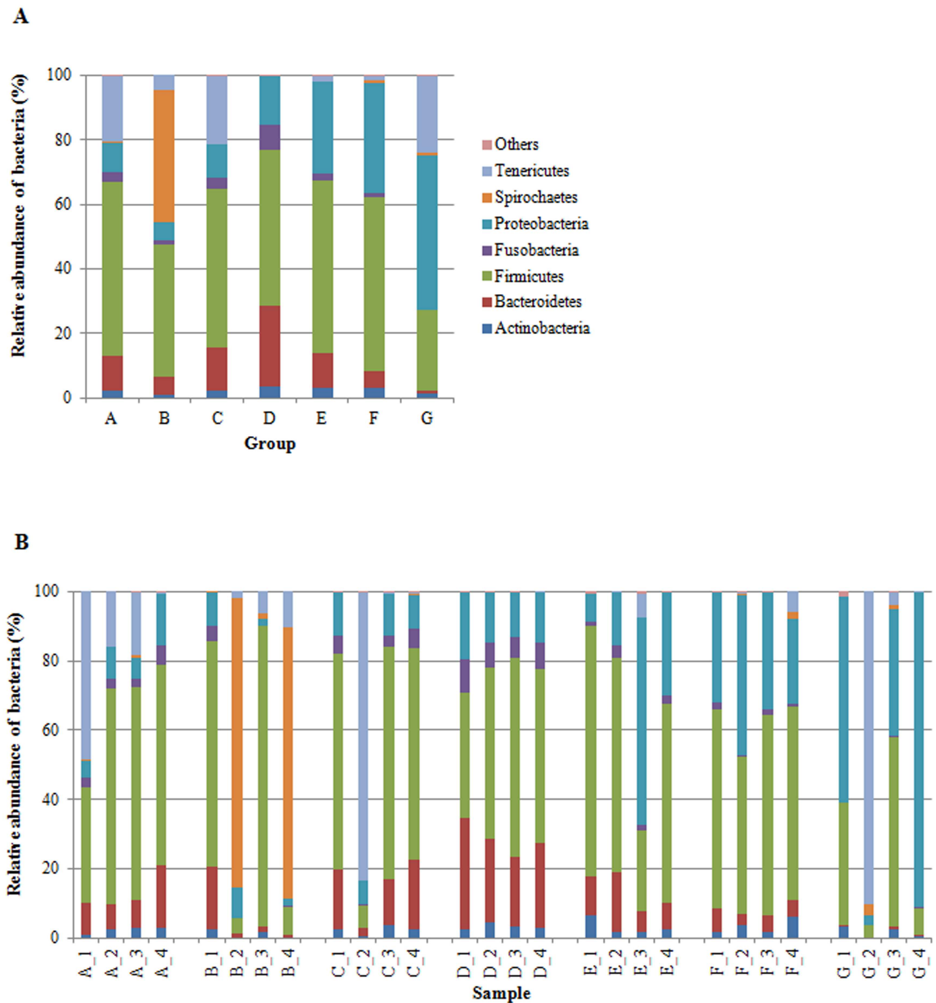

Fig 1. A, B. Relative abundance (%) of the overall most prevalent phyla in the different dietary groups (A) and in individual fish (B). In the figures, all bacteria with an overall abundance of ≥ 1% were reported. Bacteria with an abundance of ≤ 1% were pooled and indicated as "Others".

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diet (S2 Fig, S2 Dataset). Among them, 42 OTUs were common to 100% of samples, showing a dominance of *Firmicutes* (28 OTUs) (S2 Dataset). Results of metagenomic analysis of trout faecal samples revealed that, at phylum level, *Fusobacteria* and *Bacteroidetes* were influenced by the diet. Indeed, in trout fed diet D the relative abundance of *Fusobacteria* was significantly higher than in other groups ($P > 0.05$) (Fig 1A). This was due to a significantly higher presence of bacteria assigned to *Fusobacteriaceae* family (7.58 ± 1.27%) of *Fusobacteriales* order (Fig 2A and 2B). Fish fed diets C and D had high amounts ($P > 0.05$) of bacteria belonging to *Bacteroidia* class (C: 12.28 ± 6.31%; D: 23.1 ± 4.04%). Specifically, trout fed these diets presented higher

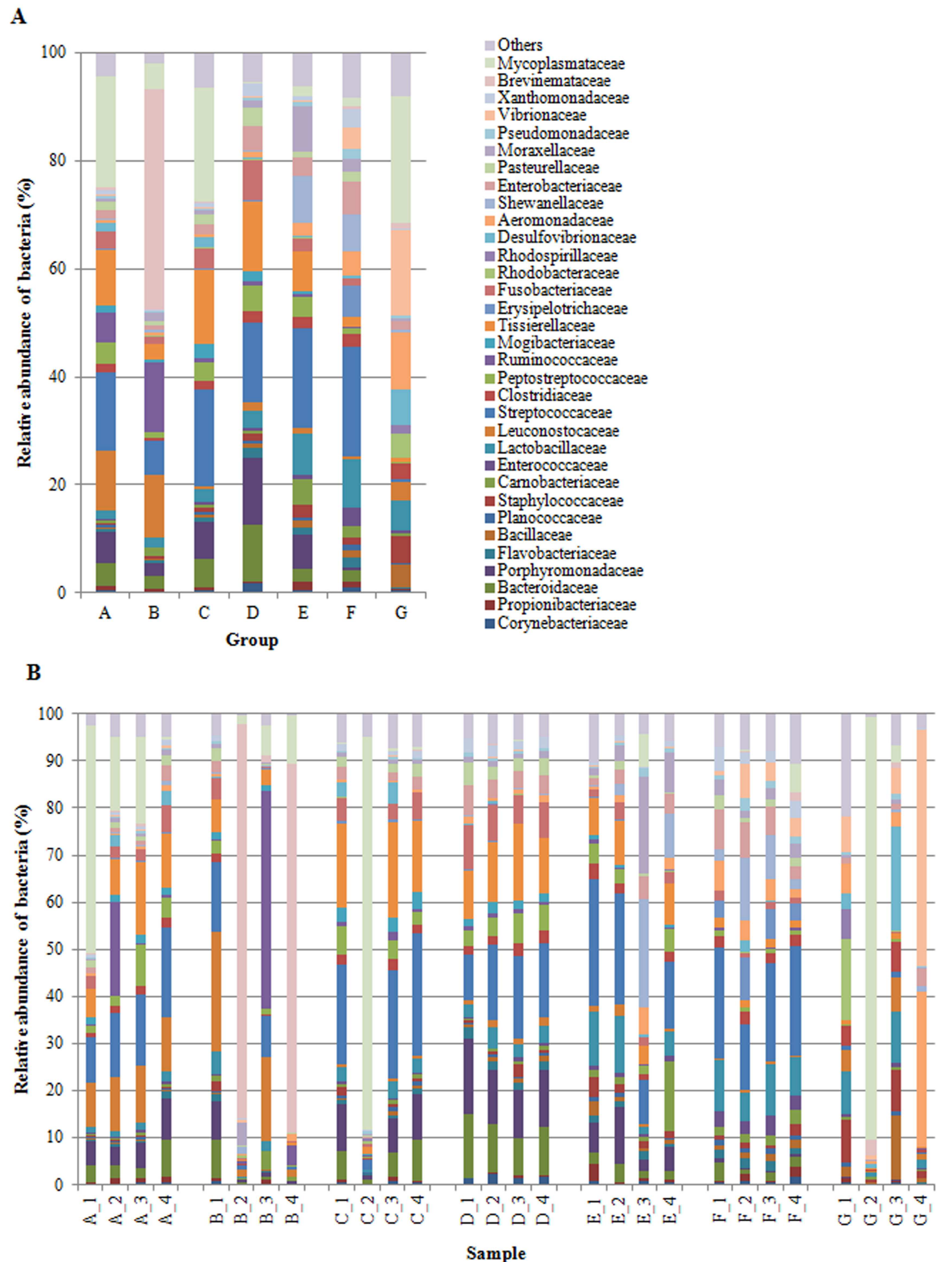

Fig 2. A, B. Relative abundance (%) of the overall most prevalent classes in the different dietary groups (A) and in individual fish (B). In the figures, all bacteria with an overall abundance of ≥ 1% were reported. Bacteria with an abundance of ≤ 1% were pooled and indicated as "Others".

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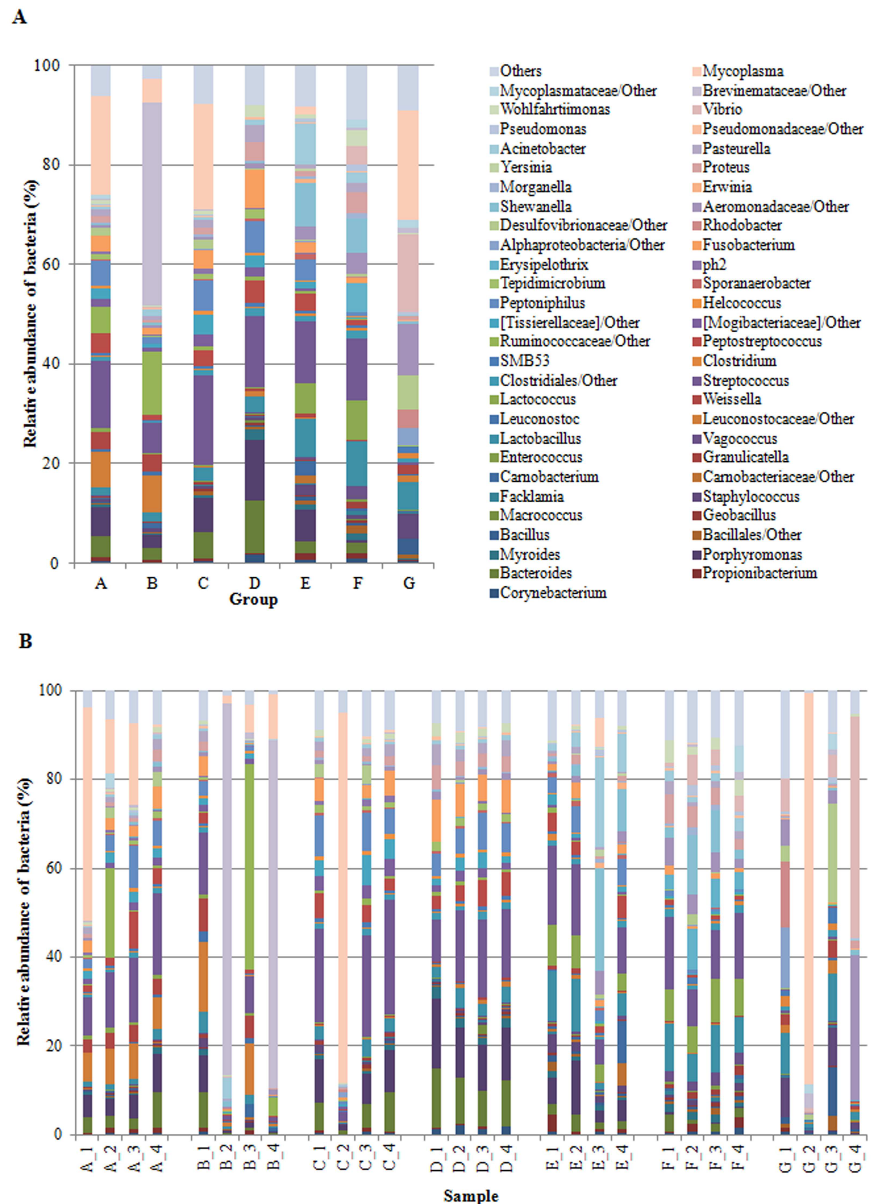

Fig 3. A, B. Relative abundance (%) of the overall most prevalent genera in the different dietary groups (A) and in individual fish (B). In the figures, all bacteria with an overall abundance of ≥ 0.5% were reported. Bacteria with an abundance of ≤ 0.5% were pooled and indicated as "Others".

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abundances of bacteria assigned to *Porphyromonadaceae* (C: 6.85 ± 3.53%; D: 12.46 ± 2.08%) and *Bacteroidaceae* (C: 5.33 ± 2.86%; D: 10.53 ± 1.98%) than other experimental groups (Fig 2A and 2B). A high percentage of bacteria belonging to *Bacteroidaceae* (from 2 to 4%) was also present in fish fed diets A, B, F, and E (control). Conversely, this bacterial family was

Table 7. Mean relative abundance (%) ± SD of phyla, classes, orders, families and genera that were influenced by the diet. Statistical Analysis of Metagenomics Profiles (STAMP) software was used to test statistical significance between taxonomic groups abundances, unclassified reads were retained only for calculating frequency profiles. One-way ANOVA ($P < 0.05$), with an effect size (ETA-squared) and multiple test correction using the Benjamini-Hochberg FDR method, was applied followed by Tukey-Kramer post-hoc test. The result of post hoc multiple comparisons is reported in supplementary S1 Table.

Phylum	A	B	C	D	E	F	G	p-value (corr.)	Effect size
Fusobacteria	3.30 ± 1.27	1.34 ± 1.80	3.60 ± 1.93	7.59 ± 1.28	2.29 ± 0.92	1.30 ± 0.52	0.12 ± 0.12	3.8E-04	0.76
Bacteroidetes	10.75 ± 4.36	5.37 ± 7.17	13.15 ± 6.65	25.19 ± 4.37	10.65 ± 4.35	4.84 ± 1.34	0.53 ± 0.36	1.1E-03	0.71
Class									
Erysipelotrichi	0.24 ± 0.05	0.07 ± 0.06	0.38 ± 0.23	0.24 ± 0.08	0.20 ± 0.05	5.77 ± 2.30	0.07 ± 0.04	2.0E-05	0.83
Flavobacteriia	0.78 ± 0.45	0.42 ± 0.49	0.86 ± 0.37	2.08 ± 0.34	1.39 ± 0.39	1.99 ± 0.15	0.20 ± 0.12	1.0E-04	0.79
Fusobacteriia	3.30 ± 1.27	1.34 ± 1.80	3.60 ± 1.93	7.59 ± 1.28	2.29 ± 0.92	1.30 ± 0.52	0.12 ± 0.11	2.5E-04	0.76
Bacteroidia	9.94 ± 3.91	4.93 ± 6.66	12.28 ± 6.31	23.10 ± 4.04	9.05 ± 4.44	2.85 ± 1.34	0.21 ± 0.18	1.0E-03	0.71
Order									
Erysipelotrichales	0.24 ± 0.05	0.07 ± 0.06	0.38 ± 0.23	0.24 ± 0.08	0.20 ± 0.05	5.77 ± 2.30	0.07 ± 0.04	3.5E-05	0.83
Flavobacteriales	0.78 ± 0.45	0.42 ± 0.49	0.86 ± 0.37	2.08 ± 0.34	1.39 ± 0.39	1.99 ± 0.15	0.20 ± 0.12	1.8E-04	0.79
Xanthomonadales	0.82 ± 0.34	0.34 ± 0.43	1.02 ± 0.52	2.36 ± 0.47	0.74 ± 0.38	3.37 ± 1.15	0.11 ± 0.13	1.3E-04	0.79
Fusobacteriales	3.30 ± 1.27	1.34 ± 1.80	3.60 ± 1.93	7.59 ± 1.28	2.29 ± 0.92	1.30 ± 0.52	0.12 ± 0.11	3.4E-04	0.76
Bacteroidales	9.94 ± 3.91	4.93 ± 6.66	12.28 ± 6.31	23.10 ± 4.04	9.05 ± 4.44	2.85 ± 1.34	0.21 ± 0.18	1.5E-03	0.71
Enterobacteriales	1.61 ± 0.91	0.75 ± 0.98	1.82 ± 0.93	4.43 ± 1.10	3.41 ± 1.28	6.17 ± 2.16	1.58 ± 1.34	7.8E-03	0.65
Pasteurellales	1.42 ± 0.61	0.80 ± 1.12	1.81 ± 0.91	3.51 ± 0.96	0.88 ± 0.50	1.83 ± 0.72	0.03 ± 0.03	1.0E-02	0.64
Family									
Enterococcaceae	0.38 ± 0.17	0.12 ± 0.12	0.37 ± 0.19	0.46 ± 0.27	0.87 ± 0.27	3.36 ± 0.48	0.45 ± 0.31	6.6E-09	0.93
Erysipelotrichaceae	0.24 ± 0.05	0.07 ± 0.06	0.38 ± 0.23	0.24 ± 0.08	0.20 ± 0.05	5.77 ± 2.30	0.07 ± 0.04	3.5E-05	0.83
Xanthomonadaceae	0.82 ± 0.34	0.34 ± 0.43	1.02 ± 0.52	2.36 ± 0.47	0.74 ± 0.38	3.37 ± 1.14	0.11 ± 0.13	2.5E-04	0.79
Fusobacteriaceae	3.30 ± 1.27	1.33 ± 1.79	3.60 ± 1.93	7.58 ± 1.27	2.26 ± 0.93	1.28 ± 0.49	0.11 ± 0.11	6.3E-04	0.76
Flavobacteriaceae	0.69 ± 0.48	0.40 ± 0.48	0.78 ± 0.34	2.00 ± 0.34	1.25 ± 0.35	1.77 ± 0.36	0.14 ± 0.07	6.9E-04	0.75
Aerococcaceae	0.08 ± 0.03	0.03 ± 0.04	0.18 ± 0.10	0.13 ± 0.03	0.27 ± 0.27	0.85 ± 0.23	0.26 ± 0.14	8.0E-04	0.74
Porphyromonadaceae	5.82 ± 1.80	2.55 ± 3.29	6.85 ± 3.53	12.46 ± 2.08	6.52 ± 3.53	0.48 ± 0.24	0.10 ± 0.10	1.5E-03	0.72
Corynebacteriaceae	0.35 ± 0.16	0.27 ± 0.30	0.49 ± 0.27	1.71 ± 0.37	0.57 ± 0.23	0.87 ± 0.46	0.35 ± 0.26	2.7E-03	0.70
Mogibacteriaceae	1.50 ± 0.23	0.66 ± 0.62	2.54 ± 1.31	1.86 ± 0.39	0.53 ± 0.29	0.05 ± 0.03	0.01 ± 0.01	2.5E-03	0.70
Bacteroidaceae	4.07 ± 2.23	2.35 ± 3.33	5.33 ± 2.86	10.53 ± 1.98	2.38 ± 0.95	2.29 ± 1.16	0.03 ± 0.02	3.0E-03	0.69
[*Tissierellaceae*]	10.13 ± 3.60	2.79 ± 2.74	13.76 ± 7.18	12.75 ± 2.15	7.36 ± 2.12	1.80 ± 0.23	0.58 ± 0.54	3.6E-03	0.68
Enterobacteriaceae	1.61 ± 0.91	0.75 ± 0.98	1.82 ± 0.93	4.43 ± 1.10	3.41 ± 1.28	6.17 ± 2.16	1.58 ± 1.34	7.7E-03	0.65
Pasteurellaceae	1.42 ± 0.61	0.80 ± 1.12	1.81 ± 0.91	3.51 ± 0.96	0.88 ± 0.50	1.83 ± 0.72	0.03 ± 0.03	1.1E-02	0.64
Lachnospiraceae	0.42 ± 0.19	0.16 ± 0.20	0.58 ± 0.30	0.62 ± 0.14	0.19 ± 0.09	0.57 ± 0.14	0.03 ± 0.03	1.8E-02	0.61
Streptococcaceae	14.41 ± 3.43	6.28 ± 5.91	18.12 ± 9.42	14.71 ± 3.01	18.55 ± 7.09	20.42 ± 3.84	0.60 ± 0.41	2.2E-02	0.60
Pseudomonadaceae	0.50 ± 0.19	0.26 ± 0.26	0.41 ± 0.07	0.73 ± 0.10	0.95 ± 0.48	1.62 ± 0.62	0.55 ± 0.45	4.1E-02	0.57
Genus									
Vagococcus	0.20 ± 0.06	0.06 ± 0.07	0.20 ± 0.10	0.26 ± 0.14	0.51 ± 0.24	2.59 ± 0.35	0.13 ± 0.09	1.7E-10	0.96
Lactococcus	0.85 ± 0.18	0.32 ± 0.28	0.20 ± 0.10	0.18 ± 0.06	6.07 ± 2.43	7.79 ± 1.44	0.11 ± 0.08	1.4E-06	0.89
Erysipelothrix	0.12 ± 0.02	0.03 ± 0.03	0.17 ± 0.10	0.13 ± 0.07	0.17 ± 0.04	5.69 ± 2.32	0.05 ± 0.04	5.6E-05	0.83
Sporanaerobacter	0.31 ± 0.11	0.09 ± 0.10	0.36 ± 0.20	0.50 ± 0.06	0.98 ± 0.26	0.11 ± 0.11	0.02 ± 0.01	6.9E-05	0.82
Tepidimicrobium	0.98 ± 0.36	0.26 ± 0.31	1.01 ± 0.61	1.79 ± 0.16	0.07 ± 0.09	0.02 ± 0.02	0.14 ± 0.16	1.8E-04	0.80
Ignatzschineria	0.02 ± 0.01	0.00 ± 0.01	0.02 ± 0.01	0.06 ± 0.02	0.01 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	4.10E-04	0.78
Wohlfahrtiimonas	0.76 ± 0.34	0.32 ± 0.40	0.93 ± 0.51	2.21 ± 0.42	0.67 ± 0.35	3.24 ± 1.17	0.09 ± 0.13	4.1E-04	0.78
Fusobacterium	3.22 ± 1.22	1.33 ± 1.79	3.56 ± 1.93	7.53 ± 1.27	2.15 ± 0.81	1.26 ± 0.48	0.03 ± 0.02	5.7E-04	0.77
Granulicatella	0.24 ± 0.10	0.23 ± 0.34	0.57 ± 0.32	0.27 ± 0.08	0.23 ± 0.10	1.42 ± 0.34	0.13 ± 0.18	8.1E-04	0.75
Porphyromonas	5.79 ± 1.78	2.54 ± 3.28	6.79 ± 3.50	12.37 ± 2.05	6.42 ± 3.51	0.41 ± 0.22	0.08 ± 0.08	2.2E-03	0.72
Myroides	0.67 ± 0.46	0.39 ± 0.46	0.72 ± 0.38	1.97 ± 0.34	1.07 ± 0.32	1.41 ± 0.39	0.07 ± 0.07	2.1E-03	0.72
Proteus	1.35 ± 0.77	0.57 ± 0.78	1.33 ± 0.67	3.68 ± 0.89	1.11 ± 0.52	4.36 ± 1.48	0.63 ± 0.71	2.2E-03	0.72

(*Continued*)

Table 7. (Continued)

Phylum	A	B	C	D	E	F	G	p-value (corr.)	Effect size
Helcococcus	0.51 ± 0.16	0.22 ± 0.20	0.77 ± 0.43	0.76 ± 0.09	0.17 ± 0.09	0.04 ± 0.05	0.00 ± 0.00	3.0E-03	0.71
Corynebacterium	0.35 ± 0.16	0.27 ± 0.30	0.49 ± 0.27	1.71 ± 0.37	0.57 ± 0.23	0.87 ± 0.46	0.35 ± 0.26	3.4E-03	0.70
Bacteroides	4.07 ± 2.23	2.35 ± 3.33	5.33 ± 2.86	10.53 ± 1.98	2.38 ± 0.95	2.18 ± 1.08	0.03 ± 0.02	4.0E-03	0.69
pH2	0.43 ± 0.10	0.09 ± 0.10	0.97 ± 0.56	0.24 ± 0.11	0.10 ± 0.11	0.01 ± 0.01	0.00 ± 0.00	8.8E-03	0.67
Enterococcus	0.12 ± 0.09	0.04 ± 0.04	0.10 ± 0.06	0.13 ± 0.09	0.25 ± 0.08	0.50 ± 0.19	0.21 ± 0.13	1.6E-02	0.64
Pasteurella	1.33 ± 0.56	0.71 ± 1.01	1.67 ± 0.85	3.25 ± 0.92	0.80 ± 0.51	1.72 ± 0.65	0.01 ± 0.01	1.6E-02	0.64
Peptoniphilus	5.20 ± 2.71	1.18 ± 1.28	5.97 ± 3.34	6.34 ± 1.26	4.42 ± 1.46	0.79 ± 0.17	0.08 ± 0.04	1.5E-02	0.64

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practically absent in the gut of fish fed with diet G (0.03 ± 0.02). Similarly, *Porphyromonadaceae* family was scarcely represented ($P < 0.05$) in the gut of fish receiving diets F (0.48 ± 0.24%) and G (0.10 ± 0.10%) (Fig 2A and 2B). Several taxa belonging to *Firmicutes* and *Proteobacteria* phyla differed quantitatively between groups, thus resulting discriminatory for diet type. The *Enterococcaceae* family of the *Lactobacillales* order was significantly enriched ($P < 0.001$) in fish fed diet F (3.36 ± 0.48%) in comparison to other feeding groups (Fig 2A and 2B). Similarly, in the same dietary group, bacteria from *Erysipelotrichaceae* were more abundant (5.77 ± 2.30%) than in others ($P < 0.001$). Fish fed diets A, C, D, F, and control diet E showed a significantly higher amount of *Streptococcaceae* (from 14% to 20%) than fish of group G (0.6 ± 0.41%). Interestingly, the relative abundance of bacteria assigned to the [*Tissierellaceae*] family of the *Clostridia* class, was significantly higher in fish receiving diets with high content of plant proteins, i.e. diets A, C, and D (Table 1, Fig 2A and 2B), whereas bacteria corresponding to the *Clostridia* class were less abundant or almost absent in the gut of fish fed diets F (1.80 ± 0.23%) and G (0.58 ± 0.44%), which contained high levels of animal proteins, mainly PBM (Table 1).

