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TOWARDS A MORE SUSTAINABLE AQUACULTURE: DIETS EFFECTS AND ENVIRONMENTAL IMPACT OF AQUACULTURE FROM A MICROBIOME PERSPECTIVE

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ABSTRACT

Today, there is a lot of interest to optimize aquaculture production due to its overexploitation of marine resources, ocean pollution and habitat destruction. Since feed production is one of the greatest costs in aquaculture production and one of the greatest issues in term of overexploitation of marine resources, feeding strategy optimization is becoming increasingly important. For this reason, the study of several different feed additives or supplementation is taking an increasing interest in the scientific community to secure optimal growth, gut health and function, and generally a wellbeing in farmed fish. Generally, feed additives are used for their nutritional value and for their promoting and disease preventing properties. These functional feeds are typically supplied to ensure good health and to help the animal ward off pathogens during both normal and challenging conditions, the latter of which could stress the animals and promote the insurgence of pathologies or pathogens invasions. In this context has an increasing interest the study of host associated microbiome to better understand the influence of novel functional feed on the health and physiology of farmed fish. In addition to achieve a more sustainable and efficient aquaculture sector, also show a great importance the understanding of the environmental impact of this human activity in terms of habitat destruction, ocean pollution and reduction marine environments biodiversity. Marine microbiomes either free-living or associated with multicellular hosts, is acquiring an increasing interest in marine biology because their determinant role in supporting the functioning and biodiversity of marine ecosystems, providing essential ecological services, and promoting the health of the entire biosphere. Becoming extremely important to understand how these potentially ecosystem-damaging activities can affect marine microbiomes by altering their function and diversity.

In this thesis work, here we were able to present a comprehensive evaluation of different functional feeds, with different feeds or supplementations, assessing their effects in terms of growth and gut health of farmed fish in normal or challenging conditions, in three species Rainbow Trout (*Oncorhynchus mykiss*), Gilthead seabream (*Sparus aurata*) and Zebrafish (*Danio rerio*). We also explored the impact of Aquaculture on the surrounding marine microbiomes, using *Patella caerulea* as a model holobionts. Finally, we provided a synoptical study on the microbiomes of the water column and surface sediments in a 130 km^2 located 13.5km afar from the coast in North-Western Adriatic Sea (Italy), providing the finest-scale mapping of marine microbiomes in the Mediterranean Sea.

~ CHAPTER 1 ~

1. INTRODUCTION

1.1 Aquaculture and related environmental impact

The term aquaculture defines the farming of marine organisms such as fish, crustaceans, mollusks, and seaweed in delimited environments controlled by humans. Based on the entity and type of human intervention were defined three aquaculture types: *extensive* (fish feeding consist of only natural resources and farmer do not take part during the breeding process, except for the seeding of juveniles' individuals), *intensive* (fish are directly fed by farmer and are bred in marine cage or artificial basin) and *semi-intensive* (Ottinger et al., 2016). In the last decades aquaculture was involved in a remarkable development, with an increasing mean production per year of 8.3% from 1970 to 2008 (Martinez-Porchas & Martinez-Cordova, 2012). Due the rapidly world demographic increase, in 2020 the world's proteins consumption was derived from foods of marine origin (Jones et al., 2020)., with global aquaculture production of fish and shellfish providing around the 52% of the total fish human consumption (FAO, The State of World Fisheries and Aquaculture 2020), while in Europe is around 20%. The development of this sector is favored by the low feed conversion ratio (FCR), defined as the ratio between the feed quantity and obtained biomass (Fraga-Corral et al., 2022). Taken together, these aspects show that aquaculture could replace fishing for human needs in the future. However, a number of related issues are to be overcome, including (I) the destruction of natural habitats and ecosystems to provide space for the construction of aquaculture facilities, (II) the use of bioactive compounds on farms, such as antibiotics and hormones, which can cause contamination of fresh or marine waters, (III) the changes caused by farms to the surrounding ecosystems mediated by nutrient and organic discharge in waters (*eutrophication*) and chemical alterations of water and sediments, and (IV) the overexploitation of marine resources for feed production (Ottinger et al., 2016) (Figure 1).

This last aspect is one of the most important to improve the sustainability of aquaculture production and to reduce its environmental impact since the produced feeds for aquaculture use are mainly constituted by ingredients derived from fishing. Indeed, fish bred through aquaculture, especially carnivorous species, needed in their diet the presence of fishmeal (FM) and fish oil (FO) as, respectively, source of protein and fatty acids (especially omega 3) (EUMOFA, Fishmeal and Fish Oil: Production and Trade Flows in the EU, 2021). This implies that globally every year 15 million tons of fished fish (over 90 million) are used for FM and FO production (EUMOFA, 2021). This emergency led in the last decades increasing research to reduce the use of FM and FO in fish feeds to reduce the environmental impact of the aquaculture production, through using of several replacement products (e.g., plant-based products or insect-based products, used of by-products from fishing and aquaculture) (Roques et al., 2020). However, these plant-based products used as a substitute for FM and FO often have some negative aspects, including an incorrect ratio of amino acids needed for the animal's development, difficulties in digestion, and reduced palatability (Serra et al., 2021). In addition, the use of FM and FO for aquaculture production is becoming more and more unsustainable due to their increasing price, indeed in the last 12 years the price of FM grew by 37% while that of FO grew by 85%, in Europe (EUMOFA, 2021). All these aspects clearly show how it is now essential to find a way to progressively reduce and eventually substitute these two ingredients in fish feeds.

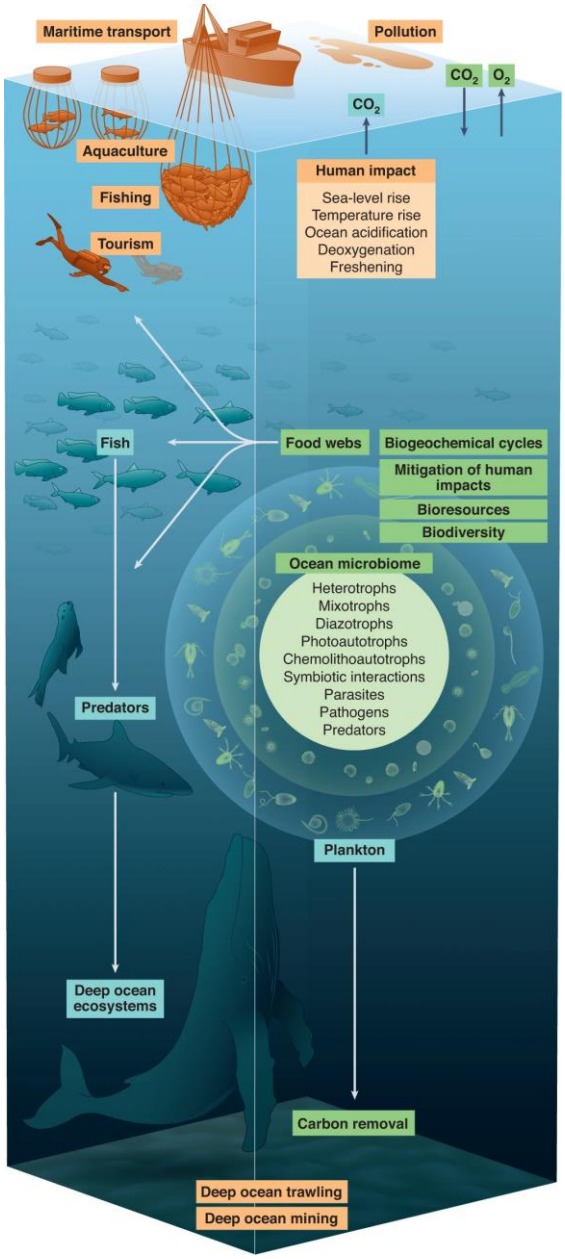

Figure 1. The ocean microbiome (centre) is composed of a range of prokaryotes, eukaryotic microbes and viruses, which have a range of different lifestyles driving microbial interactions. The essential ecosystem services they provide, such as biogeochemical cycling (for example CO_2 capture, O_2 generation and carbon removal), mitigation of human activities, bioresources, biodiversity and resources for the entire ocean food web, are shown in green. Human impacts on the ocean are indicated in orange. Other aspects of the ocean environment are indicated in blue, and interconnections between different components are shown with arrow

Another main aspect regarding the aquacultures' environmental impact are the direct negative effects on the surrounding waters and sediments, including an organic pollution and *eutrophication* (described as a buildup of excess nutrients, primarily organic nitrogen, and phosphorus) of the surrounding environment and the release of chemicals used during breeding (e.g., antibiotics, pesticides, hormones, anesthetics, pigments, minerals, and vitamins (Goldburg et al., 2001; JSA, 2007). For example, it was described that sediments below aquaculture cages (and in the proximity) showed an increase in organic matter, due to the sedimentation of uneaten feed and fish feces (Kalantzi et al., 2021; Moncada et al., 2019), causing shifts in nutrients and carbon fluxes, pH decline, ammonia and hydrogen sulfide accumulation (Holmer et al., 2003; San Diego-McGlone et al., 2008). Concerning the chemicals used in aquaculture activities, the most important on which it was posed more attention were antibiotics, indeed a concern about antibiotic use is that it may leading to the

diffusion of antibiotic resistance (across the environmental microorganisms) and other toxic effects (Brown, 1989; Cole et al., 2009). These problems together can cause, in the surrounding environment, algal bloom, depletion of oxygen, general reduction in water quality, death of corals and habitat destruction (Boesch et al., 2001; Aubin et al., 2006). In addition, it was seen that certain microorganisms that thrive in these specific conditions could be directly harmful to fish (both farmed and wild fish) through biologic and neurologic toxins (Aubin, 2006). Lastly, another crucial aspect of aquaculture impact is the diseases and parasites which could proliferate in farms, and which could diffuse in wild fish stock, causing a depletion of natural fish stocks (Finstad et al., 2000; Krkošek et al., 2007).

As described in the previous paragraphs, microbiome plays a crucial role as a life support system for the planet biosphere (both animals and environments), there is now a huge attention to understanding the impact of local and global anthropogenic factors on the planet microbiomes (Cavicchioli et al., 2019), making microbiome assessment a central point for a holist evaluation of environmental health. Specifically, for what concern aquaculture, the impact on seafloor microbiomes has recently been explored, highlighting the overgrowth of microbial groups able to live in anaerobic and carbon enriched conditions, a general bacterial biodiversity reduction, as well as an accumulation of fecal bacteria (G. M. Luna et al., 2013; Moncada et al., 2019; Zhang et al., 2020). Even the water column within and outside the aquaculture cages appeared to be different in terms of microbial community (Haro-Moreno et al., 2020), however we knew very few things about the effect of the presence of farming cages on the microbiome of nearby wild organisms. Knowing that host microbiome is directly linked to microorganisms inhabiting the same ecosystem, so it is reasonable to hypothesize that microbiome associated with marine organisms living near to the sea cages are affected by them. This influence could take place both directly, by the transfer of fish microorganisms to the water column and/or sediments, and indirectly, by changing the surrounding bacterial environmental community caused by chemical or organic alterations (Palladino et al., 2021). Taken together, these events could be result in a compositional change of the microbiomes associated with wild holobionts causing cascade impacts on the health and safety of the marine environment. For example, a study recently reported that sponges living in proximity to fishing farm presents a microbial community enriched in microorganisms involved in ammonia oxidation compared to the same sponge species sampled in pristine waters (Baquiran & Conaco, 2018).