Contrariwise, fish fed diets rich in PBM were characterized by a higher abundance of bacteria assigned to *Proteobacteria* phylum (Fig 1A and 1B). This phylum constituted 34.04 ± 7.76% and 47.44 ± 32.28% of the entire intestinal microbiome of trout fed with diets F and G respectively. Specifically, *Enterobacteriaceae*, *Xanthomonadaceae*, and *Pseudomonadaceae* families of the γ-*Proteobacteria* class were enriched in the intestine of fish fed diet F (Fig 2A and 2B).

At genus level (Fig 3A and 3B) the percentage of unassigned sequences was remarkable, in particular for fish of groups B (64.93%) and G (30.58%). Nevertheless, by considering the samples in their entirety, forty-one genera were identified. Of these, twenty-four genera belonged to *Firmicutes* phylum, eleven to *Proteobacteria*, three to *Bacteroidetes*, two to *Actinobacteria*, and only one genus belonged to *Actinobacteria* (Fig 3A and 3B). Among *Firmicutes*, the most abundant genera identified in all fish, except for those fed diet G, were *Streptococcus*, *Lactobacillus*, *Peptostreptococcus*, and *Peptoniphilus*. The latter, a member of *Clostridiales* order, was more abundant ($P > 0.05$) in faecal samples of trout receiving diets A (5.20 ± 2.71%), C (5.97 ± 3.34%), and D (6.34 ± 1.26%). Genus *Lactococcus* was enriched ($P < 0.001$) in fish fed with diets E (6.07 ± 2.43) and F (7.79 ± 1.44). Besides *Lactococcus*, other two genera of *Lactobacillales* order, i.e. *Vagococcus* (2.59 ± 0.35%) and *Enterococcus* (0.5 ± 0.19%), were more abundant in F than in other dietary groups. The *Proteobacteria* phylum was mainly represented by the genera *Proteus* and *Pasteurella* (Fig 3A and 3B), which were, together with *Wohlfahrtiimonas* genus, significantly affected by diet. In fish fed diet G, several *Proteobacteria* were identified in the gut, but they belonged to different genera such as: *Vibrio* (15.84%), unclassified *Aeromonadaceae* (10.53%), and *Rhodobacter* (3.79%) (Fig 3A and 3B). Bacteria from *Vibrio* genus were also found in faecal samples of diet F fed trout in which they represented about

4.0%. In addition, trout fed with diet E and F showed a high abundance of genus *Shewanella*, amounting to 8.77% and 6.77%, respectively (Fig 3A). Fish of group E also had a relatively high percentage of bacteria from *Acinetobacter* genus (Fig 3A and 3B). The phylum *Actinobacteria* was mainly represented by genera *Propionibacterium* and *Corynebacterium*. *Corynebacterium* genus resulted more abundant ($P < 0.05$) in fish fed diet D (1.71 ± 0.37) in comparison to other groups. Within *Bacteriodetes* phylum, *Bacteroides*, *Porphyromonas* and *Myroides* were the most abundant genera observed in our samples, and fish fed with diets C, D, and E generally showed the higher percentage ($P < 0.05$) of these genera in comparison to other groups (Table 7). Finally, *Mycoplasma* genus (Fig 3B) was identified in all samples, but in much lower quantities in fish fed with diets D, F and control diet E.

Principal coordinate analysis (PCoA) of intestinal bacterial communities

QIIME pipeline was used to compute microbial beta diversity metrics; both weighted and unweighted UniFrac analyses were performed (Fig 4A and 4B). Data of UniFrac matrices were projected onto three-dimensional plots using principal coordinates analysis (PCoA). Weighted PCoA showed that most of samples were broadly indistinguishable and clustered together except for fish D and F which clustered according to diet (Fig 4A). Conversely, diet definitely affected unweighted UniFrac. Indeed, unweighted UniFrac PCoA revealed a clear clustering of samples by diet (Fig 4B). High animal-to-animal variation was observed in the group G, whose individual microbiomes appeared, indeed, to be more widely distributed on the first principal coordinate PC1 (14.16%).

The statistical analysis (permutation multivariate analysis PERMANOVA and Adonis test) totally reflected PCoA plots results, indicating a significant divergence between groups for both weighted ($P = 0.002$; $R^2 = 0.45$; Pseudo-F = 2.82) and unweighted ($P = 0.001$; $R^2 = 0.33$; Pseudo-F = 1.74) UniFrac distance matrices (Table 8). Pairwise test on the weighted UniFrac data showed that only fish fed diet D significantly diverged ($P < 0.05$) from all other groups, while fish fed with diet F was similar only to control group E (Table 8). Result of pairwise test on unweighted UniFrac data revealed, that samples clustered in three distinct groups, one constituted by A, B, C and D samples, one by F and control E, and the last by samples G ($P < 0.05$) (Table 8).

Discussion

Animal by-product meals from the rendering industry could be the most promising and suitable alternative to FM ingredients in aquaculture practice [19] due to their high content of essential amino acids and water-soluble proteins [16]. To date, several data are available on the effect of animal by-product meals on fish growth performances [16,17,27,45,56–58], but still very few studies have been conducted on their effect on fish gut microbiota [45,59,60]. Therefore, the information obtained in this study on the effects of substitution of FM with animal by-product meals on both, fish growth performance and intestinal microbiota biodiversity, represents a contribution to our knowledge.

During the feeding trial, trout survival rate was over 99% in all groups and no disease outbreaks occurred. Despite this, a clear diet effect was observed on fish growth. Indeed, trout fed commercial diets F and G grew as well as fish fed diet E (control) that was rich in FM (37.3%) and PBM free. The same fish displayed the best FCR and SGR values, too. These formulations were characterized by the highest content in animal proteins, of which PBM constituted the majority, whereas FM accounted for only 20% and 11%, respectively. Our findings are in agreement with previous studies conducted on rainbow trout [16,17,56,61] and other fish species, such as largemouth black bass (*Micropterus salmoides*) [62], hybrid striped bass (*Morone*

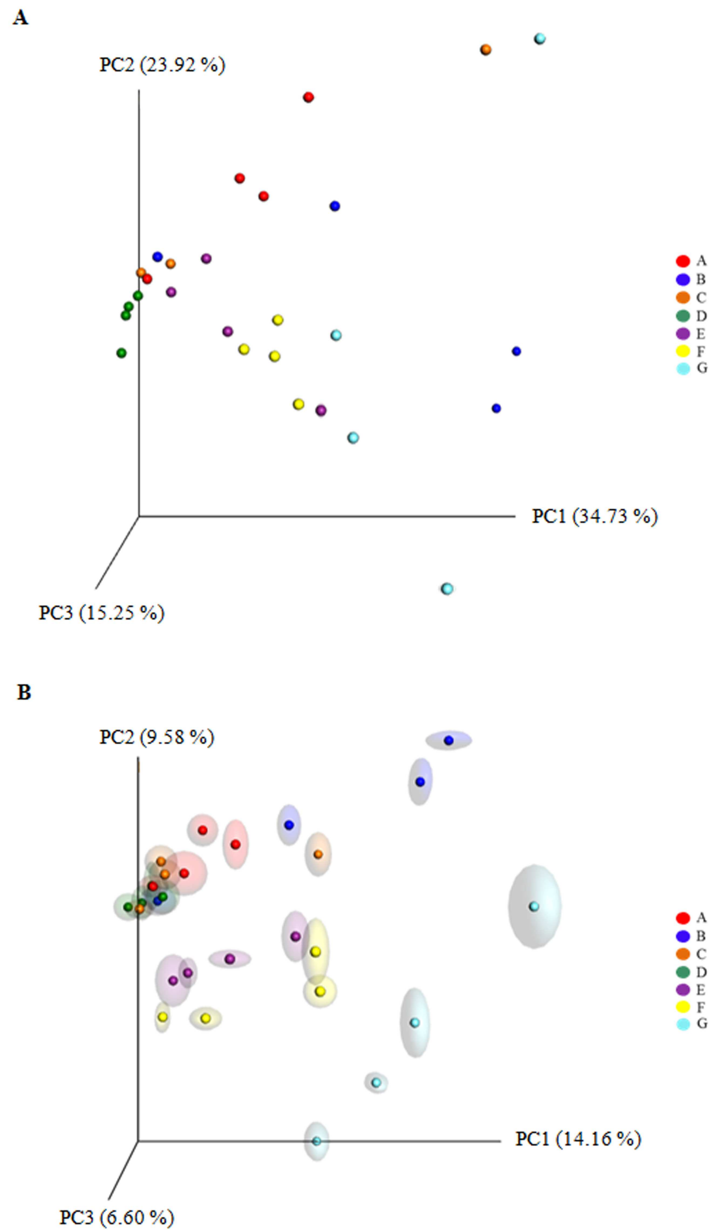

Fig 4. A, B. Beta diversity metrics. Principal coordinate analysis (PCoA) of Weighted (A) and Unweighted (B) Unifrac distances of gut microbial communities associated to different diet. The figures show the plot of individual fish (4 fish/diet) according to their microbial profile at genus level. Red = diet A; blue = diet B; orange = diet C; green = diet D; violet = control diet E; yellow = diet F; light blue = diet G.

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Table 8. Permutation multivariate analysis PERMANOVA and Adonis test on weighted and unweighted UniFrac data of intestinal microbiomes of trout fed with different experimental diets.

Adonis analysis	**Unweighted UniFrac**		**Weighted UniFrac**	
	P-value	R^2	*P*-value	R^2
	0.001	0.33	0.002	0.45
PERMANOVA analysis	**Unweighted UniFrac**		**Weighted UniFrac**	
	P-value	Pseudo-F	*P*-value	Pseudo- F
One-way				
Diet	0.001	1.74	0.001	2.82
PERMANOVA Pairwise test:				
A *vs* B	0.103		0.156	
A *vs* C	0.231		0.573	
A *vs* D	0.123		0.028	
A *vs* E	0.034		0.067	
A *vs* F	0.026		0.030	
A *vs* G	0.028		0.057	
B *vs* C	0.087		0.197	
B *vs* D	0.101		0.028	
B *vs* E	0.051		0.130	
B *vs* F	0.026		0.036	
B *vs* G	0.062		0.152	
C *vs* D	0.058		0.033	
C *vs* E	0.037		0.200	
C *vs* F	0.029		0.012	
C *vs* G	0.025		0.100	
D *vs* E	0.036		0.027	
D *vs* F	0.037		0.029	
D *vs* G	0.024		0.034	
E *vs* F	0.090		0.190	
E *vs* G	0.026		0.109	
F *vs* G	0.024		0.032	

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chrysops x *M*. *saxatilis*) [57,58], cobia (*Rachycentron canadum*) [63] and Atlantic salmon (*Salmo salar*) [18]. In these species, good results in terms of growth rate were reported when PBM was used to replace FM in the diet. In particular, in Atlantic salmon, a test diet with 20% of FM replaced by PBM did not affect weight gain, feed intake and FCR with respect to a FM-based diet [18]. Similarly, in rainbow trout, a 30% replacement of FM with PBM yielded growth performances indexes comparable to those of fish fed with a FM-based control diet [61]. Burr and colleagues [16] reported that FM levels in rainbow trout feeds could be reduced to 10% without affecting fish growth if blends of animal and plant proteins are used. Always in trout [17], even a total substitution of FM with PBM, led to a high protein retention without apparent effects on growth. Contrariwise, Asian seabass (*Lates calcarifer*) fed a diet based on a mix of animal and plant proteins with FM inclusion rate reduced to 6%, showed a higher FCR value than control fish fed with a FM-based diet [45]. An increase in FCR was also found in Coho salmon (*Oncorhynchus kisutch*), when PBM was the primary protein-providing ingredient [64].

In terms of growth and feeding efficiency parameters, A and B definitely proved to be the worst formulations among all diets tested in the present feeding trial, followed by diet C. Better growth and feeding performances were obtained in fish receiving diet D, though their growth

parameters were significantly lower than the control group E. The reduced growth rate observed in these trout was not due to an amino acid deficiency or imbalanced levels of essential amino acid (EAA). Indeed, despite varying proportion of different dietary protein sources, the amino acid profile was quite similar among experimental diets and the EAA levels exceeded the estimated EAA requirements for rainbow trout [15]. In fact, compared to control diet E, the content of EAA (such as Lys, Met and Thr) was 80% or above for all the diets. The amount and proportion of different fatty acids in the diet are important issues, too. In rainbow trout, it has been proved by time that n-3 fatty acids are essential for good growth and survival [65,66], and the substitution of FM with alternative terrestrial animal or plant proteins could alter the dietary n-3:n-6 fatty acid ratio. Actually, diets C and D, which were characterized by high vegetable and PBM inclusion, and low FM and fish oil content, showed clear differences in terms of fatty acids composition. In comparison to other formulations, C and D diets contained a higher amount of linoleic acid (18:2n-6), a lower content of eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22:6n-3) and consequently, a lower n-3:n-6 fatty acid ratio. Although the n-3 LC-PUFA requirement for trout is low (0.4–0.5%) [15,67] and it was fulfilled by diets C and D, dietary levels of EPA + DHA, as well as those of linoleic acid could have affected fish growth performances. Indeed, reduced growth rates were observed in trout receiving diets containing high concentration of linoleic acid (18:2n-6) (5% of diet) [68], whereas in salmon, better performances were obtained with dietary EPA + DHA levels between 2.7 and 3.4% of total fatty acids [69] and concentrations of linoleic acid (18:2n-6) lower than 1% [64]. However, beyond the AA and FA profiles, several other factors could be responsible of the reduced fish growth including nutrient digestibility and antinutritional factors. In this regard, a recent study reported that salmon fed a diet with a mix of soy protein concentrate (30%) and poultry meal (6%) or a diet with 58% poultry meal showed reduced apparent digestibility of crude protein, amino acids, and lipids as compared to fish receiving FM-based diet [70]. Moreover, the same study showed that certain plant protein ingredients, such as soybean meal and soy protein concentrate, increased faecal water content in the distal intestine creating a diarrhoea-like condition that impaired gut function and reduced fish growth.

Even more interesting were the results obtained from our metagenomic analysis. Up to date, several studies have used cutting edge technologies, such as NGS, to evaluate the effect of substitution of FM with plant proteins on fish intestinal microbiota [43,44,71–73]. However, to the best of our knowledge, the present study is one of the very few researches to have investigated the effects of a diet with alternative terrestrial animal protein sources on fish gut microbiome [45,59,60] and the first one in rainbow trout. We analysed trout intestinal microbiome was by means of Illumina MiSeq sequencing of 16S rRNA gene. Fish used in our research were all female, obtained from a single supplier and grown under the same environmental conditions of an aquaculture facility, thereby limiting the variations due to environment and sex. Although diet is one of the main factors affecting the intestinal microbial composition of vertebrates, including fish, gut microbiota is also affected by fish developmental stage, gender, and farming conditions [28,74,75].

In line with previous studies on rainbow trout, our results indicated that gut microbiota of this species was dominated by *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* taxa. These phyla usually constitute the "core gut microbiota" of rainbow trout regardless of the diet type [37,38,43,44,71,74]. Actually, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* represent up to 90% of fish intestinal microbiota in different marine and freshwater species [40,45,76,77]. The presence of similar bacterial taxa in the gut microbiota of multiple fish species indicates that these bacteria are involved in important host gut functions, such as digestion, nutrient absorption, and immune response [37]. However, recent studies reported that

Tenericutes were the prominent phylum, being *Mycoplasma* the dominant genus in the distal intestinal microbiome of rainbow trout [73,78]. In our study, *Mycoplasma* was detected in all samples, but the quantification was often several magnitudes lower than the other genera examined. As suggested by Harviksen *et al*. [60] it may be due to the difficulty in extracting DNA from bacteria with no cell wall.

The number of reads per sample did not differ between groups and no overall effect on bacterial richness and diversity was observed in response FM substitution with different protein blends. Similarly, replacing FM with a mix of terrestrial animal and plant proteins did not induce significant changes in gut microbial richness, alpha diversity indices, and observed number of species in Asian seabass (*Lates calcarifer*) [45]. In salmon, instead, the observed species parameter of alpha diversity metric presented higher value in fish fed poultry meal-based diet than in fish fed a control FM-based diet, whereas, in agreement with our study, Shannon's diversity index did not show significant differences between dietary groups [59]. The lack of an effect on bacterial diversity should be considered as a positive result since the reduction in diversity may provide less competition for opportunistic or invading pathogens, which could thus easily colonize the gastrointestinal tract of fish [45].

Although all the rainbow trout used in this nutritional study showed similar intestinal bacterial communities, the relative abundance of several taxa displayed a significant statistical interaction with the diet. Both weight and unweighted UniFrac PCoA of bacterial communities revealed a relationship between diet type and microbiota associated to fish intestine, showing clustering of samples by diet, especially in the PCoA plot of the unweighted UniFrac data. However, some groups showed greater dispersion than others did. This was an expected result given that large individual variations even between fish of similar genetic background fed with the same diet and maintained under the same environmental conditions, has been described in previous reports [42,44,79].

Several studies have demonstrated the impact of marine versus terrestrial plant-derived ingredients on gut microbiota of rainbow trout [11,38,43,44]. These studies revealed that plant ingredients in the diet were often associated with a higher *Firmicutes*:*Proteobacteria* ratio in comparison to FM-based diet, which favoured instead, the presence of *Proteobacteria*. The inclusion of at least 25% of plant proteins in the diet of our fish favoured the presence of genera from the *Firmicutes* phylum regardless of the content level of animal proteins. Conversely, gut microbiota of fish fed diet G, with the lowest plant protein percentage (20%) and the highest content of animal proteins (80%), was found to be rich in *γ-Proteobacteria*. Similarly, previous studies in trout reported that the presence of *Proteobacteria* was favoured by an animal protein-based diet [38,43,44]. Different genera of lactic acid bacteria such as *Streptococcus*, *Lactobacillus*, *Leuconostoc*, and *Carnobacterium* belonging to *Firmicutes*, constitute a normal part of the intestinal microbiota of fish and are generally considered beneficial microorganisms associated with a healthy intestinal epithelium [80,81]. These bacterial genera, indeed, were used as probiotics for fish as well as for other vertebrates [82–85]. Several genera belonging to *Lactobacillales* and *Clostridiales*, orders were significantly affected by feeding formulations tested in our study. This was in line with recent literature data reporting that, although the microbiota composition of cultured rainbow trout was very resistant to diet changes, dietary variations were associated with changes in the relative abundance of *Lactobacillaceae*, *Streptococcaceae*, *Staphylococcaceae*, and *Clostridiales* [71]. In particular, the relative abundance of bacteria belonging to *Streptococcaceae*, *Enterococcaceae*, *[Tissierellaceae]*, and *Carnobacteriaceae* families varied between our feeding groups. *Lactobacillales* order was highly represented in the intestine of trout fed diet E (control) and F as well as in fish fed diets A, C, and D. Conversely, bacteria belonging to this order were present, to a lesser extent, in faecal samples of fish fed with diets B and G. Similarly, digesta (faecal) samples of Atlantic salmon fed a diet containing

soy protein concentrate (30%) and poultry meal (6%), as partial replacements of FM, presented significantly higher abundance of *Lactobacillales* genera *Streptococccus*, *Carnobacterium*, and *Lactococcus* [59]. In the same study, in accordance with our results, fish fed with a high percentage of poultry meal (58%) showed higher abundance of *γ-Proteobacteria*. In a previous study, in salmon, PBM inclusion led instead to a significant increase of *Corynebacteriaceae* and a significant decrease of *β-Proteobacteria*, *Bacilli*-like, *Streptococcaceae*, and *Peptostreptococcacea* in allochthonous bacterial community in comparison to a FM-based control group, whereas in autochthonous community, dietary PBM caused an increase in *Corynebacteriaceae* and *Streptococcaceae* [60]. In our study, we found a significant enrichment of *Corynebacteriaceae* family, represented by genus *Corynebacterium*, only in trout fed with diet D. It is interestingly to note that intestinal microbiome of group B, which showed the worst performances in terms of growth and feeding efficiency, was characterized not only by scarce amount of lactic acid bacteria but also by low abundance of bacteria assigned to *Clostridiales*. These differences could partly explain the poor growth performances observed in this fish group. In European sea bass (*Dicentrarchus labrax*), for example, changes in the composition of cecal microbiota deeply influenced weight gain, suggesting the involvement of bacterial community in energy harvesting from feed [86]. Actually, members of *Streptococcaceae*, *Lactobacillaceae*, *Enterobacteriacea* and [*Tissierellaceae*] families include several bacterial species that participate to anaerobic degradation of complex carbohydrates. The end products of such degradation are short chain fatty acids (SCFAs), which are then readily absorbed by the host thus contributing to the more efficient food energy utilization [87–90].

Fusobacterium genus was enriched in the intestine of trout fed diet D in comparison to all other dietary groups. It is known that bacteria belonging to *Fusobacteria* phylum can excrete butyrate [91] and synthesize vitamins [81]. Among the SCFAs, butyrate is considered the most important due to its numerous positive and well-documented effects on the health of intestinal tract and peripheral tissues in vertebrates [87,92–94]. Butyrate has, indeed, anti-inflammatory properties and the potential to stimulate the immune system [95–99]. For these reasons, we hypothesized that the intestinal presence of *Fusobacterium* could exert a beneficial effect on fish health. Actually, trout fed on diet D grew well and showed good feed efficiency parameters, although FM content in their diet was only at 11%. Therefore, a positive effect due to their gut microbiota composition could be reasonably hypothesised. These data represent a contribution if we consider that up to date, no other studies have established which are the microbial taxa that play a dominant role in SCFAs production in fish. Moreover, if we limit the comparison between gut microbiota only to groups G and F (trout fed the two formulations with the highest percentage of animal by-product meals), an adequate number (above 0.5%) of bacterial genera assigned to *Carnobacteriaceae*, *Streptococcaceae*, and *Enterococcaceae* families were found only in trout fed with diet F. Unweighted UniFrac PCoA analysis clearly showed that intestinal microbiome profile of fish fed diet F was more similar to that of control fish (diet E) than to other groups. This is a promising and encouraging result toward the use of animal by-product meals in aquaculture. On the other hand, although the severely reduced amount of *Lactobacillales* in fish fed diet G did not negatively affect SGR and FCR, it could have influenced the susceptibility to pathogens or opportunistic bacterial species. Indeed, microbiota of this group was dominated by *γ-Proteobacteria*, mainly represented by members belonging to *Aeromonadales* and *Vibrionales* orders, which include potential pathogen genera, such as *Photobacterium* and *Aeromonas.* Furthermore, the presence in the same fish group of an imbalanced microbiota, in which *Proteobacteria* phylum represented the dominant clade, could alter immune regulatory functions of the gut and contribute to development of diseases [100].

Conclusions

In summary, taken together, our data revealed that animal by-product meals, particularly PBM, could be a valid alternative protein source for aquafeed production. These ingredients do not negatively affect fish growth performances, but rather could reduce the negative impacts of high inclusion rates of dietary plant proteins on fish growth. Adding PBM to trout diet introduced no changes in the total microbial diversity or richness. Changes to the intestinal microbiome composition that we found were actually due to the ratio between vegetable and animal proteins regardless of the animal proteins sources. In particular, intestinal abundance of specific taxa belonging to *Firmicutes* and *Proteobacteria* was discriminatory for diet type in trout. Among tested diets, formulation D provided the best results in terms of percentage of FM replacement, growth performance, and intestinal microbiota composition, whereas experimental feed B and commercial feed G had an adverse effect on the gut microbial community by reducing the abundance of *Lactobacillales*. By manipulating fish diet, it is possible to obtain positive effects on the composition of gut microbiota and, hence, on the host's physiology. However, further experiments are needed to elucidate which are the feed ingredients that have the highest impact on the gut microbiota changes.

Supporting information

S1 Fig. Alpha diversity metrics. Rarefaction curves of faecal microbial communities from trout fed different diets. (**A**) Observed species, (**B**) species richness (Chao1), (**C**) Shannon's diversity index. Data points represent the mean values (n = 4).
(TIF)

S2 Fig. The common core microbiota. The *x*-axis represents the percentage of prevalence in all samples (n = 28) regardless of the diet type, the *y*-axis represents the number of shared OTUs.
(TIF)

S1 Table. Result of Tukey-Kramer post-hoc test on relative abundance data of phyla, classes, orders, families and genera that were influenced by the diet. Significance codes: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.
(DOCX)

S1 Dataset. OTU table generated by QIIME pipeline.
(XLSX)

S2 Dataset. Core microbiota, list of shared OTUs at 80% and 100%.
(XLSX)

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SUPPORTING INFORMATION

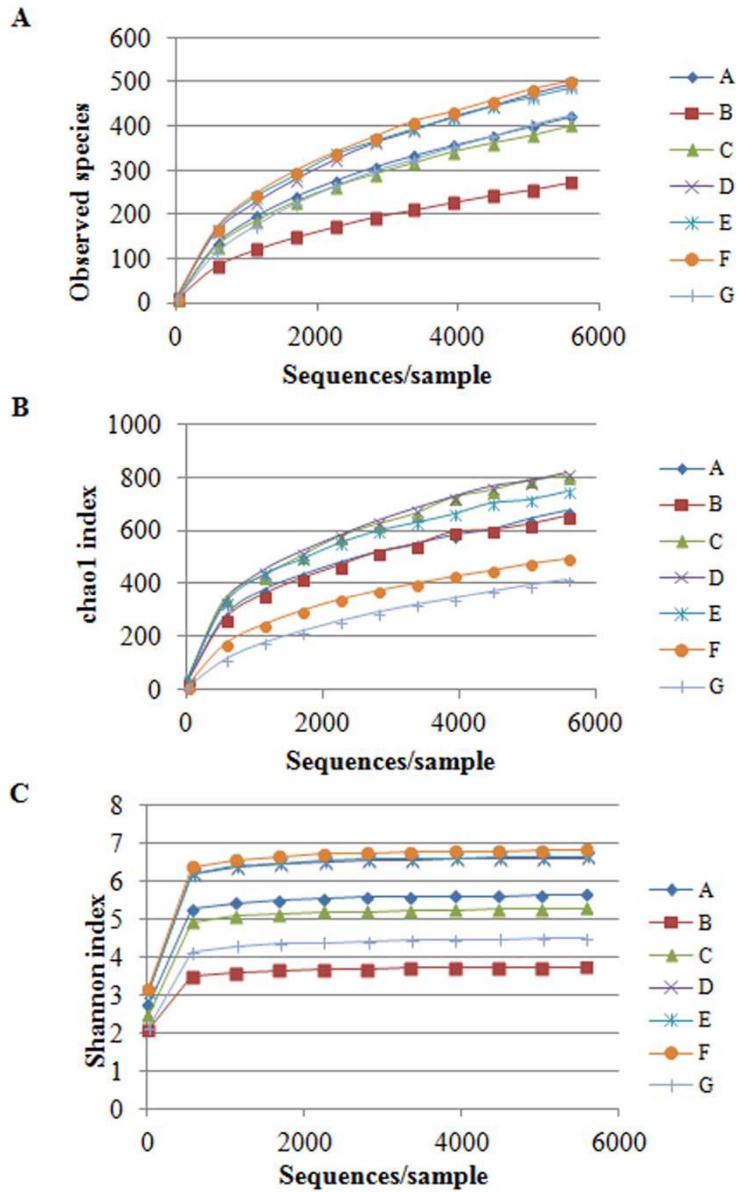


Fig S1. Alpha diversity metrics.

Rarefaction curves of faecal microbial communities from trout fed different diets. (A) Observed species, (B) species richness (Chao1), (C) Shannon's diversity index. Data points represent the mean values (n = 4).

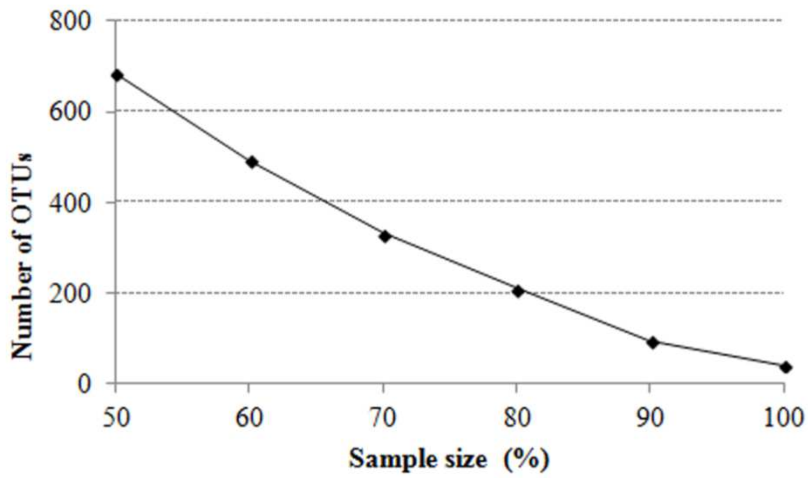


Fig S2. The common core microbiota.

The x-axis represents the percentage of prevalence in all samples (n = 28) regardless of the diet type, the y-axis represents the number of shared OTUs.

S1 Table Result of Tukey-Kramer post-hoc test on relative abundance data of phyla, classes, orders, families and genera that were influenced by the diet. Significance codes: *P < 0.05, **P < 0.01, ***P < 0.001.

	A:D	A:E	A:F	A:G	B:D	B:E	B:F	C:B	C:D	C:E	C:F	C:G	D:E	D:F	D:G	E:F	E:G	F:G
Phylum																		
Fusobacteria	**				***				*			*	***	***	***			
Bacteroidetes	*				***							*	*	***	***			
Class																		
Erysipelotrichia			***				***				***			***		***		***
Flavobacteriia	**		**		***	*	***		**		*				***		**	***
Fusobacteriia	**				***				*			*	***	***	***			
Bacteroidia	*				***							*	*	***	***			
Order																		
Erysipelotrichales			***				***				***			***		***		***
Flavobacteriales	**		**		***	*	***		**		*				***		**	***
Xanthomonadales	*		***		**		***				***		*		**	***		***
Fusobacteriales	**				***				*			*	***	***	***			
Bacteroidales	*				***							*	*	***	***			
Enterobacteriales			**		*		***				**							**
Pasteurellales	*				**								**		***			
Family																		
Enterococcaceae			***			*	***				***			***		***		***
Erysipelotrichaceae			***				***				***			***		***		***
Xanthomonadaceae	*		***		**		***				***		*		**	***		***
Fusobacteriaceae	**				***				*			*	***	***	***			
Flavobacteriaceae	**		*		***		**		**		*				***		*	***

Porphyromonadaceae	*				**							*		***	***			
Corynebacteriaceae	***				***				**				**	*	***			
[Mogibacteriaceae]								*		**	***	***		*	*			
Bacteroidaceae	*				**								**	**	***			
[Tissierellaceae]				*	*			*			**	**		*	**			
Enterobacteriaceae			**		*		***				**							**
Pasteurellaceae	*				**								**		***			
Streptococcaceae				*								*			*		**	**
Pseudomonadaceae			*				**				**							*
Genus																		
Vagococcus			***				***				***			***		***		***
Lactococcus		***	***			***	***			***	***		***	***			***	***
Erysipelothrix			***				***				***			***		***		***
Sporanaerobacter		***			*	***				***			**	*	**	***	***	
Tepidimicrobium		*	*	*	***					*	*	*	***	***	***			
Wohlfahrtiimonas			***		**		***				***		*		**	***		***
Fusobacterium	**				***				*			*	***	***	***			
Granulicatella			***				***				**			***		***		***
Porphyromonas	*				**							*		***	***			
Myroides	**				***		*		**						***		*	**
Proteus			**		**		***		*		**		*		**	**		***
Helcococcus					*			*		*	**	**	*	**	**			
Corynebacterium	***				***				**				**	*	***			
Bacteroides	*				**								**	**	***			
pH2								**	*	**	***	***						
Enterococcus			**				***				**			**				*
Pasteurella	*				**								**		***			
Peptoniphilus				*	*						*	*		*	**			

CHAPTER 3

EFFECTS OF DIETS SUPPLEMENTED WITH AN AUTOLYZED YEAST EXTRACT SELECTED FOR USE IN THE ANIMAL FEED INDUSTRY ON THE INTESTINAL MICROBIOME OF A MARINE FISH SPECIES: GILTHEAD SEA BREAM (*SPARUS AURATA*)

In summary, the present study aimed to investigate the effects of partial replacement of dietary fishmeal with a commercial feed additive named HiCell, consisting of autolyzed yeast from *Saccharomyces cerevisiae*, on the gut microbiota richness and composition, and on the growth and feeding efficiency parameters of the marine species gilthead sea bream (*Sparus aurata*). HiCell (producted by Biorigin Europe, a company specialized in the production of feed additives) is an autolyzed dry yeast obtained from the fermentation of a strain of *Saccharomyces cerevisiae*, selected for use in the animal feed industry.

Single cell proteins are actually being used in aqua feeds as fishmeal replacements. Autolyzed yeast seems a better choice than yeast because the autolysis process hydrolyses proteins into oligo-peptides or free amino acids and degrades yeast cell walls thus increasing bioavailability of yeast content (Tacon, 2014). Indeed, HiCell product results a good source of highly digestible proteins and has an excellent amino acids profile. Moreover, as described in chapter 1, yeast cell wall contains molecules that can be very efficient in protecting aquatic animals either by directly stimulating the immune system or by binding pathogens. Finally, yeast extracts, which represents the content of autolyzed yeast deprived of yeast cell walls, are potentially very good feed attractants for fish (Tacon, 2014).

To achieve this goal, we used Illumina MiSeq High-throughput sequencing of 16S rRNA gene.

3.1 Materials and methods

3.1.1 Feeding trial and diets

About 800 gilthead sea bream, with a mean initial weight of 107 ± 2.5 g, were randomly distributed into 9 experimental tanks connected to a flow-through fish culture system. Experimental tanks were supplied with degassed ground water with an approximately constant temperature of 24.5°C.

Three dietary formulations (A, B and C, table 3.1) were produced by Naturalleva VMR S.r.l. (Italy). The diet based on commercial fishmeal (FM)/vegetable meal containing 46% crude protein and 16% fat) was used as the control diet (A). The other two diets were characterized by FM replacement with fish hydrolysate (diet B) and with the commercial additive HiCell (diet C). In tables 3.2 and 3.3 the proximal composition and the amino acid content of the diets, respectively, are shown. Data in the tables represent analyzed values and the company that carried out the analysis was Naturalleva VMR S.r.l. (Italy). Fish were fed with the experimental diets in triplicate (3 tanks/diet) for 90 days.

During the study, collective weights were assessed monthly and used to calculate the Specific Growth Rate (SGR = 100 (ln final weight - ln initial weight) / (t2−t1)) and Feed Conversion Ratio (FCR = Total feed supplied / (Final weight – Initial weight)) for each dietary fish group.

At the end of the feeding trial, intestines of five fish from each tank (fifteen fish/dietary group) were aseptically removed and the fecal content was collected by squeezing out and scrapping the intestinal

mucosa with a sterile spatula, in order to collect the luminal and the mucosa associated microbiota.

Table 3.1 Experimental diets formulation.

Ingredients (%)			
	Diet A	**Diet B**	**Diet C**
Fishmeal	22	18	18
Fish protein, hydrolysate	-	5	-
HiCell® - autolysed yeast	-	-	5
Fish oil 92	7	7	7
Soybean meal	11	11	11
Guar germ meal	15	14	18
Wheat middling	7	9	7
Corn gluten meal	18	17	18
Pea meal	4	4	2
Soy Protein Concentrate	9	10	10
Rapeseed oil	1	1	1
***Camelina sativa* oil**	2	2	2
Mineral / Vitamin supplement	2	2	2
	100	**100**	**100**

Table 3.2 Experimental diet proximal composition.

Proximal composition (g/kg diet)			
	Diet A	**Diet B**	**Diet C**
Protein	46.02	46.06	46.07
Fat	16.20	16.08	16.07
Ash	6.18	5.82	6.00
Crude Fibre	2.05	2.04	2.16
Gross Energy	18.70	18.80	18.60
Digestible Energy	17.16	17.25	16.81
Digestible protein	40.90	40.96	40.75
EPA - $C_{20}H_{30}O_2$	0.58	0.55	0.52
DHA - $C_{22}H_{32}O_2$	0.50	0.51	0.47
n-3/n-6	1.03	1.02	0.97
DHA/EPA	0.86	0.94	0.89

Table 3.3 Aminoacid composition of the experimental diets.

Aminoacid	Diet		
	A	B	C
Cysteine	0.5	0.5	0.45
Methionine	1.35	1.38	1.35
Aspartic acid	4.11	4.09	4.12
Threonine	1.84	1.83	1.85
Serine	2.13	2.15	2.13
Glutamic acid	7.74	7.68	7.74
Proline	2.49	2.5	2.44
Glycine	2.24	2.35	2.15
Alanine	2.65	2.64	2.62
Valine	2.23	2.2	2.24
Isoleucine	1.85	1.84	1.87
Leucine	4.13	4.06	4.09
Tyrosine	1.65	1.64	1.65
Phenylalanine	2.35	2.31	2.39
Lysine	2.54	2.59	2.56
Histidine	1.15	1.13	1.17
Arginine	2.97	2.97	2.93
Tryptophan	0.41	0.42	0.41

3.1.2 Microbial DNA extraction and microbiome analysis

Microbial DNA was extracted from 250 mg of feces from each fish. Feces were added to plastic tube containing beads provided in the DNeasy PowerSoil Kit (Qiagen, Italy) and then shaken with Qiagen TissueLyser II for 2 min at 25 Hz. Next extraction steps were carried out following the instructions of the kit. All the extraction process was done in parallel without addition of any biological sample as negative control. The extracted DNA concentration was measured by using NanoDrop^{TM} 2000 Spectrophotometer (Thermo Fisher Scientific, Italy)

and DNA was then stored at -20°C until analysis.

3.1.3 Amplification of 16S rRNA gene and library preparation

For the characterization of the whole gut microbiome of gilthead sea bream, we used Illumina MiSeq high-throughput sequencing of the 16S rRNA gene. The first step of the analysis was the amplification of the V3–V4 regions of the 16S rRNA gene in a 25 µl reaction volume containing 500 ng of the extracted bacterial DNA, buffer 10X, dNTPs 0.2 mM, $MgSO_4$ 1.5 mM, forward (5'AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTAC ACGACGCTCTTCCGATCTCCTACGGGAGGCAGCAGCCTACGG GNBGCASCAG-3') and reverse (5'CAAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGA GTTCAGACGTGTGCTCTTCCGATCTGACTACNVGGGTATCTA ATCC-3') primers (400 nM each), and Platinum Taq DNA Polymerase High Fidelity 1U (Thermo Fisher Scientific, Italy). Both primers were selected by Takahashi et al. (2014) and contain the Illumina adapters at 5' end. After DNA purification, a library of microbial communities was produced as described in Rimoldi et al. (2018) (chapter 2 of this thesis).

3.1.4 Output data analysis

The analysis of data for the taxonomic classification and analysis of bacterial diversity were preprocessed (quality control) with QIIME (Quantitative Insights into Microbial Ecology) pipeline (Caporaso et al., 2010). Reads possessing an average quality score of <25 or with lengths shorter than 36 bases, were removed. The remaining high-quality sequences were grouped by diet based on their barcodes and the sequences with 97% or higher identity, were binned into Operational Taxonomic Units (OTUs). Representative sequences from each OTU

were then aligned and compared with Greengenes database (http://greengenes.lbl.gov). OTUs assigned to *Chloroplast* class (*Cyanobacteria* phylum), *Rickettsiales* order, and *Mitochondria* family were excluded from the analysis being considered plant-derived contaminants.

Alpha and beta diversity statistics were carried out as described in Rimoldi et al. (2018). The alpha diversity metrics included observed OTUs and Chao1 index (species richness estimator), PD whole tree, Shannon and Simpson index. Beta diversity metrics were indicated by weighted and unweighted UniFrac distance matrices, which are indexes of bacterial community diversity among experimental groups.

The common core microbiome (OTUs shared regardless of the diet and found in at least 9 out of the 15 samples per dietary group) was identified and visualized by a Venn diagram drawn using the web tool http://bioinformatics.psb.ugent.be/webtools/Venn/.

3.1.5 Statistical analysis

All statistical analyses were performed using STATISTICA v.7 (StatSoft, Inc). Statistical significance was set at P-value < 0.05.

The number of reads for each sample was normalized and the relative abundance of each taxon was calculated. Only taxa with an overall abundance of more than 1% at phylum, class, and order level, and more than 0.5% at family, genus and species level, were considered for statistical analysis. Significant differences in the relative abundance of gut bacterial community components were obtained by non-parametric Kruskall-Wallis H test (or one-way ANOVA on ranks). For the alpha–diversity metrics indices (observed OTUs, PD whole tree, Chao1, Shannon and Simpson index) one-way ANOVA analysis was used, followed by Tukey HSD post hoc test.

3.2 Results

3.2.1 Fish growth performances

At the end of the feeding trial, gilthead sea bream of the three dietary groups doubled their body weight, showing an efficient growth despite the substitution of FM with HiCell product. Moreover, fish growth performances indexes, such as SGR and FCR (Table 3.4), did not reveal statistically significant differences between the control and the other two dietary groups.

Table 3.4 Growth and feed indices.
Legend: BWi – Initial body weight, BWf – Final body weight, BWg – body weight gain, SGR – specific growth rate, FCR – feed conversion rate. The values are reported as mean ± SD. No statistically differences among groups are detected ($p < 0.05$).