All together these aspects pose the necessity to optimize aquaculture farms both in terms of feed production and direct environmental impact. Clearly these optimizations must take place without affecting the fish health status and production, in this context is emerging the study of microbiome associated with fish as a key aspect to evaluate the animals' wellbeing, with a focus on the gut microbiome.

1.2 Fish gut microbiota and its importance

Living organisms are associated with million microorganisms which populate several niches defining different microbiota associated with several tissues. For example, the human being offers multiple niches for these microbial communities (e.g., skin, gastrointestinal tract, reproductive organs etc.), in which microorganisms have a symbiotic interaction offering multiple features and functions to living organisms (Adair & Douglas, 2017). Similarly, fish host more than one microbiota associated with different tissues (e.g., gills, skin, gastrointestinal mucosa, gastrointestinal tract etc.) and all these microorganisms have a crucial role in several aspects of organisms' life (Vatsos, 2017). Among all of them, the gut microbiota (GM) is one of the most studied in last years due to its important role in several animals' physiological aspects and the importance regarding the onset of pathological conditions (Nicholson et al., 2012). Indeed, GM have a strict

symbiotic interaction with host, this interaction leads to positive aspects to both parts (both microorganisms and animal), specifically host give to microorganisms habitat and nutrients for development and sustain, while microorganisms provide to animals a set of additional functionalities (Nicholson et al., 2012). Among these, microorganisms exert functions connected to host metabolism for the production of several secondary metabolites. For example, specific bacteria denominates LAB, *Lactic Acid Bacteria*, are able to metabolize through fermentation plant origin carbohydrates and transform them into secondary metabolites utilized by host such as SCFA, *Short Chain Fatty Acids* (Refstie et al., 2005). Furthermore, bacteria of Fusobacteria phylum are able to synthesize B12 vitamin. In addition, the fish gut microbiome acts directly to prevent the host invasion by pathogens with several mechanisms such as the secretion of antimicrobial molecules, direct competition for nutrients and space, improvement of the host immune response and interaction with endothelial cells with the main goal to modulate the cytokine expression (´ Omez et al., 2008). The connection between the GM and the host immune system is extremely intimate and begins to consolidate as early as the animal's birth. The GM is able to influence the development of the host immune system and the host susceptibility to several pathologies, while at the same time the microbial composition is shaped by the host immune system itself (Nicholson et al., 2012). All these aspects emphasize the crucial role of the GM to maintain the host physiological functions, so understanding the GM composition and interaction with the animals are becoming essential, this knowledge could pose the basis for the manipulation of GM to improve the growth performance and to boost the immune system of the host, especially for aquaculture species (Vargas-Albores et al., 2021). The fish GM could be influenced by several factors including the environment and the diet (especially through using of probiotics) (Egerton et al., 2018). This manipulation regards generally the microbiome portion called "transient", while the *core gut microbiota* is not affected by these changes because are generally microorganisms essential for the host life (Serra et al., 2021). Indeed, thanks to several Next Generation Sequencing (NGS) studies it was observed that the fish core GM is shared between individuals of the same species, and in some cases even between different species (Ghanbari et al., 2015), represented by 5 dominant Phyla Proteobacteria, Firmicutes, Actinobacteria, Bacteroidota and Fusobacteria (Izvekova et al., 2007).

~ CHAPTER 2 ~

2. The study of microbiomes in the aquaculture production and the surrounding environment in the presented Thesis

2.1 Assessment of the effect of several diets in aquaculture production and related effect on the surrounding environment

Nowadays there is an increasing interest in the evaluation of several feed additives or different diets in aquaculture to both optimize the exploitation of marine resources and the growth and health conditions of fish species in aquaculture production. To secure optimal growth, gut health and function in farmed fish, there is now particular focus on various feed additives used for their nutritional value, and also for their health-promoting and disease-preventing properties. Functional feeds are typically supplied to ensure good health and to help the animal ward off pathogens during both normal and challenging farming conditions (Hernández et al., 2012). In addition, feed production is the greatest cost in the aquaculture sector and can account for up to 60-80% of the overall expenses (Hasan, 2007; M. Luna et al., 2019). So, in the last decades there is an increasing interest to reduce feed cost and reduce the using of limited and expensive protein ingredients in many farmed fish species including sea bass (*Dicentrarchus labrax*), rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) some of the most important finfish species farmed in the Mediterranean area (Guillen, 2019).