	Diet A	**Diet B**	**Diet C**
BWi (g)	107.35 ± 2.75	107.08 ± 3.07	106.67 ± 2.85
BWf (g)	245.95 ± 9.61	243.56 ± 3.14	237.54 ± 9.26
BWg (g)	138.60 ± 9.53	136.48 ± 0.58	130.88 ± 8.77
SGR	0.71 ± 0.06	0.68 ± 0.02	0.62 ± 0.06
FCR	1.51 ± 0.08	1.53 ± 0.00	1.58 ± 0.06

3.2.2 Microbiome sequencing

The number of reads taxonomically classified according to the Greengene database was 2,327,049.00 corresponding to an average number of 51,712.2 ± 15,620.2 reads per sample (Table 3.5). One hundred and two OTUs at 97% identity were identified in gilthead sea bream fecal samples collected at the end of feeding trial. The relative abundance (%) of each bacterial taxon was determined for every individual fish (45 bacterial community profiles) and for every

experimental group (3 bacterial community profiles). Good's coverage in all the three dietary groups reached a value of 0.99 (Table 3.5), indicating that the sequencing coverage was achieved and that the OTUs found in the samples were representative of all gut microbial communities.

Different alpha diversity metrics were utilized to calculate-diversity (Table 3.5). The minimum number of reads was fixed at 19,500 in order to normalize taxa counts based on OTUs number observed in the experimental samples. In particular, microbial richness index (Chao 1) that estimates the number of rare classes found in a sample, was not affected by diet type (Fig 3.1A), whereas phylogenetic diversity characterized by the "PD whole tree value" resulted significantly higher in samples of group C than in those of group B (Fig 3.1B). Shannon index was instead significantly higher in the group A than in C (Table 3.5).

Table 3.5 Alpha diversity metrics of gilthead sea bream gut microbial communities. Number of reads, observed OTUs, Good's coverage, PD whole tree, species richness (Chao1), Shannon's diversity index and Simpson index are reported as mean values (n=15) ± SD. The means were compared by ANOVA. Different superscript letters on the same column indicate significant differences after post-hoc Tukey's test.

	Diet A	**Diet B**	**Diet C**
Reads	52,248 ± 14,709	49,553 ± 17,950	53,334 ± 15,413
Observed OTUs	53.0 ± 4.2	47.4 ± 8.8	49.0 ± 6.1
Good's coverage	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00
PD whole tree	6.1 ± 0.4^{ab}	5.4 ± 1.0^{b}	6.5 ± 0.9^{a}
Chao1	56.2 ± 4.9	49.6 ± 9.4	52.5 ± 7.9
Shannon	1.8 ± 0.2^{a}	1.7 ± 0.1^{ab}	1.6 ± 0.2^{b}
Simpson	0.5 ± 0.1	0.5 ± 0.0	0.5 ± 0.1

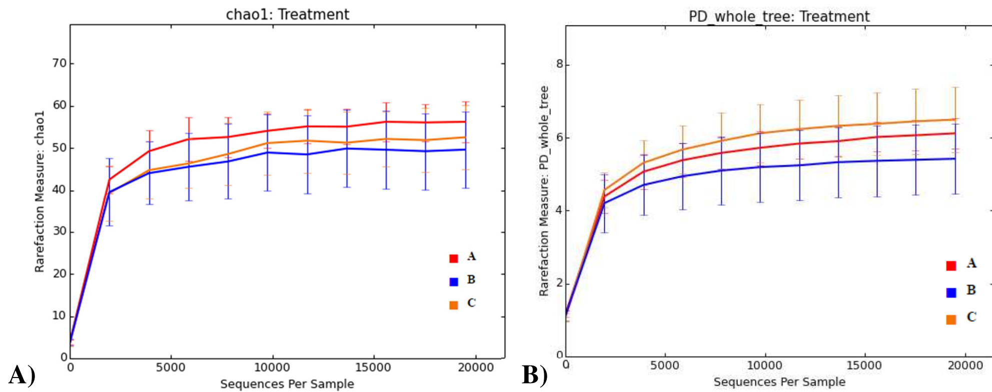


Figure 3.1 Alpha diversity metrics. Rarefaction curves of faecal microbial communities from gilthead sea bream fed different diets. (**A**) species richness (Chao1), (**B**) PD whole tree. Data points represent the mean values (n=15).

3.2.3 Microbial communities characterization

The microbial community structures for each fish and dietary group were outlined at the phylum, class, order, family, genus, and species level. The microbiome profile considering all samples comprised 7 different phyla, 10 classes, 19 orders, 29 families, 40 genera and 19 species.

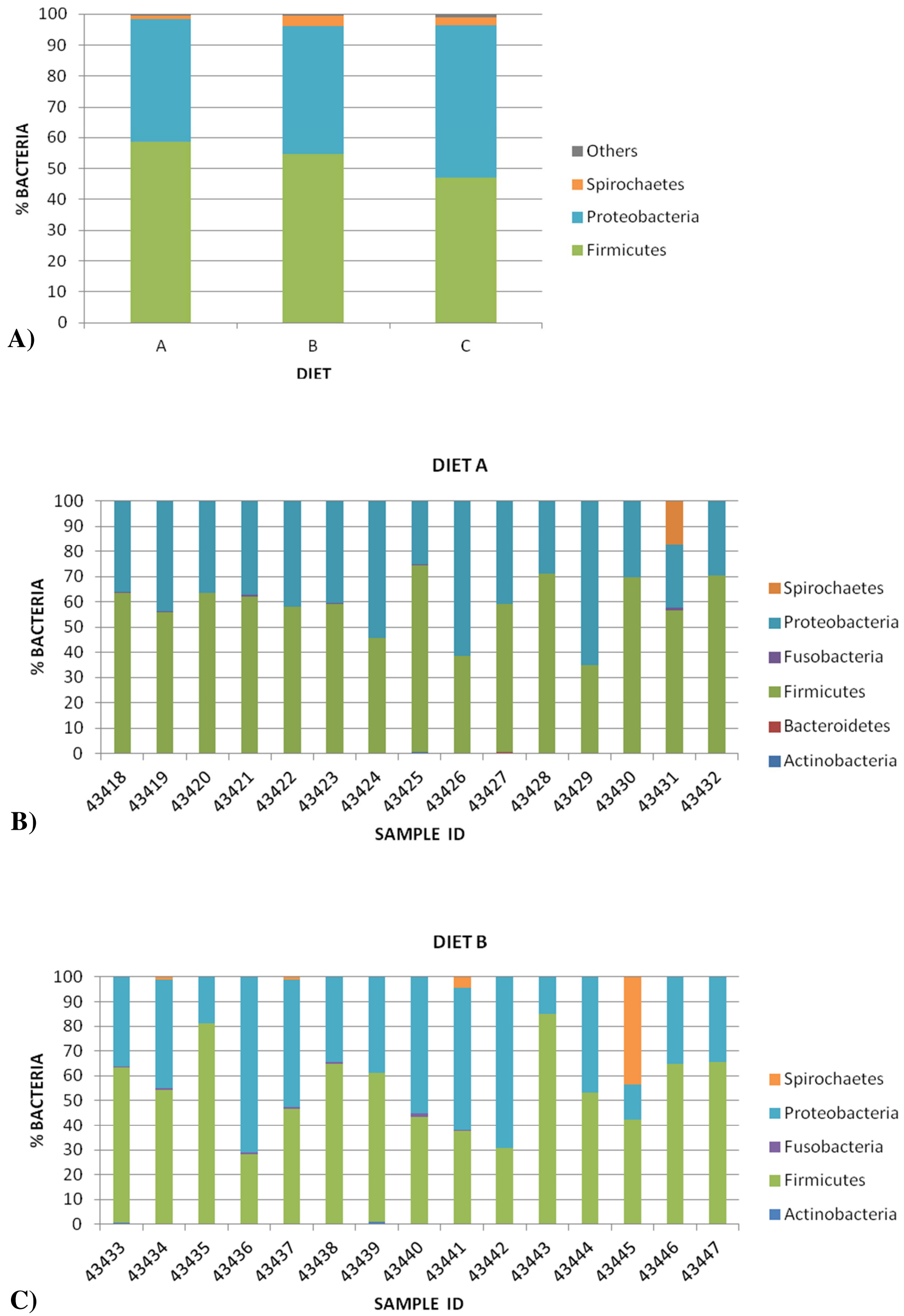
Gut microbiota of fish resulted mainly composed by two phyla *Firmicutes* (41-58%) and *Proteobacteria* (40-49%) (Fig 3.2), whereas *Bacteroidetes* were scarce in fish fed diet C or absent in fish fed diets A and B.

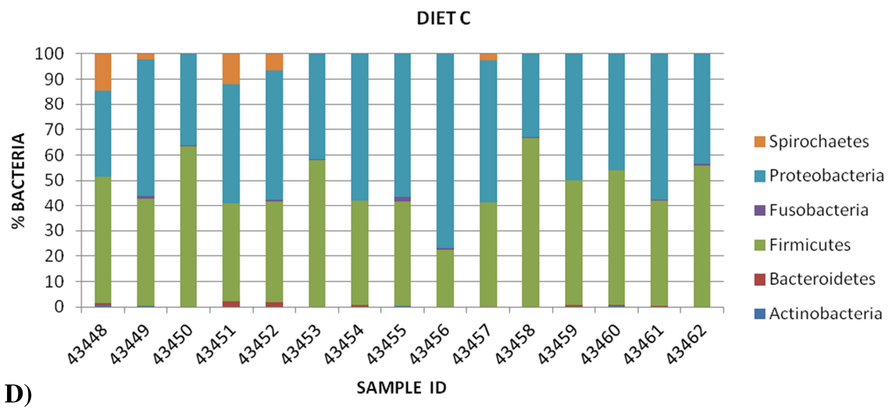
In figure 3.3 and 3.4 are shown the microbial communities for dietary groups and for individual fish at family and genus levels, respectively.

The statistical analysis (Kruskal-Wallis analysis) was performed on the most abundant taxa found in the three experimental dietary groups and in table 3.6 are reported the taxa that were significantly affected by diet.

At the species taxonomical level, the percentage of unassigned sequences was remarkable (comprised between 78-85%) for all analyzed samples, therefore statistical analysis was performed up to genus level.
The major differences in terms of relative abundance of bacterial taxa were found between the groups A (control diet) and C (HiCell-supplemented diet). For example, at order level (Table 3.6), the relative abundance of *Clostridiales* belonging to *Firmicutes* phylum, resulted significantly higher in the group C than in A ($p<0.05$), as well as *Prevotellaceae*, *Bacillaceae*, *Veillonellaceae* families ($p<0.001$, $p<0.01$, $p<0.001$ respectively). *Enterobacteriacea*e, instead, were higher in C ($p<0.001$) than in B group, whereas the percentage of bacteria assigned to this family in group A, was of an intermediate level. Conversely, *Lactobacillaceae,* represented by genus *Lactobacillus,* constituted the most abundant taxon (63-70%) found in our samples, irrespective of the diet. The genus *Photobacterium*, mainly represented by *Photobacterium damselae*, a marine bacterium of the family *Vibrionaceae*, resulted the second most abundant genus in all dietary groups (Fig. 3.4).
The most noticeable difference at genus taxonomical level (Fig 3.4; Table 3.6) regarded genera *Prevotella* and *Megasphera*, which were detected only in fecal samples from fish receiving HiCell (diet C). Furthermore, in comparison to other dietary groups, fish fed diet C showed also higher abundance of OTUs assigned to genus *Bacillus* ($p<0.01$). Conversely, diet B (containing fish hydrolysate) leaded to a significant decrease of bacteria belonging to *Shewanella* genus in comparison to diets A and C.
The core gut microbiota, i.e. OTUs present in at least 60% of fecal samples and shared regardless of the diet, was constituted by 14 OTUs

(Fig. 3.5), showing 8 OTUs belonging to Firmicutes and 6 to Proteobacteria phylum.

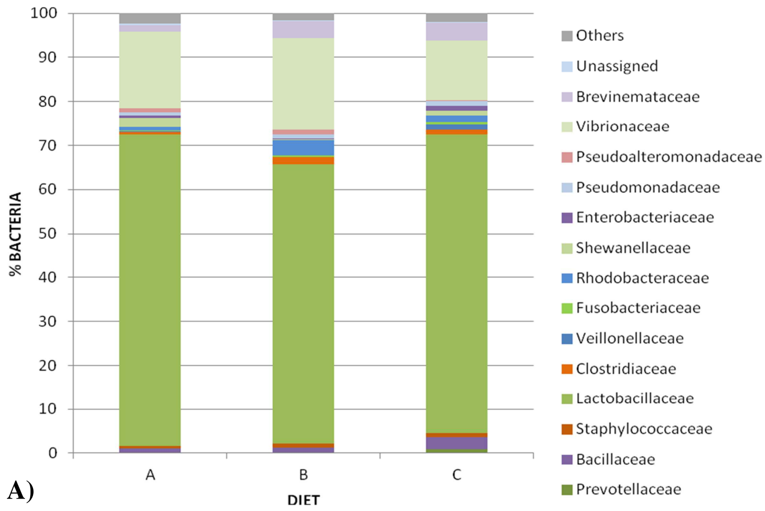




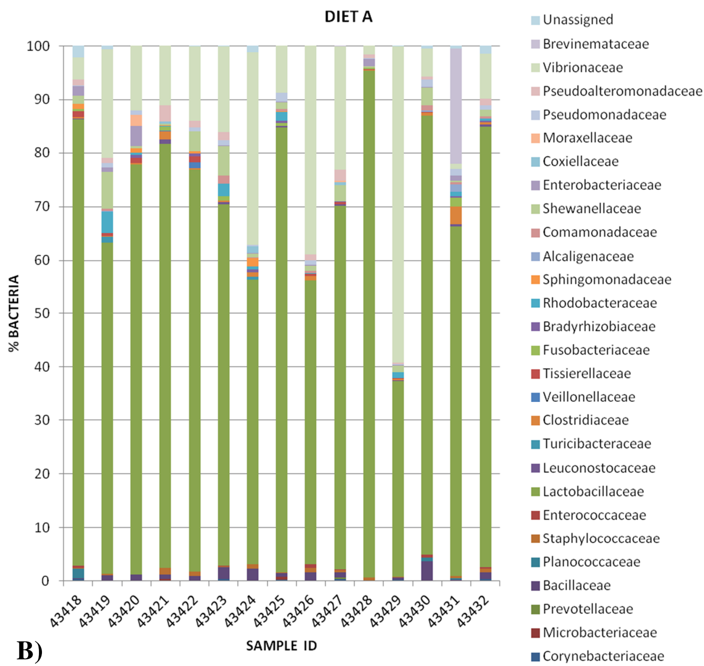
D)

Figure 3.2 Relative abundance (%) of the overall most prevalent bacteria in each dietary group (**A**) and in individual fish (**B, C, D**), at phylum taxonomical level.

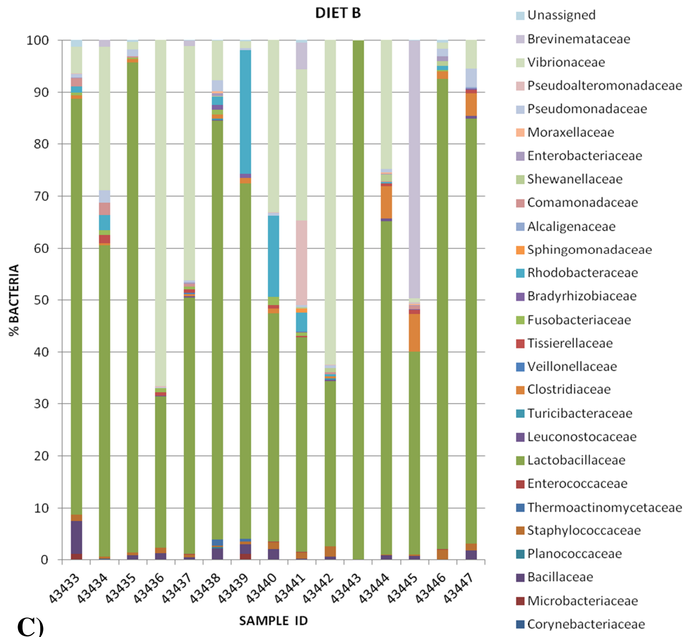
All bacteria with an overall abundance of ≥1% are reported, whereas bacteria with an abundance of ≤1% are regrouped and indicated as "Others".



A)



B)



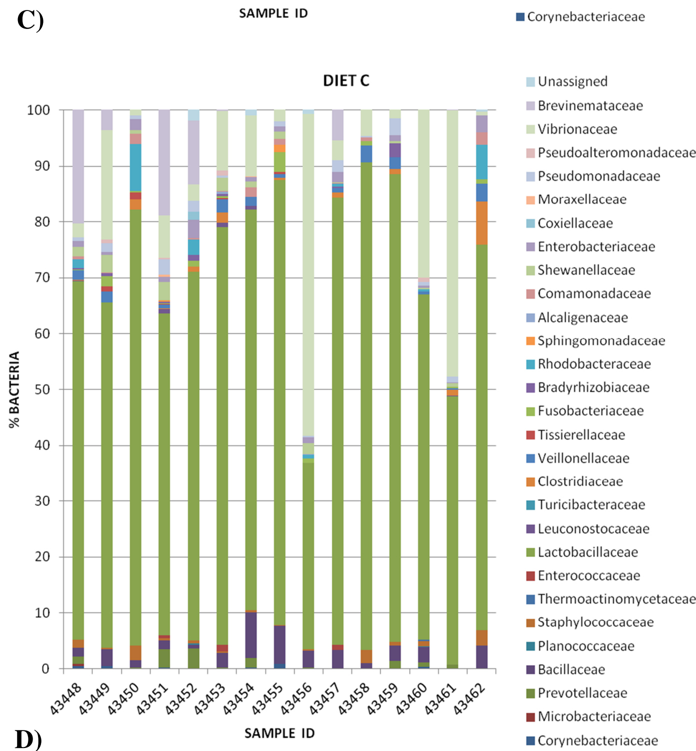
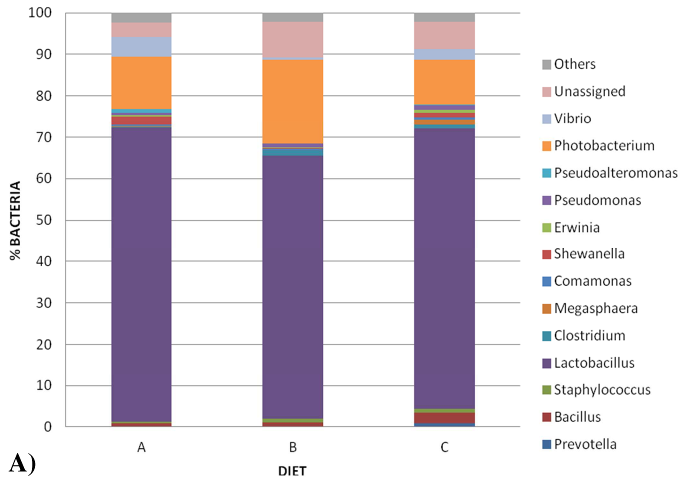
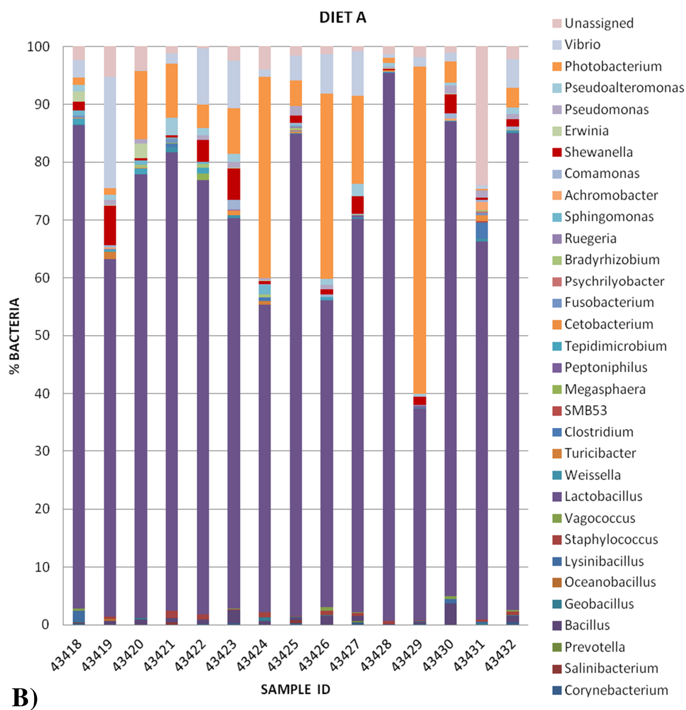


Figure 3.3 Relative abundance (%) of the overall most prevalent bacteria in each dietary group (**A**) and in individual fish (**B, C, D**), at family taxonomical level.

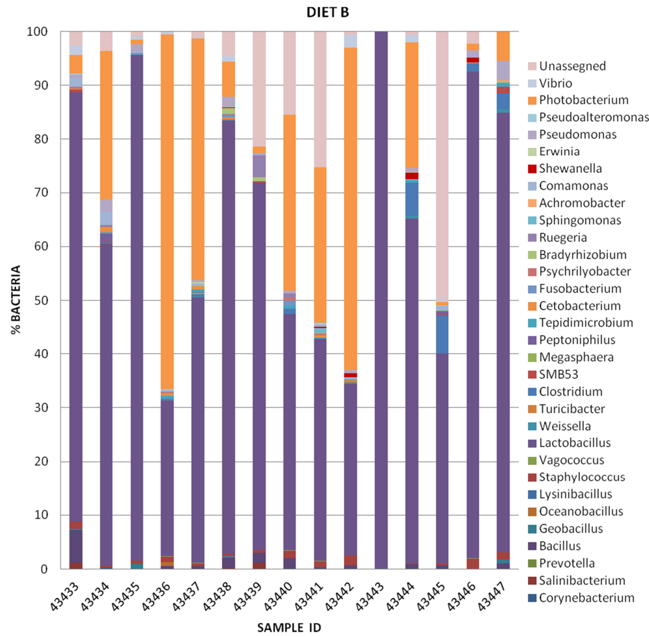
All bacteria with an overall abundance of ≥1% are reported, whereas bacteria with an abundance of ≤1% are regrouped and indicated as "Others".



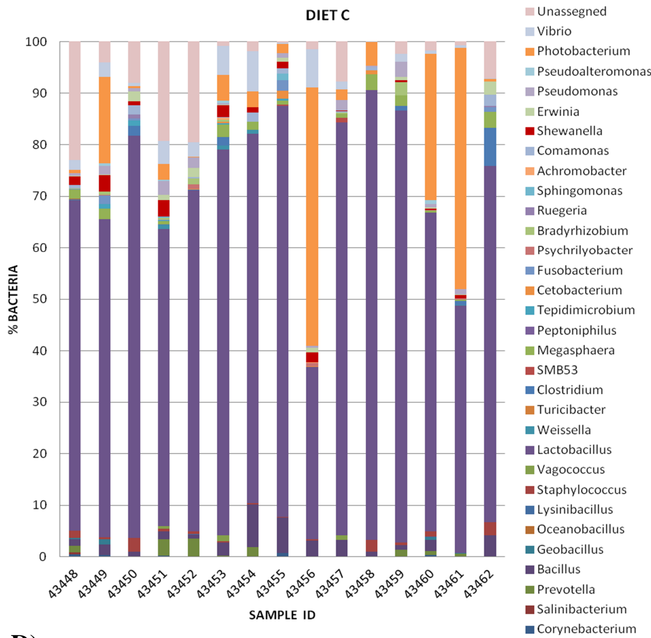
A)



B)



C)



D)

Figure 3.4 Relative abundance (%) of the overall most prevalent bacteria in each dietary group (**A**) and in individual fish (**B, C, D**), at genus taxonomical level.

All bacteria with an overall abundance of ≥1% are reported, whereas bacteria with an abundance of ≤1% are regrouped and indicated as “Others”.

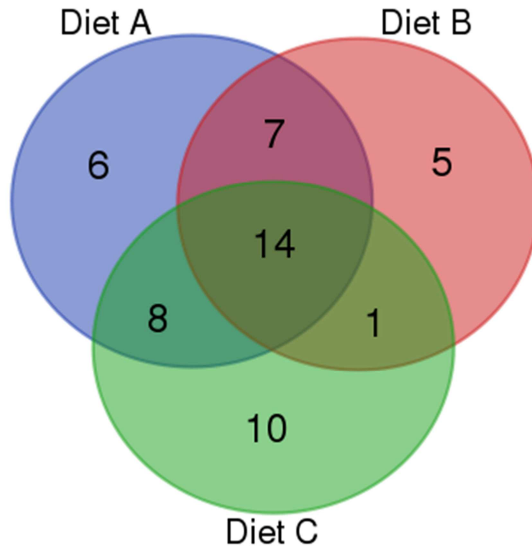


Figure 3.5 Venn diagram representing unique and shared OTUs among all dietary groups.

Table 3.6 Percentage of most abundant taxa (mean ± SEM) found in different dietary groups. "n.d." means not detected. Statistical significance: (*) p<0.05; (**) p<0.01; (***) p<0.001.

	A	**B**	**C**	***p*-value**
Phylum				
Firmicutes	58.6 ± 2.9	54.6 ± 4.3	41.5 ± 2.9	
Proteobacteria	39.7 ± 3.1	41.5 ± 4.5	49.4 ± 2.9	
Spirochaetes	1.1 ± 1.1	3.3 ± 2.8	2.5 ± 1.2	
Class				
Bacilli	57.9 ± 3.0^{a}	53.0 ± 4.4^{ab}	45.3 ± 2.7^{b}	*
Clostridia	< 1.0	1.6 ± 0.5	1.6 ± 0.4	
Alphaproteobacteria	21.8 ± 1.6^{b}	21.3 ± 2.2^{b}	37.5 ± 2.9^{a}	**
Gammaproteobacteria	17.6 ± 3.5	19.8 ± 5.3	11.5 ± 3.3	
Brevinematae	1.1 ± 1.1	3.3 ± 2.8	2.5 ± 1.2	
Order				
Bacillales	1.9 ± 0.2^{b}	2.3 ± 0.4^{ab}	3.7 ± 0.5^{a}	**
Lactobacillales	71.5 ± 3.9	63.9 ± 6.1	68.4 ± 3.6	
Clostridiales	< 1.0^{b}	2.0 ± 0.6^{ab}	2.5 ± 0.6^{a}	*
Rhodobacterales	< 1.0	3.3 ± 1.7	1.3 ± 0.6	
Alteromonadales	2.0 ± 0.5^{a}	< 1.0^{b}	1.1 ± 0.2^{a}	***
Enterobacteriales	0.6 ± 0.2^{ab}	< 1.0^{b}	1.1 ± 0.2^{a}	***
Pseudomonadales	0.8 ± 0.2	1.0 ± 0.2	1.0 ±0.2	
Vibrionales	18.2 ± 4.1	21.9 ± 6.1	13.6 ± 4.6	
Brevinematales	1.4 ± 1.4	3.8 ± 3.2	3.9 ± 1.2	
Family				
Prevotellaceae	< 0.5^{b}	n.d.	0.8 ± 0.3^{a}	***

Bacillaceae	1.1 ± 0.2^{b}	1.2 ± 0.4^{b}	2.8 ± 0.5^{a}	**
Staphylococcaceae	0.4 ± 0.1	0.8 ± 0.1	0.8 ± 0.2	
Lactobacillaceae	70.9 ± 3.9	63.6 ± 6.1	67.8 ± 3.7	
Clostridiaceae	0.5 ± 0.2	1.6 ± 0.6	1.0 ± 0.5	
Fusobacteriaceae	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.2	
Veillonellaceae	n.d.	n.d.	1.2 ± 0.2^{a}	***
Rhodobacteraceae	0.7 ± 0.3	3.3 ± 1.8	1.3 ± 0.6	
Shewanellaceae	2.0 ± 0.5^{a}	$< 1^{b}$	1.1 ± 0.2^{a}	***
Enterobacteriaceae	0.6 ± 0.2^{ab}	$< 0.5^{b}$	1.1 ± 0.2^{a}	***
Pseudomonadaceae	0.7 ± 0.1	1.0 ± 0.2	1.0 ± 0.2	
Pseudoalteromonadaceae	1.0 ± 0.2^{a}	1.1 ± 1.0^{b}	$< 0.5^{b}$	**
Vibrionaceae	17.2 ± 4.1	20.8 ± 5.9	13.5 ± 4.6	
Brevinemataceae	1.4 ± 1.4	3.8 ± 3.2	4.0 ± 1.8	
Genus				
Prevotella	n.d.	n.d.	0.8 ± 0.3^{a}	***
Bacillus	0.9 ± 0.2^{b}	1.5 ± 0.4^{b}	2.3 ± 0.6^{a}	**
Staphylococcus	< 0.5	0.8 ± 0.1	0.8 ± 0.2	
Lactobacillus	70.9 ± 3.9	63.6 ± 6.1	67.8 ± 3.7	
Clostridium	< 0.5	1.3 ± 0.6	0.8 ± 0.5	
Megasphaera	n.d.	n.d.	1.2 ± 0.3^{a}	***
Comamonas	< 0.5	< 0.5	0.6 ± 0.2	
Shewanella	2.0 ± 0.5^{a}	$< 0.5^{b}$	1.1 ± 0.3^{a}	***
Erwinia	$< 0.5^{ab}$	$< 0.5^{b}$	0.6 ± 0.2^{a}	**
Pseudomonas	0.7 ± 0.1	0.9 ± 0.3	1.0 ± 0.3	
Pseudoalteromonas	0.9 ± 0.2^{a}	$< 0.5^{b}$	$< 0.5^{b}$	***
Photobacterium	12.4 ± 4.2	20.2 ± 5.8	10.9 ± 4.4	
Vibrio	4.7 ± 1.3^{a}	0.5 ± 0.2^{b}	2.5 ± 0.7^{a}	***

3.2.4 Beta diversity metrics

The overall composition of intestinal microbial communities was compared using principal coordinate analysis (PCoA) that was performed by UniFrac based beta diversity analysis. Beta diversity is the difference in community structure between samples and it is represented by a distance matrix. A weighted and unweighted PCoA test was performed on the data. Both weighted and unweighted PCoA (Fig 3.6) showed a tendency of samples belonging to dietary group C to cluster separately from the other two groups. The permutational

multivariate analysis confirmed that groups A and B were more similar to each other than to group C (Table 3.7). Indeed, both ANOSIM and Adonis tests showed statistically significant differences ($p < 0.001$) only between group C and the other two groups, whereas there were not significant differences between group A and group B.

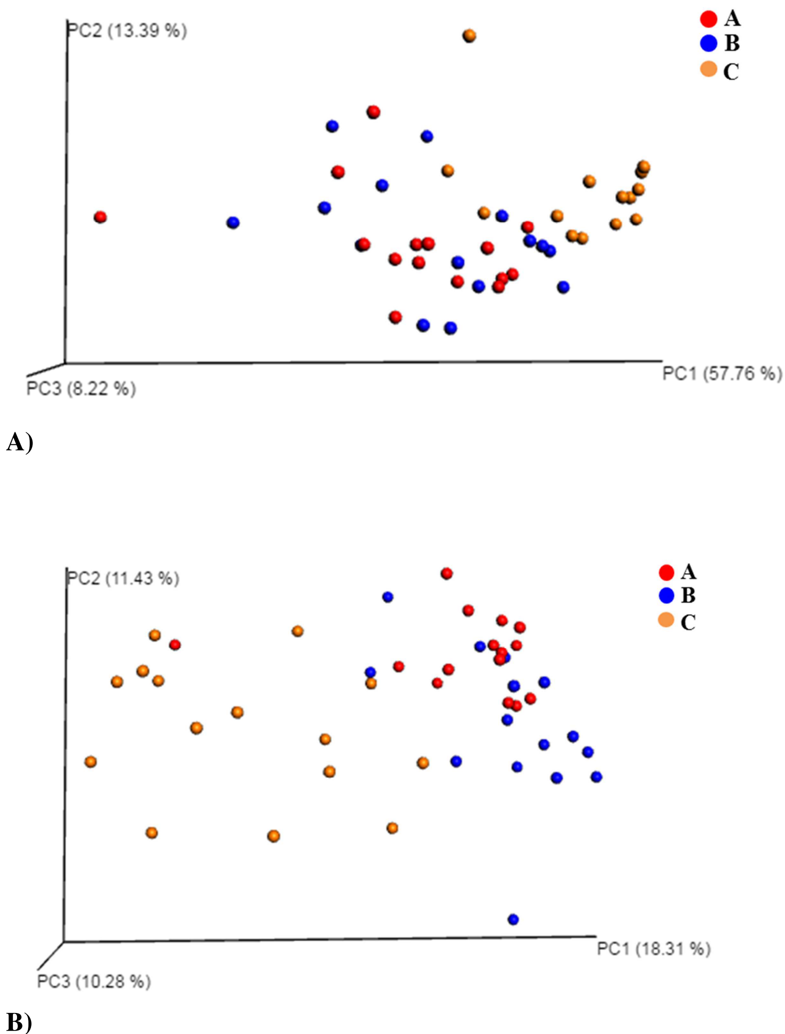


Fig 3.6 Beta diversity metrics. Principal Coordinate Analysis of Weighted (**A**) and Unweighted (**B**) Unifrac. The samples (15/diet) are colored according to the diet.

Table 3.7 Analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (Adonis). *P* value < 0.05 indicates statistically significant differences. In the ANOSIM function, a R value close to +1 indicates that the grouping of samples is strong. In the Adonis function the R^2 value indicates the % of the variation in distances, which is explained by this grouping.

	ANOSIM				Adonis			
	Unweighted		Weighted		Unweighted		Weighted	
	p value	R value	*p* value	R value	*p* value	R^2	*p* value	R^2
A vs B	0.004	0.144	0.199	0.028	0.001	0.08	0.285	0.04
A vs C	0.001	0.514	0.001	0.489	0.001	0.19	0.001	0.38
B vs C	0.001	0.480	0.001	0.341	0.001	0.18	0.001	0.30

3.3 Discussion

In this trial we tested the HiCell product, an autolyzed yeast from *Saccharomyces cerevisiae*, as a replacement for FM in gilthead sea bream diet and evaluated its effect on fish intestinal microbiota composition. Among the FM alternatives, the autolyzed yeast from *S. cerevisiae* proved to be a good choice to use as an additive, as it becomes more accessible and more digestible due to the autolyzed process that digests proteins into oligo-peptides or free aminoacids (Tacon, 2014). *S. cerevisiae*, depending on the concentration, can be used as a protein source or prebiotic. Usually, brewer's yeast is used as a beneficial additive to stimulate the host immune system, with supplementation up to 2%, while increasing this percentage would seem to offer a good protein source (Ozório et al., 2012). A study on gilthead sea bream demonstrated that the prebiotic product MOS modulates the gut microbiota, increasing the microbial species richness and diversity (Dimitroglou et al., 2010).

In addition, introducing protein hydrolysates as the source of protein could directly and indirectly modify the animal's intestinal microbiota. Indeed, short peptides would represent a suitable substrate for bacteria

thus encouraging proliferation (Egerton et al., 2018). For this reason, we also considered another diet based on fish hydrolysate that provides free highly digestible peptides and bioactive peptides, which are more easily absorbed by the intestine than fishmeal.

In our study, the dietary supplementation of HiCell (diet C) did not affect fish SGR, and this result was also observed also in sea bass (*Dicentrarchus labrax*) and in Nile tilapia (*Oreochromis niloticus*) up to a FM substitution of 30% and 10% with *S. cerevisiae*, respectively (Oliva-Teles and Goncalves, 2001; Ozório et al., 2012). In line with our results, the FCR index was not influenced by dietary yeast in the study of Yuan et al. (2017); however they found, in contrast to our study, an increased weight gain in fish fed with 1% and 3% of yeast hydrolysate. Moreover, based on the weight gain of fish, the same authors determined that 3.7% is the best percentage of *S. cerevisiae* to replace FM.

Intestinal microbiome sequencing showed that *Firmicutes* and *Proteobacteria* were the most abundant phyla in all three groups studied. The *Firmicutes:Proteobacteria* ratio was comparable between groups, indicating that the three diets had a similar composition in terms of vegetable and animal ingredients. Indeed, these phyla can usually distinguish diet type since the dominance of *Firmicutes* is related more to diets that use plant ingredients than to FM-based diets (Rimoldi et al., 2018). Alpha diversity metrics showed that HiCell supplementation affected gut microbial richness. Indeed, the Shannon diversity index, which accounts for both the number of species present (species richness) and their abundances (termed evenness), showed an increase in species richness as it was lower in group C (HiCell-based group) than in group A (control group). This observation is in contrast to the results of Liu et al. (2018) who found no significant differences

in terms of alpha-diversity between the control and yeast treatment groups analyzed. This discrepancy is probably due to the intestinal microbiota variability, which depends on the variety of host species and the environmental factors.

In order to address that difference, we detected some taxa in fish fed with diet C that were missing in the other two groups or only present to a minor extent. These taxa could have advantages for the host. For example, *Clostridiales* order that were significantly higher in the HiCell group than in the control group might include microorganisms able to degrade indigestible carbohydrates, contributing to the more efficient feed energy utilization. Furthermore, at the family level, *Prevotellaceae* and *Veillonellaceae*, which were represented at the genus level by *Prevotella* and *Megasphera*, play a central role in carbohydrate digestion. In particular, *Megasphera* is characterized by the ability to produce short-chain fatty acids (SCFAs) such as propionate, acetate, and butyrate from lactate (Tsukahara et al., 2002). Among the microbial-derived SCFAs, butyrate is considered the most important due to the numerous, well-documented positive effects it has on the intestinal health of vertebrates, including fish (Rimoldi et al., 2016; Terova et al., 2016).

The most abundant taxon found in all three groups is *Lactobacillus*, a member of the lactic acid bacteria and characterized by the production of lactic acid as the only or main product of carbohydrate metabolism. *Lactobacilli* constitute a normal part of the intestinal microbiota of fish and are generally considered beneficial microorganisms associated with a healthy intestinal epithelium. They have beneficial effects on the immune system and could protect the fish against pathogenic invasion through the intestinal surface (Askarian et al., 2011; Ringø and Gatesoupe, 1998). The second more abundant genus in experimental

groups was *Photobacterium*, classified as *Photobacterium damselae*, an autochthonous microorganism of aquatic ecosystems; it is considered a primary pathogen in several species of both wild fish and farm fish, including gilthead sea bream (Essam et al., 2016). HiCell diet also positively affected the amount of *Bacillus* genus; it is a member of the *Firmicutes* phylum, which has numerous beneficial effects, including the enhancement of immune responses and disease resistance. Finally, *Shewanella* genus, which was decreased in diet B (fish hydrolysate-based diet) remained constant in diet C as compared to the control diet. Several members of the *Shewanella* genus have been reported as being omega-3 fatty acid-producing (EPA and DHA) bacteria (Dailey et al., 2016) and have been used as probiotics in aquaculture (Cordero et al., 2016; Lobo et al., 2014).

In agreement with data described to date, the HiCell-based diet affected the relative abundance of specific taxa. Furthermore, the beta diversity metrics confirmed this result. We used the Weighted UniFrac test, which takes into account the relative abundance of different species (OTUs), and the unweighted UniFrac, which looks only at the presence/absence of the species. Both functions analyzed (ANOSIM and Adonis) showed that intestinal microbiota of sample C differed from the other two groups for the relative abundance of bacterial taxa and, more interestingly, for the presence or absence of specific OTUs.

In conclusion, this study described the beneficial effects of autolysate yeast in gilthead sea bream by using high-throughput analysis of host gut microbiota. These effects are likely due to the yeast characteristics and not only to the ready availability of the short peptides of which it is composed; in line with that finding, diet B (fish hydrolysate-based) did not show the same changes in gut microbiota composition as obtained with the HiCell supplemented diet.

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CHAPTER 4

EFFECTS OF DIFFERENT PERCENTAGES OF *HERMETIA ILLUCENS* MEAL ON RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) INTESTINAL MICROBIOTA COMPOSITION

The aim of the present research was to study the effects of substituting fishmeal with insect meal in the diet of rainbow trout (*Oncorhynchus mykiss*) on fish growth performance, and the intestinal microbiome. The work is part of a 3-year project entitled "Insect Bioconversion: from vegetable waste to Protein production for fish Feed (InBioProFeed)".

4.1 Materials and methods

4.1.1 Experimental diets

Black soldier fly (*Hermetia illucens*) larvae were grown on a substrate of fruit and vegetables provided by the wholesale market of Milano (Milano Ortomercato). At the prepupal stage, larvae were harvested and then processed under controlled conditions to be transformed into insect meal. The insect meal was produced by the Department of Food, Environmental and Nutritional Sciences (DeFENS) at the University of Milan, Italy. *H. illucens* (Hi) meal was partially defatted by using a mechanical process based on high pressure and without use of solvents. Insect meal had to be defatted because full-fat insect meal contained high amounts of lipids, which were difficult to be managed during feed preparation due to overmuch energy, proneness to oxidation and a decrease in pellet stability (for a review please see Henry et al., 2015). Four diets were formulated with increasing percentages of Hi larvae

meal as substitutes for fishmeal (FM). Specifically one control diet with 0% (Hi 0) and three experimental diets with 10% (Hi 10), 20% (Hi 20), and 30% (Hi 30) of Hi meal were formulated. All feeds were prepared at the experimental facility of the Department of Agricultural, Forest and Food Science (DISAFA) of the University of Turin (Italy) and were isonitrogenous (crude protein: about 41 g/100 g dry matter), isolipidic (ether extract: about 18 g/100 g dry matter) and isoenergetic (gross energy: between 19.47 and 19.87 MJ/kg). Tables 4.1 and 4.2 show the principal ingredients and proximate composition of the tested diets. For the proximate composition and energy level analysis, feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analysed for DM (AOAC #934.01), CP (AOAC #984.13) and ash (AOAC #942.05) contents according to AOAC International (AOAC International, 2000); EE (AOAC #2003.05) was analyzed according to AOAC International (AOAC International, 2003). The GE content was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany).

The amino acid composition of the four experimental diets is shown in table 4.3. Total amino acid composition was determined using a Jasco HPLC system (Jasco -Europe S.r.l) equipped with a quaternary pump (Model PU-2089, Jasco), connected to degasser, a programmable fluorescence detector (Model FP-4025, Jasco) (excitation 250 nm, emission 395 nm), and a temperature control module. The analysis was performed according to the method described by Rimoldi et al. (2018), with the only exception that, in the present study, tryptophan was not determined. Each diet was analyzed in triplicate.

Table 4.1. Formulation of the experimental diets (g/100g dry matter).

Ingredient (%)	Diet			
	Hi 0	**Hi 10**	**Hi 20**	**Hi 30**
Fishmeal	60	54	48	42
***Hermetia illucens* meal**	-	10	20	30
Fish oil	7	7	7	7
Soybean oil	5	4	3	2
Wheat bran	10	7	4	1
Wheat meal	4	4	4	4
Filler	11	11	11	11
Vitamin premix[a]	1.5	1.5	1.5	1.5
Mineral premix[b]	1.5	1.5	1.5	1.5
	100	**100**	**100**	**100**

Abbreviations: Hi *Hermetia illucens*; Hi 0, Hi 10, Hi 20, Hi 30: three diets, with increasing levels of replacement of FM with Hi (10%, 20% and 30%) and a control diet without Hi.

[a] DL-α tocopherol acetate 60 mg Kg^{-1}; sodium menadione bisulphate 5 mg Kg^{-1} diet; retinyl acetate 15,000 mg Kg^{-1}; DL-cholecalciferol 3000 mg Kg^{-1}; thiamin 15 mg Kg^{-1} diet; riboflavin 30 mg Kg^{-1} diet; pyridoxine 15 mg Kg^{-1} diet; B12 0.05 mg Kg^{-1} diet; nicotinic acid 175 mg Kg^{-1} diet; folic acid 500 mg Kg^{-1} diet; inositol 1,000 mg Kg^{-1} diet; biotin 2.5 mg Kg^{-1} diet; calcium panthotenate 50 mg Kg^{-1} diet.

[b] manganese 9,000 mg Kg^{-1} diet; zinc 8,000 mg Kg^{-1} diet; iron 7,000 mg Kg^{-1} diet; copper 1,400 mg Kg^{-1} diet; cobalt 160 mg Kg^{-1} diet; iodine 120 mg Kg^{-1} diet.

Table 4.2. Proximate composition of the experimental diets (g/100g dry matter).

Proximate composition	Diet			
	Hi 0	**Hi 10**	**Hi 20**	**Hi 30**
DM	91.9	91.9	91.9	91.9
CP	41.3	41.3	41.3	41.3
EE	17.8	18.1	18.3	18.5
ash	11.5	11.3	11.1	10.9
n.f.e.	15.5	15.8	16.1	16.4

Abbreviations: Hi *Hermetia illucens*; DM dry matter; CP crude protein;
EE ether extract; n.f.e nitrogen free extracts; Hi 0, Hi 10, Hi 20, Hi 30: three diets, with increasing levels of replacement of FM with Hi (10%, 20% and 30%) and a control diet without Hi.