In the presented thesis were assessed the effects of several feed additives on growth performance, feed utilization and gut bacterial community in different fish species and related to different abiotic conditions which could affect the breeding of these animals. Among these feed additives Organic Acids (OA), are well known to improve performance and health in terrestrial livestock, especially swine and poultry (Dibner & Buttin, 2002; Lückstädt, 2007). However, their utilization as potential growth promoters in aquaculture are more recent and less known (Ng & Koh, 2017). Many advantages are reported in connection with lowered pH of feed and digesta owing to diet OA addition: (i) increase in feed hygiene by inhibiting growth of microbial acid-intolerant species, (ii) increase in digestive enzyme activity, leading to higher nutrient digestibility and feed utilization, (iii) modulation of the host gut microbiota and animal health (Lückstädt, 2008; Ng & Koh, 2017). Another feed additives worth to be studied are tannins, which are important vegetable bioactive compounds for both human and animal nutrition purposes (Das et al., 2020). Tannins are phenolic compounds, secondary chemicals ubiquitous in woody plants. Recently, tannins have received more attention due to numerous beneficial actions such as antioxidant (Okuda, 2005), anticancer (Cai et al., 2017), antimicrobial and antiviral (Arapitsas & Prado, 2008) activity. However, the potential effects of tannins on animal health remain largely unexplored, especially on farmed fish.

Another crucial aspect during aquaculture production is the evaluation of abiotic factors variation during farming condition which could affect several physiological aspects of animals. Among abiotic factors, water temperature is the most important one, playing a crucial role on metabolism, nutrient utilization, fat deposition and welfare for species high susceptible to thermal seasonal changes and fluctuations, such as gilthead sea bream (Ibarz et al., 2010; Sánchez-Nuño, Sanahuja, et al., 2018). For these reasons it's very important to assess the best value of dietary lipid level during temperature fluctuations, which could take place during fish farming, to prevent negative effects on fish metabolism, digestive enzyme activity and gut bacterial community which at cascade may influence performance and fish health (Couto et al., 2012; García-Meilán et al., 2013; Guerreiro et al., 2016; Sepulveda & Moeller, 2020b; Zarkasi et al., 2016).

At the end, in order to gain a better understanding of the impact of anthropogenic stressors (such as aquaculture production) on marine host-associated microbiome response and environmental microbiome, we propose one holobionts study model and a fine assessment of the diversity and distribution of the marine microbial community in an off-shore area of the Northern Adriatic Sea. To assess the impact of anthropogenic activities and, in particular, of fish farming cages on the microbiome of the surrounding wild holobionts, we select a common grazer gastropod from the genus Patella as a representative fouling holobiont. *Patella caerulea* is a common seaweed grazing marine limpet in all Mediterranean rocky shores (Della Santina & Chelazzi, 1991). As a result of their wide distribution, abundance, and sedentary lifestyle, limpets of this species have been proposed as biomonitors for the local water quality in terms of heavy metal accumulation and organic pollutants (Reguera et al., 2018; Viñas et al., 2018). In addition, limpets are considered keystone species for the coastal ecosystem because they can regulate the degree of algal coverage and, consequently, succession processes in rocky intertidal communities (Coleman et al., 2006). In the last study of this thesis, we have been able to map the variation at the local scale of the pelagic and sediment microbiomes in the Northwestern Adriatic Sea, which could be influenced by anthropogenic activities. The coupled investigation of the pelagic and benthic microbiomes from each sampling site also allowed us to identify connections, exchanges, and isolation of microbial members in the two realms. Obtaining for the first time a granular assessment of the marine microbiome changes at the local scale.

2.2 Technical aspects: sampling and molecular analysis

In order to avoid redundancy in the next paragraphs, common techniques which were repeatedly used throughout different studies are illustrated in this section. In the "Materials and methods" section of each study, only a brief recall of the appropriate protocol is mentioned, with the indication to look in this section for further details. Only techniques that are particular of a specific study are not illustrated here, but in the correspondent study.

Sample collection and DNA extraction

Specimen collection is very different depending on the sample origin and type. Sterility is preserved at the best of the sampling conditions by using sterile containers or previously sterilized tools. After specimen collection, all samples are transported to the laboratory as fast as possible where they are kept at -80 °C until further processing, unless some preliminary step is required before freezing. Total microbial DNA extraction protocols are also dependent on the sample origin and type. Despite these differences, all the DNA extraction methods described in this thesis rely on Qiagen (Hilden, Germany) spin column-based nucleic acid purification kits. Extracted DNA samples are quantified with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at -20 °C until further processing. 16S rRNA gene amplification and sequencing Targeted gene sequencing is performed on the V3-V4 hypervariable region of the 16S rRNA gene. PCR amplification of this region is carried out in a 50-µL final volume containing 25 ng of microbial DNA, 2X KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), and 200 nmol/L of 341F and 785R primers carrying Illumina overhang adapter sequences. The thermal cycle consists of 3 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and a final 5-min step at 72°C (Biagi et al., 2020; Musella et al., 2020). PCR products are purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA). Indexed libraries are prepared by limited-cycle PCR with Nextera technology and cleaned-up as above. Libraries are normalized to 4 nM and pooled. The sample pool is denatured with 0.2 N NaOH and diluted to a final concentration of 6 pM with a 20% PhiX control. Sequencing is

performed on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA).