Table 4.3. Amino acid composition (g/100g diet) of the experimental diets.

	DIET			
	Hi 0	**Hi 10**	**Hi 20**	**Hi 30**
Asparagine	1.46	1.42	1.33	1.35
Serine	0.88	0.88	0.89	0.91
Glutamic acid	2.26	2.24	2.10	2.09
Glycine	0.34	0.32	0.33	0.31
Histidine	2.29	2.26	2.32	2.23
Arginine	1.17	1.37	1.14	1.09
Threonine	0.80	0.76	0.79	0.79
Alanine	1.00	1.03	1.06	1.10
Proline	0.75	0.76	0.84	0.84
Tyrosine	0.58	0.64	0.67	0.74
Valine	0.79	0.79	0.83	0.84
Lysine	1.25	1.19	1.17	1.14
Isoleucine	0.73	0.71	0.73	0.73
Leucine	1.27	1.23	1.25	1.25
Phenylalanine	0.75	0.71	0.76	0.75
Cysteine	0.06	0.11	0.52	1.06
Methionine	5.29	5.61	4.46	6.33

Abbreviations: Hi 0, Hi 10, Hi 20, Hi 30: three diets, with increasing levels of replacement of FM with Hi (10%, 20% and 30%) and a control diet without Hi.

4.1.2 In vivo feed digestibility trial

An in vivo digestibility experiment was performed to determine the apparent digestibility coefficients (ADC) of the diets.

Forty-eight trout (weight 70.1 ± 1.6 g) were divided into twelve 40-L cylindroconical tanks connected to the same open water system of the growth trial. After 14 days of acclimatization with the experimental diets, the fish were fed by hand to visual satiety twice a day. The apparent digestibility coefficients were measured using the indirect acid-insoluble ash method; 1% celite® (Fluka, St. Gallen, Switzerland) was added to the diets as inert marker in substitution of 1% of starch

gelatinized (D500). The feces were collected daily from each tank for three consecutive week, using a continuous automatic device, as described by Palmegiano et al. (2006). The feces were freeze dried and frozen (−20 °C) until analyzed. The ADC of DM (ADCDM), crude protein (ADCCP), ether extract (ADCEE) and gross energy (ADCGE) were calculated following Palmegiano et al. (2006).

4.1.3 Fish and feeding trial

All procedures involving rainbow trout (*Oncorhynchus mykiss*) were conducted at the experimental facility of DISAFA (University of Turin, Italy), in accordance with EU Directive 2010/63/EU for animal experiments.

A 12-week feeding trial was performed on 348 rainbow trout with an initial mean body weight of 66.5 ± 1.7 g. Fish were randomly distributed in 12 indoor rectangular fiber-glass tanks of 1 m^3 connected to a flow-through open system supplied with artesian well water (constant temperature of 13 ± 1 °C, 8 L min^{-1}). Dissolved oxygen was regularly measured and ranged between 7.6 and 8.7 mg L^{-1}. After 7 days of acclimatization, fish were fed twice daily (seven days per week) for 12 weeks with four experimental diets in triplicate (3 tanks/diet). Feed was manually distributed and feeding rate was restricted to 1.5% of biomass for the entire duration of the experiment. To calculate the daily feed ratio, fish from each tank were weighed in bulk every 14 days. Mortality was checked every day. At the end of the feeding trial all fish were individually weighted to calculate weight gain (WG = final body weight – initial body weight), specific growth rate (SGR = 100 x [ln (final body weight) – ln (initial body weight)]/days]) and feed conversion ratio (FCR = feed intake / WG).

4.1.4 Sample collection

At the end of the trial, 2 fish per replicate (6 fish/diet) were sampled and euthanized with 320 mg/L of tricaine-methasulfonate (MS-222, Sigma-Aldrich, Italy). Before dissection, the external surface of each fish was wiped with 70% ethanol to avoid any accidental contamination from external body surface microflora. The intestine (excluding pyloric caeca) was aseptically removed with alcohol-disinfected instruments from each fish and squeezed to collect the fecal content (luminal bacteria). The samples were collected in sterile tubes containing Xpedition™ Lysis/Stabilization Solution (Zymo Research) at a ratio of 1:3 and stored at room temperature until analysis.

4.1.5 DNA extraction and library preparation

The bacterial DNA was extracted from 250 mg of feces and 200 mg of each tested feeds using DNeasy PowerSoil Kit (Qiagen, Italy), according to the manufacturer's instructions. The samples were lysed in PowerBead Tube by means of a TissueLyser II (Qiagen, Italy) for 2 min at 25 Hz. As negative control of the extraction procedure, a sample with only lysis buffer was processed in parallel with samples. The concentration of extracted DNA was measured using NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Italy) and stored at -20°C until the PCR reaction was performed.

The creation of a 16S rRNA library was explained in detail in chapter 2.

4.1.6 Raw sequencing data analysis

Raw FASTQ sequencing data were processed using the open-source bioinformatics pipeline QIIME v1.9.1 (Caporaso et al. 2010) at the default setting. To reconstruct the original amplicons, overlapping

paired reads were joined using FLASH v1.2.11 software (http://sourceforge.net/projects/flashpage) and filtered for base quality (Q>30). The remaining high-quality sequences were aligned to the Greengenes reference database v.13.8 (http://greengenes.lbl.gov) using QIIME script 'pick_closed_reference_otus.py' with an identity percentage ≥ 97% to select the operational taxonomic units (OTUs). Only the OTUs that represented at least 0.005 % of total reads were kept. The taxonomical classification was performed down to the species level. The final OTU-table output files, containing the relative abundance of each OTU, were created using the custom script 'summarize_taxa_through_plots.py'. OTUs assigned to the phylum *Cyanobacteria* (class *Chloroplast*) and to mitochondria were removed from the analysis, as they are considered plant contaminants. Advanced analysis was performed using the QIIME script 'core_diversity_analyses.py' at the default setting reported by Rimoldi et al. (2018). Alpha diversity metrics were calculated based on a rarefied OTU table (rarefied at the lowest sample size) using 'Good's coverage', 'observed species', 'Chao1 index' (species richness estimator), 'PD whole tree', and 'Shannon' and 'Simpson' diversity indices. The variation in species composition between communities (beta diversity) was assessed by applying weighted (presence/absence/abundance matrix) and unweighted (presence/absence matrix) UniFrac distance matrices (Lozupone and Knight, 2005; Lozupone et al., 2007). The distance matrices were visualized by principal coordinate analysis (PCoA) three-dimensional plots.

The common core microbiome (OTUs shared regardless of the diet and found in at least five out of the six samples per dietary group) was

identified and visualized by a Venn diagram drawn using the web tool http://bioinformatics.psb.ugent.be/webtools/Venn/.

4.1.7 Statistics

All data were tested for normality and homogeneity of variances by Shapiro-Wilk's and Levene's test, respectively. Differences between two groups were analysed by one-way ANOVA followed by Tukey-Kramer post-hoc test or by non-parametric Kruskal-Wallis and Dunn's post-hoc test, depending on normality and homoscedasticity of the data. Statistical significance was set at $p<0.05$. All analyses were performed using Past3 software.

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 family level) and 0.5% at genus level were considered for statistical analysis. Before being statistically analyzed, the resulting microbial profiles were calculated as the angular transformation (arcsine of the square root). Differential abundance analysis of OTUs between groups was performed using MetagenomeSeq (R package). Significances ($p<0.05$) were identified by Fisher's test and applying the Benjamini Hochberg False Discovery Rate (FDR) correction.

Multivariate analysis of beta diversity was tested using non-parametric analysis of similarities (ANOSIM) and Adonis tests with 999 permutations. Both tests were available with the QIIME script 'compare_categories.py'.

4.2 Results

4.2.1 Fish growth performance and feed conversion indicators

During the 12 weeks of feeding fish promptly accepted the experimental diet and mortality rate was negligible, i.e., lower than 1%. At the end of the feeding trial, all fish had tripled their initial body weight, and growth performance parameters (WG and SGR) were not affected by diet composition (Table 4.4). Similarly, FCR was comparable among the treatments and remained lower than 1 in all groups, meaning that all fish grew efficiently and including the *H. illucens* meal did not negatively affect diet palatability.

Table 4.4. Initial and final mean body weight (BWi, BWf), weight gain (WG), specific growth rate (SGR), and feed conversion ratio (FCR) values of rainbow trout fed with different diets.
The values are reported as mean ± SD (n=84 fish per diet). No statistically significant differences were found between groups.

	Hi 0	**Hi 10**	**Hi 20**	**Hi 30**
BWi (g)	67.01 ± 1.71	66.38 ± 2.51	65.63 ± 0.42	66.95 ± 2.31
BWf (g)	223.20 ± 23.67	220.34 ± 29.60	216.97 ± 26.16	221.74 ± 22.25
WG (g)	156.86 ± 4.33	154.20 ± 6.04	146.89 ± 8.03	152.30 ± 10.18
SGR	1.42 ± 0.01	1.40 ± 0.06	1.38 ± 0.04	1.38 ± 0.04
FCR	0.90 ± 0.02	0.93 ± 0.04	0.95 ± 0.03	0.93 ± 0.04

Abbreviations: Hi 0, Hi 10, Hi 20, Hi 30: three diets, with increasing levels of replacement of FM with Hi (10%, 20% and 30%) and a control diet without Hi.

4.2.2 Digestibility trial

The ADC values of nutrients are presented in Table 4.5. No statistical significant differences (p<0.05) were recorded between Hi 0 diet (control) and Hi 10, Hi 20, and Hi 30 for DM and CP digestibility.

Table 4.5 Apparent digestibility coefficient of dry matter, proteins, ether extract and gross energy of rainbow trout fed the experimental diets. The values are reported as mean ± SEM (4 fish/tank and 3 tanks/diet). No statistically differences among groups are detected ($p < 0.05$).

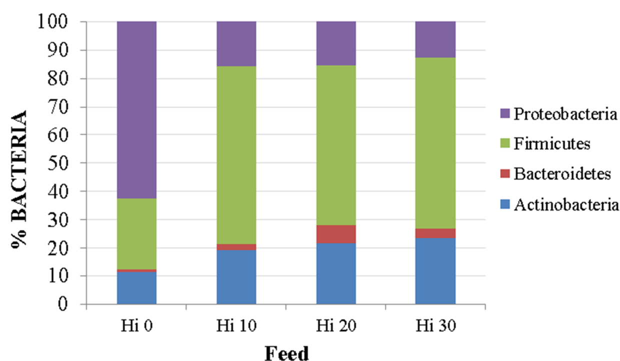
	ADC_{DM}	ADC_{CP}	ADC_{EE}
Hi 0	76.35 ± 2.89	90.70 ± 1.18	98.53 ± 0.21
Hi 10	77.43 ± 2.28	91.18 ± 0.92	98.64 ± 0.25
Hi 20	76.52 ± 0.19	90.46 ± 0.07	98.70 ± 0.10
Hi 30	79.66 ± 0.65	92.01 ± 0.12	98.85 ± 0.11

Abbreviations: Hi *Hermetia illucens*; Hi 0, Hi 10, Hi 20, Hi 30: three diets, with increasing levels of replacement of FM with Hi (10%, 20% and 30%) and a control diet without Hi; ADC_{DM} - dry matter apparent digestibility coefficient; ADC_{CP} - crude protein apparent digestibility coefficient; ADC_{EE} - ether extract apparent digestibility coefficient.

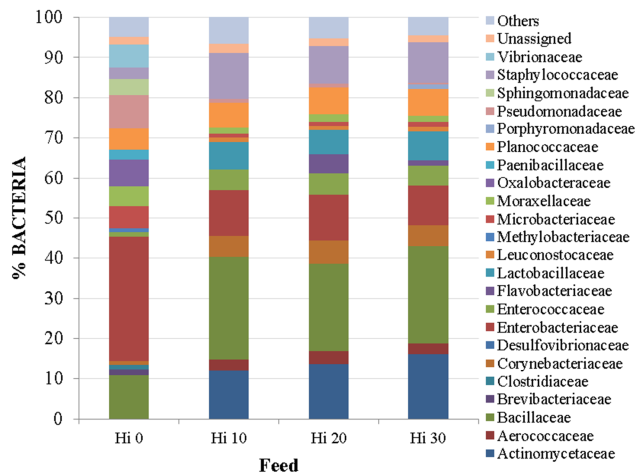
4.2.3 QIIME data of experimental diets

QIIME analysis on NGS raw data of bacteria associated to feeds revealed that the microbial profile of the Hi 0 diet (control) qualitatively and quantitatively differed from that of Hi meal-containing feeds. After filtering for quality, trimming length, and generating consensus lineages, the number of reads taxonomically classified according to the Greengenes database was 75,882, 39,896, 22,086, and 51,126 for the Hi 0, Hi 10, Hi 20, and Hi 30 diets, respectively. Based on a 97% sequence similarity cutoff, these sequences yielded a bacterial OTU number that ranged from 194 to 318 OTUs. After discarding those OTUs corresponding to eukaryotic sequences, the most abundant bacterial taxa (relative abundance > 1% until family and > 0.5% for lower taxonomic levels) were mainly comprised of 3 phyla, 8 classes, 12 orders, 23 families, 34 genera, and 19 species (Fig. 4.1 A-C). At phylum level microbiota of the Hi 0 feed was dominated by *Proteobacteria* (62%), conversely Hi 10, Hi 20, and

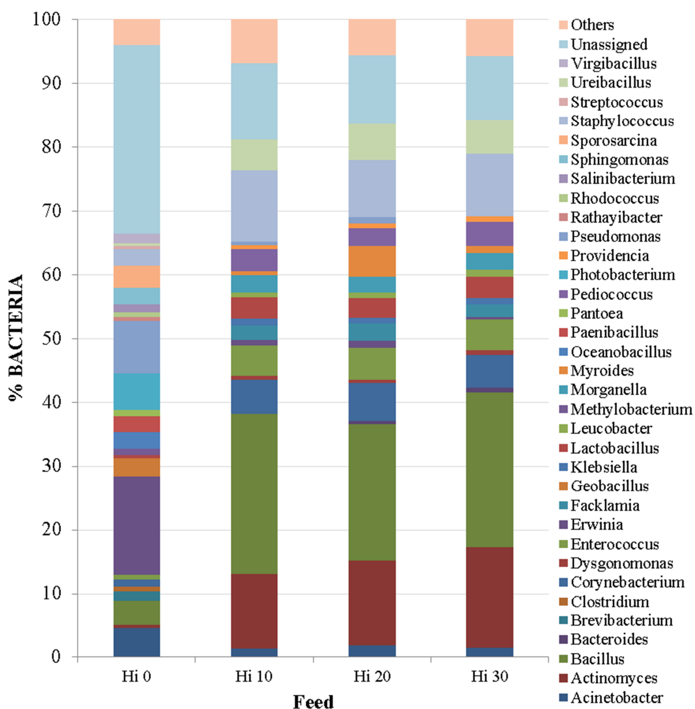
Hi 30 feeds were enriched in *Firmicutes* (56-62%) and *Actinobacteria* (19-23%) (Fig. 1A). Accordingly, a high percentage of *Enterobacteriaceae* (31%) and *Pseudomonadaceae* (8%) was found in the control diet, while feeds containing Hi meal showed high amounts of bacteria belonging to *Bacillaceae* (21-25%), *Enterobacteriaceae* (10-11%), *Lactobacillaceae* (6-7%), *Staphylococcaceae* families (9-11%), and *Actinomycetaceae* (12-16%) (Fig. 1B). At the genus level, *Erwinia* (15%), *Photobacterium* (5%) and *Pseudomonas* (8%) were the most abundant genera found in the Hi 0 diet. Feeds supplemented with Hi meal were instead characterized by a high relative abundance of *Actinomyces* (11-16%), *Bacillus* (21-25%), *Corynebacterium* (5-6%), *Lactobacillus* (3%), and *Staphylococcus* (9-11%) (Fig. 1C).



A)



B)



C)

Figure 4.1 Bacterial relative abundance (%) in the feeds.

The amount (%) of the most prevalent bacteria in Hi 0, Hi 10, Hi 20 and Hi 30 feeds at phylum (**A**), family (**B**), and genus (**C**) level. Only bacteria with an overall abundance of $\geq$ 1% (at phylum level) and $\geq$ 0.5% (at family and genus level) were reported. Bacteria with lower abundance were pooled and indicated as "Others".

4.2.4 Analysis of gut microbiota structure

The 24 fecal samples were sequenced on one paired-end MiSeq run (Illumina, Italy) and the sequencing raw generated data were analyzed using the QIIME pipeline. While analysing the bioinformatics data, one Hi 0 and two Hi 30 samples were discarded after the out selection step due to an insufficient number of sequences. The total number of filtered reads taxonomically classified was 1,140,534, which corresponded to an average number of 54,311 ± 16,607 reads per sample (Table 4.6). A total of 450 OTUs at 97% identity were identified in trout fecal samples. The Good's coverage value was >0.99, indicating that the coverage degree of the MiSeq sequencing was high and the number of OTUs identified was representative of all microbial communities in each dietary group (Table 4.6).

The core gut microbiota, i.e. OTUs present in at least 80% of fecal samples and shared regardless of the diet, was constituted by 62 OTUs (Fig. 4.2). Among these, 23 OTUs were common to 100% of samples, showing a dominance of *Firmicutes* (15 OTUs).

After removing those reads assigned to eukaryotic taxa, the whole microbial community profile of fecal samples was mainly comprised of 7 phyla, 12 classes, 26 orders, 68 families, 98 genera, and 55 species. However, considering only the most representative taxa, with an overall abundance of more than 1% at phylum, class, order, and family level and more than 0.5% at genus and species level, the overall gut microbial community consisted of 3 phyla, 6 classes, 7 orders, 21 families, 15 genera, and 8 species. The intestinal microbial community profiles of each dietary group and individual fish are presented at the phylum (Fig. 4.3 A-E), family (Fig. 4.4 A-E), and genus (Fig. 4.5 A-E) level. The mean relative abundance changes between groups at species

level were excluded from analysis, as they were not considered to be informative because of the remarkable number of unassigned sequences found (77-91%).

A sequencing depth of 25,441 reads per sample was considered to elaborate alpha rarefaction curves (Fig. 4.6 A-C) and analyze the effect of insect meal-based diets on diversity within microbial populations (alpha diversity). *H. illucens* meal administration significantly increased the number of observed species, species richness (Chao1 index) and entropy (Shannon and Simpson diversity indices) with respect to control Hi 0. Similarly, phylogenetic diversity (PD whole tree index) improved in Hi 10, Hi 20, and Hi 30 samples compared to the control group (Table 4.6). Strong differences were also detected between microbial communities (beta diversity) both in type (unweighted UniFrac) and relative abundance (weighted UniFrac) of taxa. As displayed in unweighted and weighted UniFrac PCoA plots, PC1 and PC2 together explained 56% and 83% of the variation between individuals, respectively. In particular, fecal samples from Hi 10, Hi 20, and Hi 30 groups clustered together and separately from control Hi 0 (Fig. 4.7 A,B). The permutational multivariate analysis Adonis fully confirmed the PCoA plots results, revealing a significant difference in gut microbial communities between experimental groups (Hi 10, Hi 20, and Hi 30) and their control Hi 0 ($R^2 > 0.43$, $p < 0.05$). Similarly, the ANOSIM test was significant for both the unweighted and weighted Unifrac distance matrix ($R > 0.70$, $p < 0.05$) when control Hi 0 was compared to any other feeding group. Results of pairwise statistical analysis on phylogenetic distances are summarized in table 4.7.

Table 4.6 Number of reads per sample assigned to OTUs, good's coverage value, and alpha diversity metrics values of gut microbial community of trout fed with different diets for 12 weeks. Reported data are expressed as means ± SD. The means were compared by ANOVA ($p < 0.05$). Different superscript letters on the same column indicate significant differences after post-hoc Tukey's test.

	Hi 0	**Hi 10**	**Hi 20**	**Hi 30**
Reads	44,771 ± 6,874	59,716 ± 13,723	54,600 ± 25,172	57,696 ± 16,494
Good's	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00	0.99 ± 0.00
Observed	203.2 ± 40.67^{b}	307.2 ± 15.6^{a}	304 ± 20.2^{a}	314.5 ± 6.4^{a}
Chao 1	226.7 ± 37.3^{b}	328.5 ± 18.7^{a}	322.7 ± 28.7^{a}	342.3 ± 6.2^{a}
PD whole tree	13.21 ± 1.84^{b}	18.08 ± 0.79^{a}	17.97 ± 1.32^{a}	18.86 ± 0.66^{a}
Shannon	4.01 ± 0.29^{b}	5.46 ± 0.29^{a}	5.65 ± 0.12^{a}	5.58 ± 0.15^{a}
Simpson	0.85 ± 0.05^{b}	0.94 ± 0.03^{a}	0.95 ± 0.00^{a}	0.95 ± 0.00^{a}

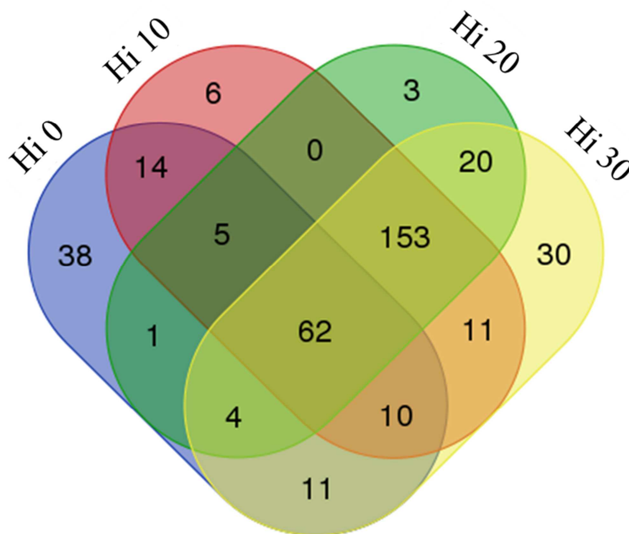
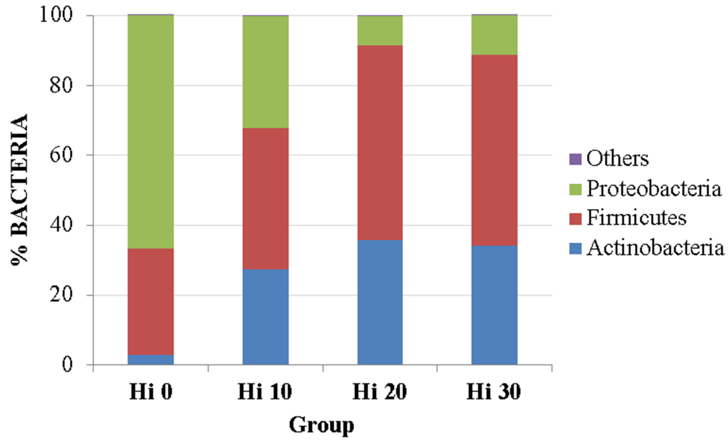
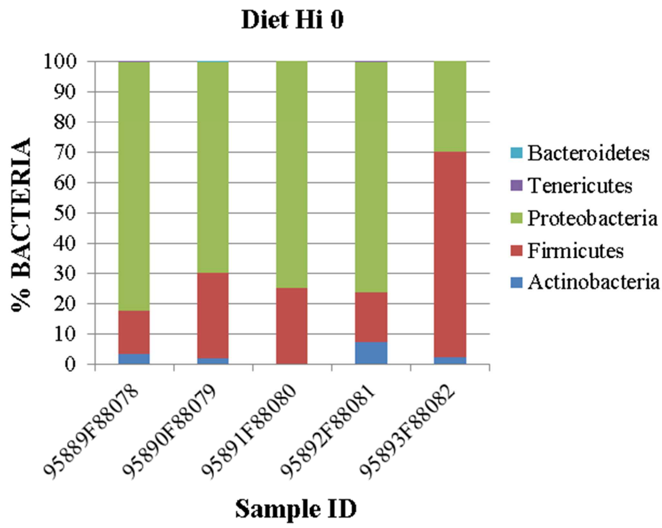


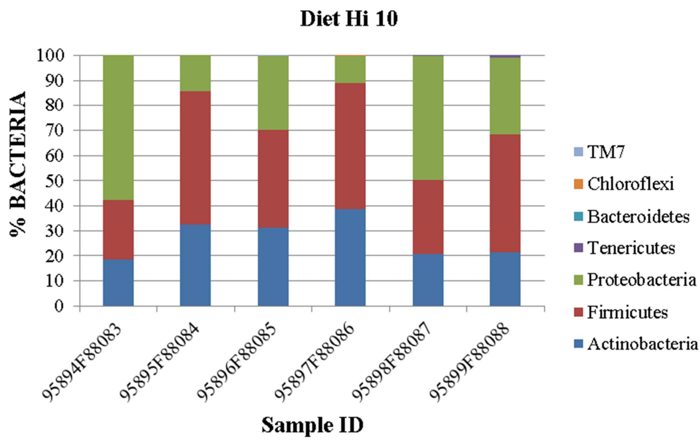
Figure 4.2 Venn diagram representing unique and shared OTUs among all dietary groups.



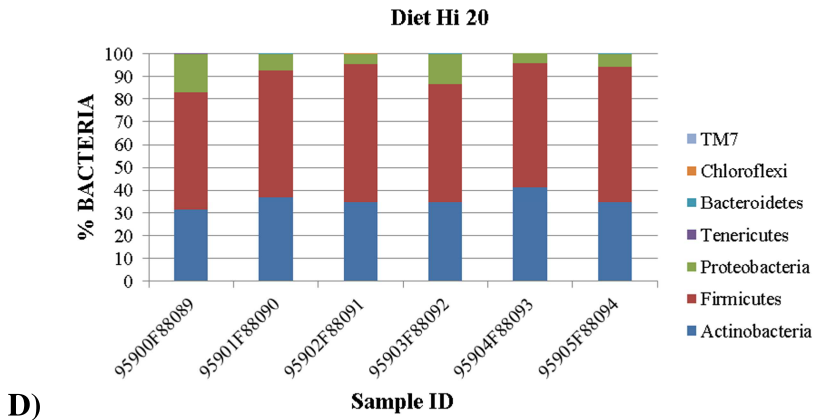
A)



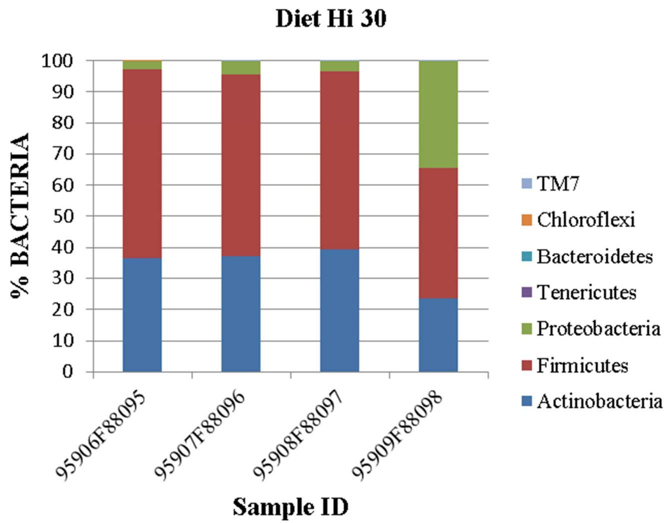
B)



C)



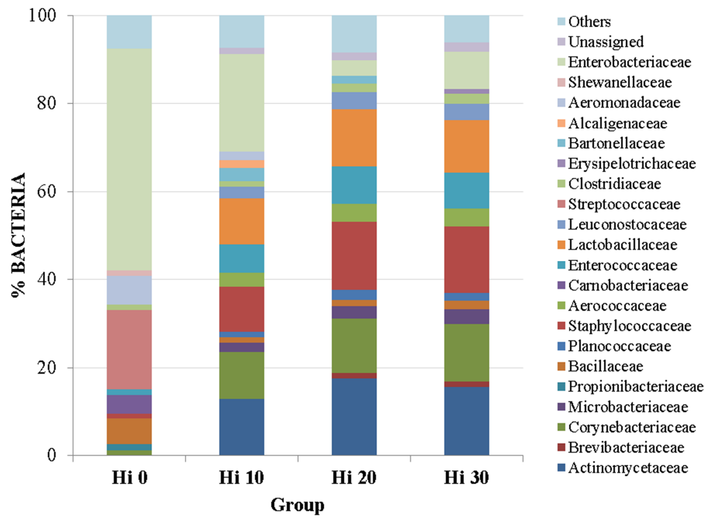
D)



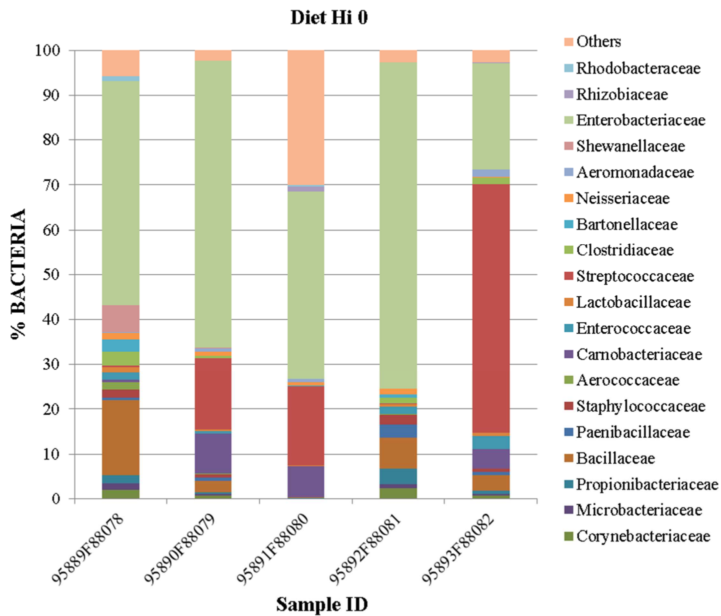
E)

Figure 4.3 Relative abundance (%) of the overall most prevalent bacteria in each dietary groups (**A**) and in individual fish (**B, C, D, E**) at phylum level.

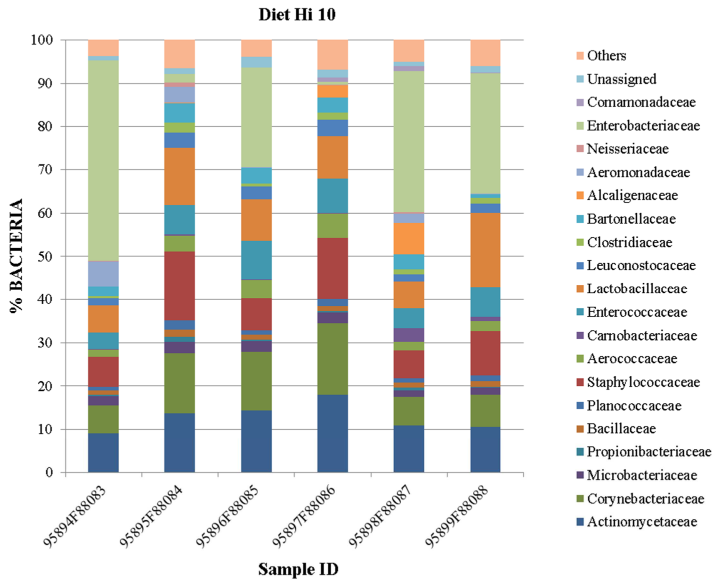
In the figure, all bacteria with an overall abundance of ≥1% were reported.



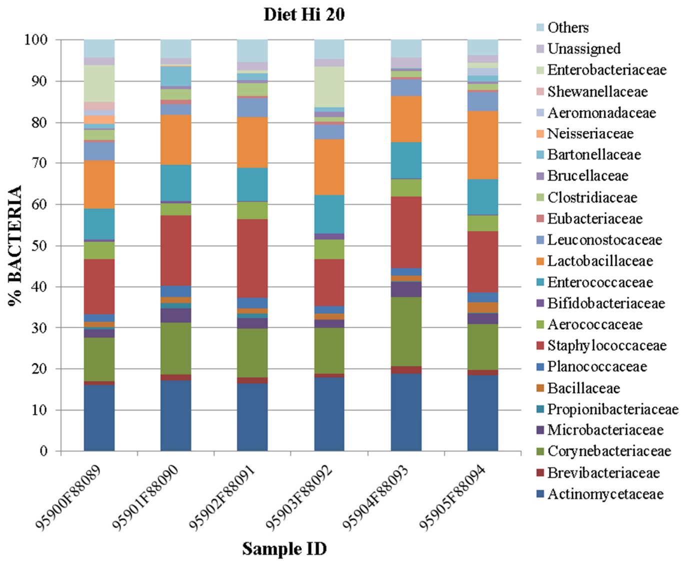
A)



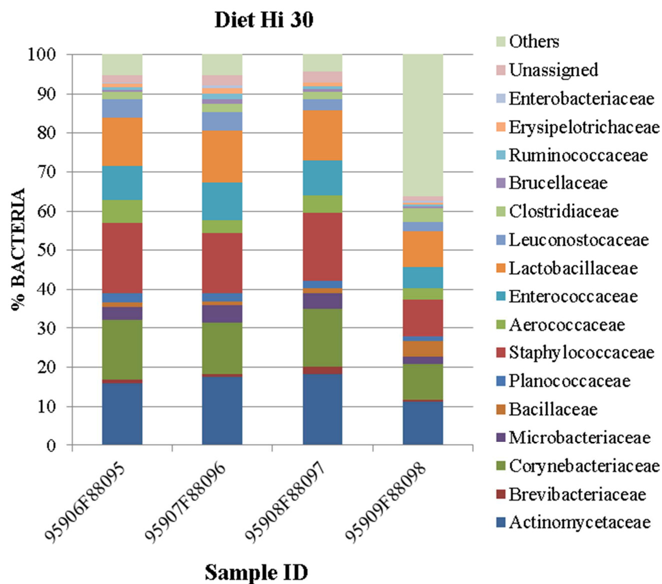
B)



C)



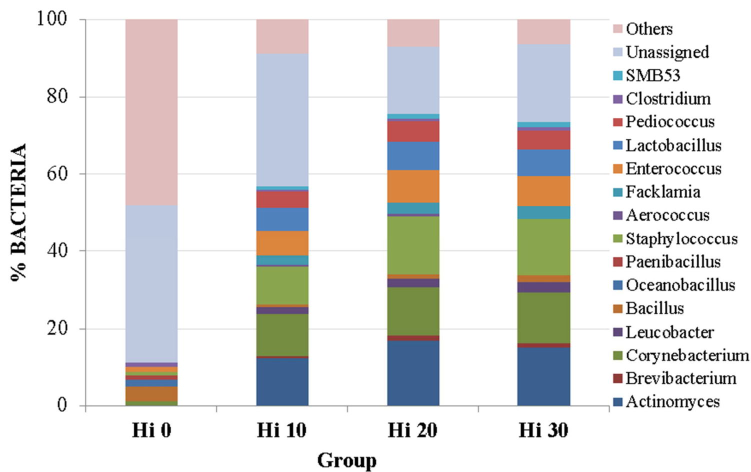
D)



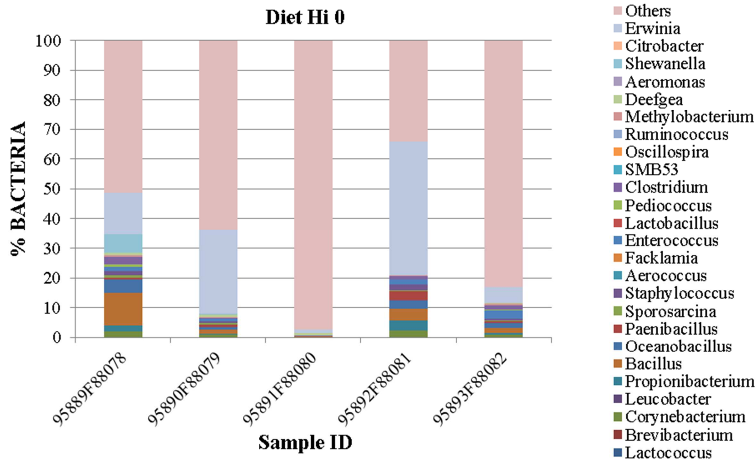
E)

Figure 4.4 Relative abundance (%) of the overall most prevalent bacteria in each dietary groups (**A**) and in individual fish (**B, C, D, E**) at family level.

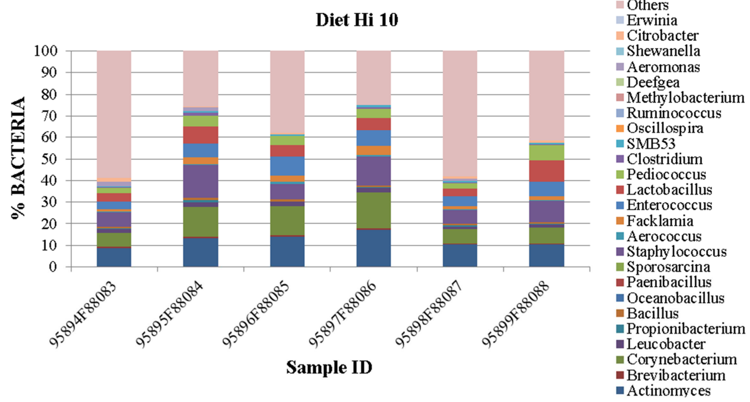
In the figure, all bacteria with an overall abundance of ≥1% were reported, while bacteria with an abundance of ≤1% were regrouped and indicated as "Others".



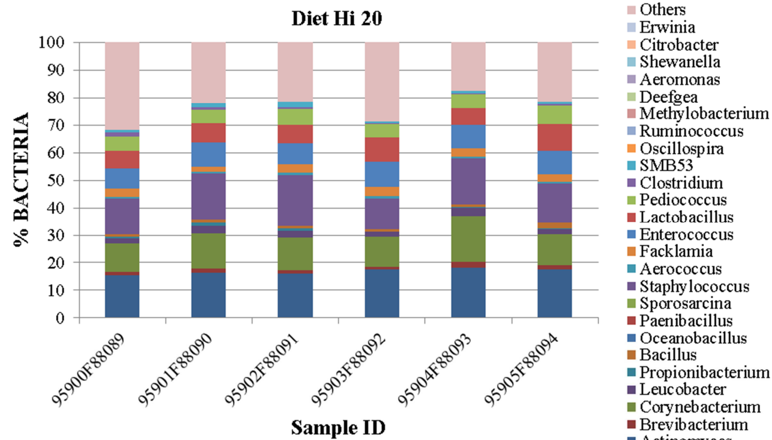
A)



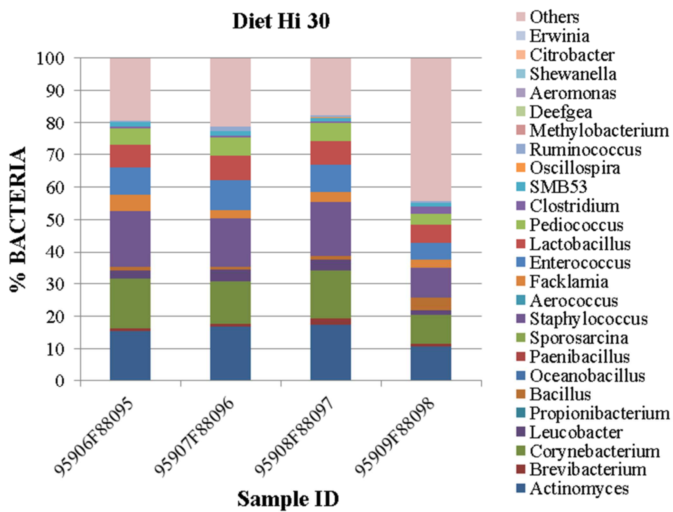
B)



C)



D)



E)

Figure 4.5 Relative abundance (%) of the overall most prevalent bacteria in each dietary groups (**A**) and in individual fish (**B, C, D, E**) at genus level.

In the figure, all bacteria with an overall abundance of ≥0.5% were reported, while bacteria with an abundance of ≤1% were regrouped and indicated as “Others”.

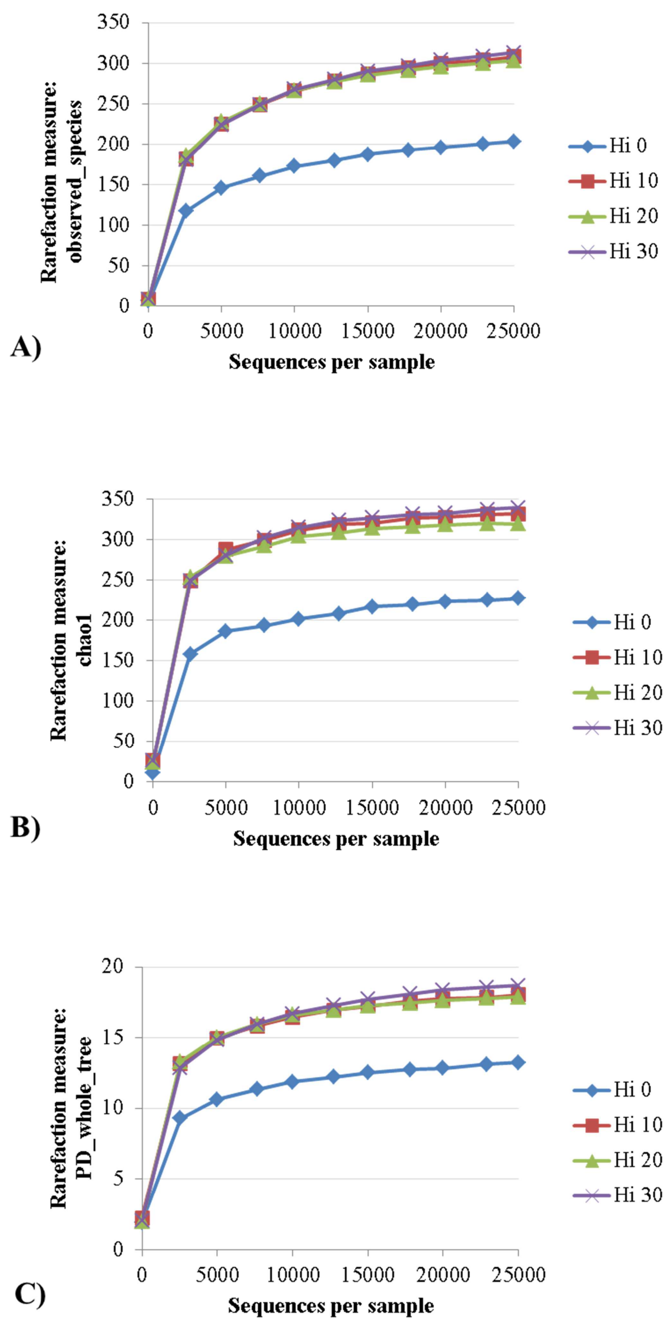


Figura 4.6 Alpha diversity metrics.

Rarefaction curves of fecal microbial communities from trout fed four tested diets, normalized at the lowest sample size (25,000 reads). (**A**) Observed species; (**B**) Species richness (Chao1); (**C**) PD whole tree. Data points represent the mean values (n=6).