Bioinformatics and biostatistics

A pipeline combining PANDAseq (Masella et al., 2012) and QIIME 2 (Bolyen et al., 2019) is used to process raw sequences. The “fastq filter” function of the Usearch11 algorithm (Edgar & Bateman, 2010) is applied to retain highquality reads (min/max length = 350/550 bp), that are then binned into amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016). Taxonomy assignment was performed using the VSEARCH algorithm (Rognes et al., 2016) and the SILVA database (December 2017 release) (Quast et al., 2013). All the sequences assigned to eukaryotes (i.e., chloroplasts and mitochondria) or unassigned are discarded. Different metrics, depending on the dataset, are used to evaluate alpha diversity, whereas beta diversity is estimated by computing the unweighted UniFrac distance. All statistical analyses are performed using the R software (R Core Team), version 3.6.1., 6 with the packages “Made4” (Culhane et al., 2005) and “vegan”7. When unweighted UniFrac distances are plotted using the vegan package, the data separation in the Principal Coordinates Analysis (PCoA) is tested using a permutation test with pseudo-F ratios (function “adonis” in the vegan package). Significant data separation was assessed by Kruskal–Wallis test or Wilcoxon rank-sum test, based on the data. When necessary, p-values were corrected for multiple testing with Benjamini-Hochberg method, with a false discovery rate (FDR) ≤ 0.05 considered as statistically significant.

References

´ Omez, G. D. G., Jos´, J., Luis, J., Balc´balcázar, B., Luis Balcázar, J., & Balcázar, B. (2008). A review on the interactions between gut microbiota and innate immunity of fish. *FEMS Immunology & Medical Microbiology*, *52*(2), 145–154. https://doi.org/10.1111/J.1574-695X.2007.00343.X

Adair, K. L., & Douglas, A. E. (2017). Making a microbiome: the many determinants of host-associated microbial community composition. *Current Opinion in Microbiology*, *35*, 23–29. https://doi.org/10.1016/J.MIB.2016.11.002

Albro, P. W., Hall, R. D., Corbett, J. T., & Schroeder, J. (1985). Activation of nonspecific lipase (EC 3.1.1.-) by bile salts. *Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism*, *835*(3), 477–490. https://doi.org/10.1016/0005-2760(85)90117-1

Anson, M. L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *Journal of General Physiology*, *22*, 79–89.

Arantzamendi, L., Roo, F., Hernández-Cruz, C. M., Fernández-Palacios, H., & Izquierdo, M. (2019). Lipid digestion capacity in gilthead seabream (Sparus aurata) from first feeding to commercial size. *Fish Physiology and Biochemistry*, *45*(1), 469–484. https://doi.org/10.1007/s10695-018-0577-y

Arapitsas, P., & Prado, A. (2008). *Antimicrobial and antiviral activity of hydrolysable tannins*. https://doi.org/10.2174/138955708786140990

Aubin, J., Papatryphon, E., Van der Werf, H. M. G., Petit, J., & Morvan, Y. M. (2006). Characterisation of the environmental impact of a turbot (Scophthalmus maximus) re-circulating production system using Life Cycle Assessment. *Aquaculture*, *261*(4), 1259–1268. https://doi.org/10.1016/J.AQUACULTURE.2006.09.008

Baquiran, J. I. P., & Conaco, C. (2018). Sponge-microbe partnerships are stable under eutrophication pressure from mariculture. *Marine Pollution Bulletin*, *136*, 125–134. https://doi.org/10.1016/J.MARPOLBUL.2018.09.011

Barton, B. A. (2002). Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integrative and Comparative Biology*, *42*(3), 517–525. https://doi.org/10.1093/icb/42.3.517

Bell, J. G., & Koppe, W. (2010). Lipids in aquafeeds. In D. R. Turchini, Giovanni M., Ng, Wing-Keong , Tocher (Ed.), *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*. CRC Press, 2010.

Bernet, D., Schmidt, H., Wahli, T., & Burkhardt-Holm, P. (2001). Effluent from a sewage treatment works causes changes in serum chemistry of brown trout (Salmo trutta L.). *Ecotoxicology and Environmental Safety*, *48*(2), 140–147. https://doi.org/10.1006/eesa.2000.2012

Bernfeld, P. (1955). Amylases, alpha and beta. *Methods in Enzymology*, *1*, 149–154.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/o59-099

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. In *Nature Biotechnology* (Vol. 37, Issue 8, pp. 852–857). Nature Publishing Group. https://doi.org/10.1038/s41587-019-0209-9

Bonaldo, A., Isani, G., Fontanillas, R., Parma, L., Grilli, E., & Gatta, P. P. (2010). Growth and feed utilization of gilthead sea bream (Sparus aurata, L.) fed to satiation and restrictively at increasing dietary energy levels. *Aquaculture International*, *18*(5), 909–919. https://doi.org/10.1007/s10499-009-9312-0

Bonvini, E., Bonaldo, A., Mandrioli, L., Sirri, R., & Dondi, F. (2018). Effects of feeding low fishmeal diets with increasing soybean meal levels on growth, gut histology and plasma biochemistry of sea bass. *Animal*, *12*(5), 923–930.

Bruce-Keller, A. J., Richard, A. J., Fernandez-Kim, S.-O., Ribnicky, D. M., Salbaum, J. M., Newman, S., Carmouche, R., & Stephens, J. M. (2020). Fenugreek counters the effects of high fat diet on gut microbiota in mice: Links to metabolic benefit. *Scientific Reports*, *10*(1).

Busti, S., Bonaldo, A., Dondi, F., Cavallini, D., Yúfera, M.,

Gilannejad, N., Moyano, F., Gatta, P., & Parma, L. (2020). Effects of different feeding frequencies on growth, feed utilisation, digestive enzyme activities and plasma biochemistry of gilthead sea bream (Sparus aurata). *Aquaculture*, *529*.

Busti, S., Rossi, B., Volpe, E., Ciulli, S., Piva, A., D'Amico, F., Soverini, M., Candela, M., Gatta, P. P., Bonaldo, A., Grilli, E., & Parma, L. (2020). Effects of dietary organic acids and nature identical compounds on growth, immune parameters and gut microbiota of European sea bass. *Scientific Reports*, *10*(1).

Cai, Y., Zhang, J., Chen, N. G., Shi, Z., Qiu, J., He, C., & Chen, M. (2017). Recent Advances in Anticancer Activities and Drug Delivery Systems of Tannins. *Medicinal Research Reviews*, *37*(4), 665–701. https://doi.org/10.1002/MED.21422

Caipang, C. M. A., Brinchmann, M. F., Berg, I., Iversen, M., Eliassen, R., & Kiron, V. (2008). Changes in selected stress and immune-related genes in Atlantic cod, *Gadus morhua* , following overcrowding. *Aquaculture Research*, *39*(14), 1533–1540. https://doi.org/10.1111/j.1365-2109.2008.02026.x

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cataldi, E., Marco, P. Di, Mandich, A., & Cataudella, S. (1998). Serum parameters of Adriatic sturgeon Acipenser naccarii (Pisces: Acipenseriformes): effects of temperature and stress. In *Comparative Biochemistry and Physiology Part A* (Vol. 121).

Cavicchioli, R., Ripple, W. J., Timmis, K. N., Azam, F., Bakken, L. R., Baylis, M., Behrenfeld, M. J., Boetius, A., Boyd, P. W., Classen, A. T., Crowther, T. W., Danovaro, R., Foreman, C. M., Huisman, J., Hutchins, D. A., Jansson, J. K., Karl, D. M., Koskella, B., Mark Welch, D. B., … Webster, N. S. (2019). Scientists' warning to humanity: microorganisms and climate change. *Nature Reviews Microbiology 2019 17:9*, *17*(9), 569–586. https://doi.org/10.1038/s41579-019-0222-5

Cole, D. W., Cole, R., Gaydos, S. J., Gray, J., Hyland, G., Jacques, M. L., Powell-Dunford, N., Sawhney, C., & Au, W. W. (2009). Aquaculture: Environmental, toxicological, and health issues. *International Journal of Hygiene and Environmental Health*, *212*(4), 369–377. https://doi.org/10.1016/J.IJHEH.2008.08.003

Coleman, R. A., Underwood, A. J., Benedetti-Cecchi, L., Åberg, P., Arenas, F., Arrontes, J., Castro, J., Hartnoll, R. G., Jenkins, S. R., Paula, J., Santina, P. Della, & Hawkins, S. J. (2006). A continental scale evaluation of the role of limpet grazing on rocky shores. *Oecologia*, *147*(3), 556–564. https://doi.org/10.1007/S00442-005-0296-9/TABLES/5

Couto, A., Enes, P., Peres, H., & Oliva-Teles, A. (2012). Temperature and dietary starch level affected protein but not starch digestibility in gilthead sea bream juveniles. *Springer*. https://doi.org/10.1007/s10695-011-9537-5

Culhane, A., Thioulouse, J., & … G. P. (2005). MADE4: an R package for multivariate analysis of gene expression data. *Bioinformatics*, *21*(11), 2789–2790.

Das, A. K., Islam, M. N., Faruk, M. O., Ashaduzzaman, M., & Dungani, R. (2020). Review on tannins: Extraction processes, applications and possibilities. *South African Journal of Botany*, *135*, 58–70. https://doi.org/10.1016/J.SAJB.2020.08.008

Davis, P. S. (1988). Two occurrences of the gilthead, Sparus aurata Linnaeus 1758, on the coast of Northumberland, England. *Journal of Fish Biology*, *33*(6), 951–951. https://doi.org/10.1111/j.1095-8649.1988.tb05545.x

Della Santina, P., & Chelazzi, G. (1991). Temporal organization of foraging in two Mediterranean limpets, Patella rustica L. and P. coerulea L. *Journal of Experimental Marine Biology and Ecology*, *153*(1), 75–85. https://doi.org/10.1016/S0022-0981(05)80007-3

Dibner, J. J., & Buttin, P. (2002). Use of Organic Acids as a Model to Study the Impact of Gut Microflora on Nutrition and Metabolism. *Journal of Applied Poultry Research*, *11*(4), 453–463. https://doi.org/10.1093/JAPR/11.4.453

Dulski, T., Kozłowski, K., & Ciesielski, S. (2020). Habitat and seasonality shape the structure of tench (Tinca tinca L.) gut microbiome. *Scientific Reports*, *10*(4460). https://doi.org/10.1038/s41598-020-61351-1

Edgar, R. C., & Bateman, A. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. https://doi.org/10.1093/BIOINFORMATICS/BTQ461

Egerton, S., Culloty, S., Whooley, J., Stanton, C., & Ross, R. P. (2018). The gut microbiota of marine fish. *Frontiers in Microbiology*, *9*(MAY), 873. https://doi.org/10.3389/FMICB.2018.00873/BIBTEX

Erlanger, B. F., Cooper, A. G., & Cohent, W. (1966). The Inactivation of Chymotrypsin by Diphenylcarbamyl Chloride and Its Reactivation by Nucleophilic Agents. *Biochemistry*, *5*(1), 190–196. https://doi.org/10.1021/bi00865a025

Erlanger, B., Kokowsky, N., & Cohen, N. (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics*, *95*(2), 271–278.