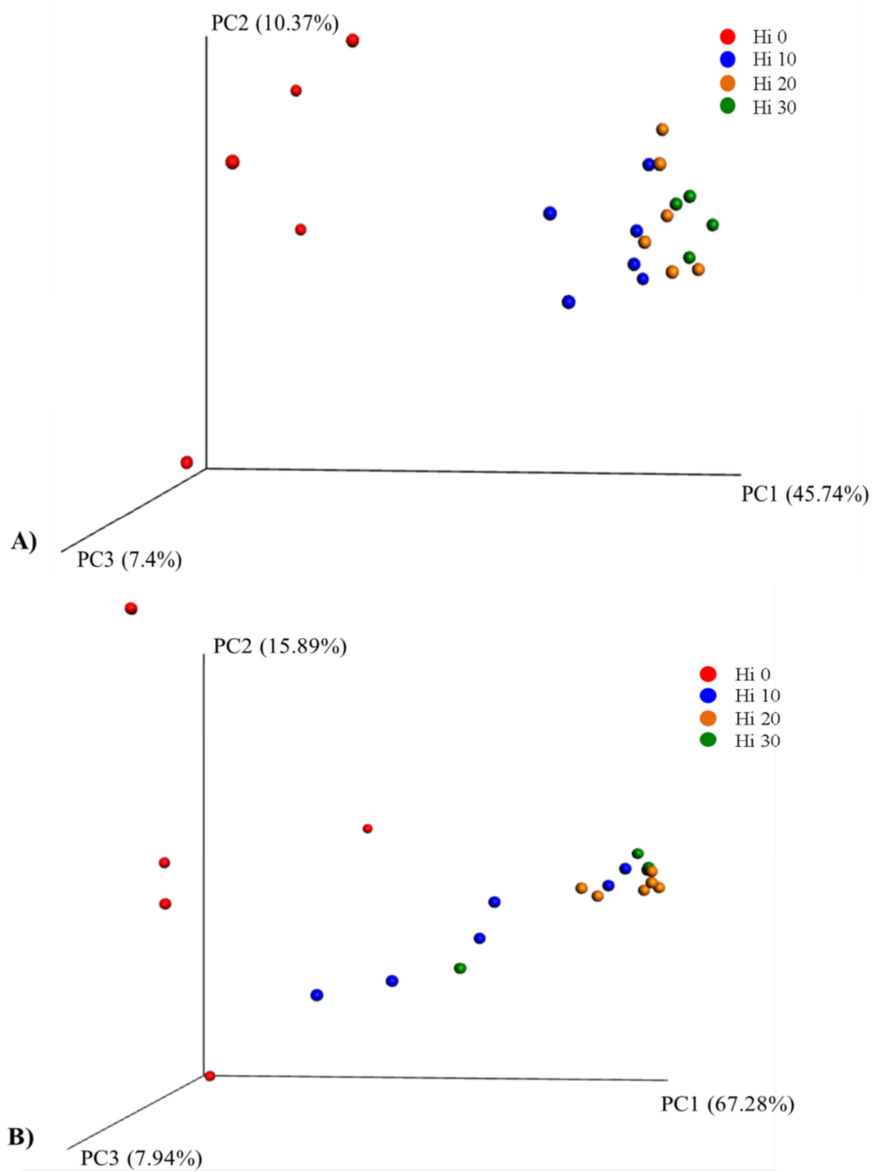


Figure 4.7 Beta diversity metrics.

Principal Coordinate Analysis (PCoA) of Weighted (**A**) and Unweighted (**B**) Unifrac distances of gut microbial communities associated to four experimental diets. Each dot represents an individual sample plots according to its microbial profile at genus level.

Table 4.7 Permutational multivariate analysis of variance (Adonis) and Analysis of similarity (ANOSIM) on weighted and unweighted UniFrac data of intestinal microbiomes of trout fed with four experimental diets.

	Adonis				**ANOSIM**			
	Unweighted		**Weighted**		**Unweighted**		**Weighted**	
	p-value	R^2	*p*-value	R^2	*p*-value	R	*p*-value	R
Hi 0 vs Hi 10	0.002	0.46	0.001	0.43	0.002	0.88	0.005	0.70
Hi 0 vs Hi 20	0.004	0.51	0.004	0.66	0.005	0.93	0.004	0.91
Hi 0 vs Hi 30	0.009	0.55	0.013	0.55	0.005	0.93	0.004	0.91
Hi 10 vs Hi 20	0.05	0.14	0.017	0.39	0.079	0.18	0.017	0.32
Hi 10 vs HI 30	0.02	0.21	0.144	0.23	0.106	0.20	0.236	0.07
Hi 20 vs Hi 30	0.161	0.14	0.668	0.06	0.783	-0.12	0.29	0.07

4.2.5 Changes in rainbow trout gut microbiota composition produced by insect-based diet

The gut microbial community of our trout was mainly dominated, regardless of the diet, by three phyla: *Actinobacteria*, *Firmicutes* and *Proteobacteria* (Fig. 4.3). Among them, amount of *Actinobacteria* and *Proteobacteria* were significantly influenced ($p < 0.05$) by including insect meal in the diet (Fig. 4.3 A, Table 4.8). Specifically, the relative abundance of *Actinobacteria* was higher in fish fed with diets containing Hi meal than in control Hi 0. Conversely, 20% and 30%, but not 10%, of FM substitution with insect meal caused a significant decrease in *Proteobacteria* abundance compared to the FM-based dietary group, whose gut microbiota was instead enriched with bacteria belonging to the *Alphaproteobacteria* and *Gammaproteobacteria* classes (Table 4.8). At the family level, Hi dietary inclusion was associated with an increased proportion of *Actinomicetaceae*, *Brevibacteriaceae*, *Corynebacteriaceae*, and *Microbacteriaceae* (Table 4.7, Fig. 4.4). Among *Actinomycetales*, only *Propionibacteriaceae*

were reduced in the intestinal bacterial microflora of the Hi 30 group of fish compared to controls. Higher percentage of bacteria assigned to *Planococcaceae* and *Staphyloccaceae* were found in gut of trout fed with diet Hi 20 and Hi 30, while the number of *Bacillaceae* was only slightly negatively affected by insect meal-based diets (Fig 4.4 A, Table 4.8).

In fish fed with insect meal, a higher percentage of *Lactobacillales* was found. The increased proportion of *Lactobacillales* was due to a significant enrichment in lactic acid bacteria (LAB) belonging to *Aerococcaceae*, *Enterococcaceae*, *Lactobacillaceae*, and *Leuconostocaceae* families in comparison to the control group. In contrast, fish fed the Hi 0 diet were characterized by a higher percentage of *Carnobacteriaceae* and *Streptococcaceae* (Fig. 4.4 A, Table 4.7). Similarly, the proportion of *Enterobacteriaceae* was significantly ($p < 0.05$) higher in the control group, so much so that they represented the dominant bacterial family (50.4 ± 8.57%) in these samples (Fig. 4.4 A, Table 4.8).

Accordingly, the number of bacteria assigned to *Actinomyces, Brevibacterium, Corynebacterium, Leucobacter,* and *Staphylococcus* genera were positively influenced by dietary Hi meal supplementation (Fig. 4.5, Table 4.7). Among LAB, *Facklamia*, *Enterococcus*, *Lactobacillus*, and *Pediococcus* genera were significantly enriched in Hi 10, Hi 20 and Hi 30 samples (Fig 4.5 A, Table 4.8). At the species level, the number of unassigned bacteria ranged between 80 and 90% thus making a comparison between the two groups meaningless at this taxonomical level.

Table 4.8 Relative abundance of most abundant taxa presented as mean ± SEM. Means in the same row with different letters are significantly different ($p < 0.05$). "n.d." means not detected.

	Hi 0	**Hi 10**	**Hi 20**	**Hi 30**
Phylum				
Actinobacteria	2.94 ± 1.16 ^{b}	27.30 ± 3.31^{a}	35.56 ± 1.37 ^{a}	34.08 ± 3.58 ^{a}
Firmicutes	30.39 ± 9.76	40.38 ± 4.79	55.77 ± 1.55	54.66 ± 4.32
Proteobacteria	66.62 ± 9.43 ^{a}	32.16 ± 7.59^{a}	8.59 ± 2.13 ^{b}	11.21 ± 7.77 ^{b}
Class				
Actinobacteria	2.94 ± 1.16 ^{b}	27.27 ± 3.30 ^{ab}	35.53 ± 1.37 ^{a}	34.05 ± 3.58 ^{a}
Bacilli	29.22 ± 9.69	37.18 ± 4.35	50.56 ± 1.44	48.69 ± 4.36
Clostridia	1.17 ± 0.36 ^{b}	2.70 ± 0.45 ^{ab}	4.43 ± 0.40 ^{a}	5.01 ± 0.36 ^{a}
Alphaproteobacteria	15.08 ± 9.86	5.99 ± 0.64	3.54 ± 0.65	2.31 ± 0.58
Betaproteobacteria	0.87 ± 0.11	2.39 ± 1.42	0.58 ± 0.30	0.17 ± 0.04
Gammaproteobacteria	50.67 ± 10.32 ^{a}	23.79 ± 7.46 ^{ab}	4.47 ± 2.21 ^{b}	8.73 ± 8.25 ^{ab}
Order				
Actinomycetales	2.94 ± 1.16 ^{b}	26.94 ± 3.34 ^{a}	35.02 ± 1.43 ^{a}	33.58 ± 3.54 ^{a}
Bacillales	6.23 ± 2.12 ^{b}	13.57 ± 1.99 ^{ab}	20.53 ± 1.32 ^{a}	20.43 ± 1.73 ^{a}
Lactobacillales	22.98 ± 10.73	23.60 ± 2.66	30.02 ± 1.12	28.26 ± 2.74
Clostridiales	1.17 ± 0.36 ^{b}	2.70 ± 0.45 ^{ab}	4.43 ± 0.40 ^{a}	5.01 ± 0.36 ^{a}
Rhizobiales	1.18 ± 0.21 ^{b}	4.08 ± 0.61 ^{a}	2.86 ± 0.66 ^{a}	1.16 ± 0.19 ^{b}
Rickettsiales	13.63 ± 9.65 ^{a}	1.63 ± 0.57 ^{b}	0.61 ± 0.37 ^{b}	1.11 ± 0.46 ^{b}
Enterobacteriales	43.44 ± 9.36 ^{a}	21.61 ± 7.09 ^{ab}	3.49 ± 1.87 ^{b}	8.41 ± 8.13 ^{ab}
Family				
Actinomycetaceae	0.26 ± 0.08 ^{b}	12.79 ± 1.33 ^{a}	17.49 ± 0.46 ^{a}	15.68 ± 1.58 ^{a}
Brevibacteriaceae	0.10 ±0.03 ^{b}	0.65 ± 0.04 ^{ab}	1.38 ± 0.16 ^{a}	1.12 ± 0.32 ^{a}
Corynebacteriaceae	1.23 ± 0.42 ^{b}	10.74 ± 1.78 ^{a}	12.33 ± 0.94 ^{a}	13.04 ± 1.40 ^{a}
Microbacteriaceae	0.59 ± 0.25 ^{b}	2.13 ± 0.20 ^{a}	2.71 ± 0.28 ^{a}	3.34 ± 0.56 ^{a}
Propionibacteriaceae	1.31 ± 0.61	0.51 ± 0.15	0.58 ± 0.18	0.14 ± 0.05
Bacillaceae	5.91 ± 2.89	1.21 ± 0.07	1.54 ± 0.19	1.94 ± 0.73
Planococcaceae	0.46 ± 0.18	1.32 ± 0.23	2.16 ± 0.15	1.83 ± 0.25
Staphylococcaceae	1.08 ± 0.38 ^{b}	10.17 ± 1.65 ^{a}	15.63 ± 1.15 ^{a}	15.13 ± 1.99 ^{a}
Aerococcaceae	0.40 ± 0.31 ^{b}	3.26 ± 0.63 ^{ab}	4.00 ± 0.25 ^{a}	4.10 ± 0.69 ^{a}
Carnobacteriaceae	4.16 ± 1.73	0.83 ± 0.47	0.33 ± 0.05	0.27 ± 0.06
Enterococcaceae	1.36 ± 0.49 ^{b}	6.48 ± 0.78 ^{a}	8.52 ± 0.27 ^{a}	8.17 ± 0.90 ^{a}
Lactobacillaceae	0.55 ± 0.20 ^{b}	10.36 ± 1.69 ^{a}	12.93 ± 0.79 ^{a}	11.89 ± 0.97 ^{a}

Leuconostocaceae	0.06 ± 0.02 [b]	2.63 ± 0.39 [a]	3.91 ± 0.31 [a]	3.71 ± 0.62 [a]
Streptococcaceae	17.96 ± 10.08 [a]	0.07 ± 0.02 [ab]	0.06 ± 0.01 [ab]	0.03 ± 0.00 [b]
Clostridiaceae	1.25 ± 0.54	1.28 ± 0.26	2.01 ± 0.32	2.31 ± 0.44
Erysipelotrichaceae	n. d. [b]	0.50 ± 0.09 [ab]	0.79 ± 0.05 [a]	0.97 ± 0.14 [a]
Bartonellaceae	0.72 ± 0.50 [b]	2.99 ± 0.53 [a]	1.67 ± 0.65 [ab]	0.33 ± 0.12 [b]
Alcaligenaceae	0.02 ± 0.01	1.70 ± 1.21	0.06 ± 0.05	0.07 ± 0.04
Aeromonadaceae	6.52 ± 5.87	2.02 ± 0.96	0.53 ± 0.32	0.06 ± 0.03
Shewanellaceae	1.23 ± 1.20	0.07 ± 0.05	0.38 ± 0.36	n. d.
Enterobacteriaceae	50.45 ± 8.57 [a]	22.10 ± 7.31 [ab]	3.49 ± 1.87 [b]	0.32 ± 0.16 [b]
Genus				
Actinomyces	0.25 ± 0.07 [b]	12.29 ± 1.30 [a]	16.86 ± 0.44 [a]	15.08 ± 1.51 [a]
Brevibacterium	0.10 ± 0.03 [b]	0.65 ± 0.04 [ab]	1.38 ± 0.16 [a]	1.12 ± 0.32 [a]
Corynebacterium	1.23 ± 0.42 [b]	10.74 ± 1.78 [a]	12.33 ± 0.94 [a]	13.04 ± 1.40 [a]
Leucobacter	0.14 ± 0.08 [b]	1.76 ± 0.16 [a]	2.27 ± 0.25 [a]	2.83 ± 0.48 [a]
Bacillus	3.60 ± 2.00	0.76 ± 0.07	1.10 ± 0.18	1.66 ± 0.75
Oceanobacillus	1.96 ± 0.75 [a]	0.24 ± 0.04 [ab]	0.19 ± 0.03 [ab]	0.10 ± 0.01 [b]
Paenibacillus	0.99 ± 0.52	0.12 ± 0.03	0.23 ± 0.05	0.27 ± 0.06
Staphylococcus	0.97 ± 0.34 [b]	9.69 ± 1.54 [a]	15.13 ± 1.11 [a]	14.66 ± 1.89 [a]
Aerococcus	n. d. [b]	0.61 ± 0.13 [a]	0.68 ± 0.04 [a]	0.42 ± 0.07 [a]
Facklamia	0.07 ± 0.02 [b]	2.34 ± 0.46 [a]	2.84 ± 0.20 [a]	3.22 ± 0.57 [a]
Enterococcus	1.32 ± 0.48 [b]	6.36 ± 0.77 [a]	8.35 ± 0.27 [a]	7.93 ± 0.87 [a]
Lactobacillus	0.32 ± 0.08 [b]	5.98 ± 1.00 [a]	7.33 ± 0.59 [a]	6.75 ± 0.43 [a]
Pediococcus	0.23 ± 0.14 [b]	4.28 ± 0.68 [a]	5.45 ± 0.29 [a]	5.03 ± 0.53 [a]
Clostridium	1.05 ± 0.43	0.49 ± 0.18	0.66 ± 0.18	0.89 ± 0.49
SMB53	0.04 ± 0.02 [b]	0.77 ± 0.12 [ab]	1.30 ± 0.23 [a]	1.32 ± 0.11 [a]

4.3 Discussion

Interest in using insects as animal feed is growing mainly in the aquaculture sector. In fact, insects, in addition to being part of the natural diet of fish, represent a good source of essential amino acids, lipids and minerals (Henry et al., 2015); they grow and reproduce quickly on low-quality organic products (agricultural waste or agro-industrial); and they demonstrate a high feed conversion efficiency and

limited environmental contamination (Makkar et al., 2014; van Huis, 2013). In particular, compared to other species, *Hermetia illucens* shows an essential aminoacid profile very similar to that of fishmeal (Henry et al., 2015) and is therefore considered a good alternative protein source. Recently, many studies investigated the effect of partial FM substitution with insect meal on fish growth performances (Henry et al., 2015; Lock et al., 2016; Magalhães et al., 2017; Renna et al., 2017; Xiao et al., 2018), showing that it effectively supports fish growth. Conversely, the data available regarding the effect of an insect-based diet on intestinal microbial composition in fish are limited (Bruni et al., 2018). Insects are rich in chitin, a polysaccharide that reduces their digestibility, but, according to Karlsen et al. (2017) dietary chitin could stimulate the growth of beneficial bacteria in the fish intestine.

The substitution of up to 30% of FM with a partially defatted Hi larva meal did not affect survival, indicating that all the experimental diets were well accepted by fish, which tripled their initial body weight. Moreover, fish performance growth rates (Table 4.4), in particular FCR values in all four groups were 0.9, which is much lower than 1.3, a mean FCR value estimated for cultured rainbow trout (Tacon and Metian, 2008). Like our results, previous studies showed that including up to 40% of Hi meal did not influence fish growth and conversion efficiency (Renna et al., 2017; Stadtlander et al., 2017). In addition, in the study conducted by St-Hilaire et al. (2007) in trout, growth performance was not affected in fish fed diets with 25% Hi prepupae, conversely to results obtained in fish fed the diet containing 50% of insect meal, which presented a higher FCR value.

As reported in Bruni et al. (2018) and in the other studies conducted in trout, intestinal microbiome sequencing showed that *Firmicutes*, *Proteobacteria* and *Actinobacteria* were the most dominant phyla

regardless of the diet administered. These phyla together with *Bacteroidetes* compose the core gut microbiota (Desai et al., 2012; Ghanbari et al., 2015; H. C. Ingerslev et al., 2014; Hans Christian Ingerslev et al., 2014; Navarrete et al., 2012; Wong et al., 2013). These taxa represent up to 90% of fish intestinal microbiota in different marine and freshwater species (Apper et al., 2016; Rimoldi et al., 2018; Ringø et al., 2016), indicating that these bacteria are involved in important host functions (Ghanbari et al., 2015).

Alpha diversity metrics showed that an insect-based diet influenced positively bacterial richness and diversity (Table 4.6), as Bruni et al. (2018) also found. Similarly, the dietary inclusion of 5-20% of chitin in salmon increased intestinal microbial community diversity (Askarian et al., 2012; Ringø et al., 2012). Conversely, replacing FM with a mixture of terrestrial animal and vegetable proteins did not cause significant changes in the intestinal microbial richness either according to in alpha diversity indices or observed species number (Apper et al., 2016; Rimoldi et al., 2018). The lack of effect on diversity could be considered beneficial, as a reduction in diversity may provide less competition for opportunistic or invading pathogens that may enter the gastrointestinal tract of fish via feed or water (Apper et al., 2016).

Insect dietary inclusion also influenced significantly the relative abundance of microbial taxa. Indeed, beta diversity measurements revealed that there was a significant relationship between diet type and microbiota associated to fish intestine. Weighted Unifrac PCoA showed a clear clustering of samples by diet, which was statistically validated by ANOSIM and Adonis tests.

In our study, the four experimental diets showed different amounts of the most highly represented taxa. In particular, control feed was characterized by a higher amount of *Gammaproteobacteria* class

(*Proteobacteria* phyla), while the three insect-based feeds contained greater percentages of *Bacilli* and *Actinobacteria* (belonging to *Firmicutes* and *Actinobacteria* phyla, respectively). As demonstrated in several studies, members of *Firmicutes* phyla are associated with plant ingredients (Desai et al., 2012; Heikkinen et al., 2006), while the amount of *Proteobacteria* is higher with animal protein ingredients (Desai et al., 2012; H. C. Ingerslev et al., 2014; Hans Christian Ingerslev et al., 2014). This can be explained by the fact that insect larvae were reared on vegetable substrates. Clearly, we found the same trend in studying gut microbiota in our experimental samples. In particular, fish fed Hi meal presented an increase in the relative abundance of lactic bacteria belonging to *Staphylococcaceae*, *Lactobacillaceae*, and *Leuconostocaceae* families, which play an important role in degrading complex carbohydrates that are otherwise indigestible (cellulose, hemicellulose, and pectin), producing metabolic end products such as short chain fatty acids. In accordance with our results, Bruni et al. (2018) found lactic bacteria in the fecal samples of trout fed with Hi meal, but not in the control group. In their study, the total absence of this microbial group in the trout fed a FM-based diet was probably due to the low of detection quality of the DGGE technique compared to the NGS approach used in our research. Nevertheless, the increasing number of lactic bacteria could be promoted by chitin, similarly to that observed with a vegetable meal-based diet (Desai et al., 2012). In our study, many microbial genera belonging to *Lactobacillales*, *Actinomycetales*, and *Bacillales* were affected by diet. In particular, *Aerococcus*, *Enterococcus*, *Lactobacillus*, and *Pediococcus* genera increased in trout fed the Hi supplement, regardless of the amount. The increased number of lactic bacteria could produce essential defense effects against the proliferation

of pathogenic bacteria, such as *Staphylococcus aureus*, *Streptococcus agalactiae*, and *Pseudomonas aeruginosa*, producing bactericidal compounds, such as lactic acid and hydrogen peroxide or surfactants, that create a biofilm able prevent pathogens from adhering to the intestinal surface (Gudiña et al., 2015). Moreover, we found in fish fed with insect meal a strong increase in genera belonging to the *Actinobacteria* phylum, specifically, *Actinomyces*, known as cellulose-degrading bacteria (Liu et al., 2018), and *Corynebacterium*, which produces secondary metabolites with antibacterial activity against potential pathogens (Gladysheva et al., 2017). These observations indicate that the dietary treatment might be beneficial for potentially favorable bacteria.

In conclusion, the present study demonstrates that substituting FM by up to 30% with Hi meal is well tolerated, without affecting growth performance, and positively modifies gut microbial composition, increasing its richness and diversity. In particular, partial substitution increases the amount of lactic bacteria and other taxa that promote healthy activity in the host.

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CHAPTER 5

CONCLUSIONS

The continuous search for alternative protein sources in the aquafeed industry has generated numerous questions regarding the effects of these alternatives to fishmeal (FM) protein sources on fish physiology and health. Many studies have focused on the effects of dietary substitution in term of fish growth performance, fillet quality, intestinal health, and transcriptional response. Recently, interest in fish intestinal microbial composition has increased as bacteria associated with the epithelium of an animal's digestive tract play a crucial role in establishing and maintaining their host's health.

Molecular methods such as high-throughput sequencing of the 16S rRNA gene will undoubtedly facilitate the definition of microbial diversity in the normal gut and how the bacterial communities respond to changes in diet. In addition, better knowledge on the gut microbiota response in farmed fish could contribute to being better able to apply pre- and probiotic products in aquafeed.

In summary, our results showed no negative effects on fish growth performance after partial FM replacement with poultry by-products, autolyzed yeast, or prepupal insect meal. Conversely, strong effects on intestinal microbial community composition were found. Specifically, including autolyzed yeast and insect meal in the diet affected positively the gut microbiota biodiversity, increasing the amount of beneficial bacteria, such as lactic bacteria.

In conclusion, our results comprise a valuable contribution to the aquaculture feed industry and provide the knowledge needed to design appropriate feed formulations for farmed fish.