Faggio, C., Piccione, G., Marafioti, S., Arfuso, F., Fortino, G., & Fazio, F. (2014). Metabolic response to monthly variations of Sparus aurata reared in mediterranean on-shore tanks. *Turkish Journal of Fisheries and Aquatic Sciences*, *14*(2), 567–574. https://doi.org/10.4194/1303-2712-v14_2_28

Falcinelli, S., Picchietti, S., Rodiles, A., Cossignani, L., Merrifield, D., & Taddei, A. (2015). Lactobacillus rhamnosus lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. *Scientific Reports*, *5*(9336).

Finstad, B., Bjørn, P. A., Grimnes, A., & Hvidsten, N. A. (2000). Laboratory and field investigations of salmon lice [Lepeophtheirus salmonis (Krøyer)] infestation on Atlantic salmon (Salmo salar L.) post-smolts. *Aquaculture Research*, *31*(11), 795–803. https://doi.org/10.1046/J.1365-2109.2000.00511.X

Fountoulaki, E., Alexis, M. N., Nengas, I., & Venou, B. (2005). Effect of diet composition on nutrient digestibility and digestive enzyme levels of gilthead sea bream (Sparus aurata L.). *Aquaculture Research*, *36*(13), 1243–1251. https://doi.org/10.1111/j.1365-2109.2005.01232.x

Fraga-Corral, M., Ronza, P., Garcia-Oliveira, P., Pereira, A. G., Losada, A. P., Prieto, M. A., Quiroga, M. I., & Simal-Gandara, J. (2022). Aquaculture as a circular bio-economy model with Galicia as a study case: How to transform waste into revalorized by-products. *Trends in Food Science & Technology*, *119*, 23–35. https://doi.org/10.1016/J.TIFS.2021.11.026

Gallardo, M. A., Sala-Rabanal, M., Ibarz, A., Padrós, F., Blasco, J., Fernández-Borras, J., & Sánchez, J. (2003). Functional alterations associated with "winter syndrome" in gilthead sea bream (Sparus aurata). *Aquaculture*, *223*(1–4), 15–27.

García-Meilán, I., Valentín, J. M., Fontanillas, R., & Gallardo, M. A. (2013). Different protein to energy ratio diets for gilthead sea bream (Sparus aurata): Effects on digestive and absorptive processes. *Aquaculture*, *412–413*, 1–7. https://doi.org/10.1016/j.aquaculture.2013.06.031

Ghanbari, M., Kneifel, W., & Domig, K. J. (2015). A new view of the fish gut microbiome: Advances from next-generation sequencing. *Aquaculture*, *448*, 464–475. https://doi.org/10.1016/J.AQUACULTURE.2015.06.033

Givens, C. E. (2012). *A fish tale: comparison of the gut microbiome of 15 fish species and the influence of diet and temperature on its composition*. University of South Carolina.

Goldburg, R. J., Pew Oceans Commission, A., Elliott, M. S., & Naylor, R. L. (2001). *Marine aquaculture in the United States: environmental impacts and policy options*. https://doi.org/10.3/JQUERY-UI.JS

Gómez-Milán, E., & Lozano, M. J. S. M. (2007). Daily and annual variations of the hepatic glucose 6-phosphate dehydrogenase activity and seasonal changes in the body fats of the gilthead seabream Sparus aurata. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *307*(9), 516–526. https://doi.org/10.1002/jez.406

Gras, J. (1983). *Proteínas plasmáticas: fisicoquímica, metabolismo, fisiopatología y clínica de las proteínas extracelulares* (4th ed.). Editorial JIMS, Barcelona.

Guerreiro, I., Enes, P., Rodiles, A., Merrifield, D., & Oliva-Teles, A. (2016). Effects of rearing temperature and dietary short-chain fructooligosaccharides supplementation on allochthonous gut microbiota, digestive enzymes activities and intestine health of turbot (Scophthalmus maximus L.) juveniles. *Aquaculture*

Nutrition, *22*(3), 631–642. https://doi.org/10.1111/anu.12277

Guijarro, A. I., Lopez-Patiño, M. A., Pinillos, M. L., Isorna, E., De Pedro, N., Alonso-Gómez, A. L., Alonso-Bedate, M., & Delgado, M. J. (2003). Seasonal changes in haematology and metabolic resources in the tench. *Journal of Fish Biology*, *62*(4), 803–815. https://doi.org/10.1046/j.1095-8649.2003.00066.x

Guillen, J. (2019). European and Mediterranean Aquaculture data collection and reporting under the STECF. In *medaid-h2020.eu*.

Hani, Y. M. I., Marchand, A., Turies, C., Kerambrun, E., Palluel, O., Bado-Nilles, A., Beaudouin, R., Porcher, J. M., Geffard, A., & Dedourge-Geffard, O. (2018). Digestive enzymes and gut morphometric parameters of threespine stickleback (Gasterosteus aculeatus): Influence of body size and temperature. *PLoS ONE*, *13*(4). https://doi.org/10.1371/journal.pone.0194932

Haro-Moreno, J. M., Coutinho, F. H., Zaragoza-Solas, A., Picazo, A., Almagro-Moreno, S., & López-Pérez, M. (2020). Dysbiosis in marine aquaculture revealed through microbiome analysis: reverse ecology for environmental sustainability. *FEMS Microbiology Ecology*, *96*(12), 218. https://doi.org/10.1093/FEMSEC/FIAA218

Hasan, M. R. (2007). *Global synthesis of feeds and nutrients for sustainable aquaculture development Food and Agriculture Organization of the United Nations*.

Hernández, A. J., Satoh, S., & Kiron, V. (2012). Supplementation of Citric Acid and Amino Acid Chelated Trace Elements in Low-Fish Meal Diet for Rainbow Trout Affect Growth and Phosphorus Utilization. *Journal of the World Aquaculture Society*, *43*(5), 688–696. https://doi.org/10.1111/J.1749-7345.2012.00589.X

Holmer, M., Duarte, C. M., Heilskov, A., Olesen, B., & Terrados, J. (2003). Biogeochemical conditions in sediments enriched by organic matter from net-pen fish farms in the Bolinao area, Philippines. *Marine Pollution Bulletin*, *46*(11), 1470–1479. https://doi.org/10.1016/S0025-326X(03)00281-9

Ibarz, A., Beltrán, M., Fernández-Borràs, J., & Gallardo, M. (2007a). Alterations in lipid metabolism and use of energy depots of gilthead sea bream (Sparus aurata) at low temperatures. *Aquaculture*, *262*(2–4), 470–480. https://doi.org/10.1016/j.aquaculture.2006.11.008

Ibarz, A., Beltrán, M., Fernández-Borràs, J., & Gallardo, M. (2007b). Alterations in lipid metabolism and use of energy depots of gilthead sea bream (Sparus aurata) at low temperatures. *Aquaculture*.

Ibarz, A., Blasco, J., Beltrán, M., Gallardo, M., & Sánchez, J. (2005). Cold-induced alterations on proximate composition and fatty acid profiles of several tissues in gilthead sea bream (Sparus aurata). *Aquaculture*.

Ibarz, A., Blasco, J., Sala-Rabanal, M., Gallardo, Á., Redondo, A., & Fernández-Borràs, J. (2007). Metabolic rate and tissue reserves in gilthead sea bream (Sparus aurata) under thermal fluctuations and fasting and their capacity for recovery. *Canadian Journal of Fisheries and Aquatic Sciences*, *64*(7), 1034–1042. https://doi.org/10.1139/F07-079

Ibarz, A., Padrós, F., Ngeles Gallardo, M. A. ´, Fernández-Borrà, J., Blasco, J., & Tort, L. (2010). Low-temperature challenges to gilthead sea bream culture: review of cold-induced alterations and "Winter Syndrome." *Springer*. https://doi.org/10.1007/s11160-010-9159-5

Infante-Villamil, S., Huerlimann, R., & Jerry, D. R. (2020). Microbiome diversity and dysbiosis in aquaculture. In *Reviews in Aquaculture*. Wiley-Blackwell. https://doi.org/10.1111/raq.12513

Izvekova, G. I., Izvekov, E. I., & Plotnikov, A. O. (2007). Symbiotic microflora in fishes of different ecological groups. *Biology Bulletin 2007 34:6*, *34*(6), 610–618. https://doi.org/10.1134/S106235900706012X

Jesus, G. F. A., Vieira, F. D. N., Silva, B. C., Junior, M. M. D. S., Ushizima, T. T., Schmidt, E. C., Bouzon, Z. L., Pereira, S. A., Pereira, G. V., Martins, M. L., & Mouriño, J. L. P. (2017). Probiotic bacteria may prevent haemorrhagic septicaemia by maturing intestinal host defences in Brazilian native surubins. *Aquaculture Nutrition*, *23*(3), 484–491. https://doi.org/10.1111/anu.12416

Jobling, M., & Peruzzi, S. (2010). Seabreams and Porgies (Family: Sparidae). In M. Jobling & C. Carter (Eds.), *Finfish Aquaculture Diversification* (p. 681). CABI.

Jones, S. W., Karpol, A., Friedman, S., Maru, B. T., & Tracy, B. P. (2020). Recent advances in single cell protein use as a feed ingredient in aquaculture. *Current Opinion in Biotechnology*, *61*, 189–197. https://doi.org/10.1016/J.COPBIO.2019.12.026

Kalantzi, I., Rico, A., Mylona, K., Pergantis, S. A., & Tsapakis, M. (2021). Fish farming, metals and antibiotics in the eastern Mediterranean Sea: Is there a threat to sediment wildlife? *Science of The Total Environment*, *764*, 142843. https://doi.org/10.1016/J.SCITOTENV.2020.142843

Kim, A., Kim, N., Roh, H. J., Chun, W.-K., Ho, D. T., Lee, Y., & Kim, D.-H. (2019). Administration of antibiotics can cause dysbiosis in fish gut. *Aquaculture*, *512*.

Koven, W. (2002). Gilthead sea bream, Sparus aurata. In C. Webster, Carl D., Lim (Ed.), *Nutrient Requirements and Feeding of Finfish for Aquaculture* (p. 448). CABI.

Krkošek, M., Ford, J. S., Morton, A., Lele, S., Myers, R. A., & Lewis, M. A. (2007). Declining wild salmon populations in relation to parasites from farm salmon. *Science*, *318*(5857), 1772–1775. https://doi.org/10.1126/SCIENCE.1148744/SUPPL_FILE/KRKOSEK_SOM_REV1.PDF

Leaver, M. J., Bautista, J. M., Björnsson, T., Jönsson, E., Krey, G., Tocher, D. R., Torstensen, B. E., Jos´, J., Bj¨ Bj¨orn Thrandur Bj¨ornssonbj¨ Bj¨ornsson, B., & Onsson, E. J. ¨. (2008). *Reviews in Fisheries Science Towards Fish Lipid Nutrigenomics: Current State and Prospects for Fin-Fish Aquaculture Towards Fish Lipid Nutrigenomics: Current State and Prospects for Fin-Fish Aquaculture*. https://doi.org/10.1080/10641260802325278

Lückstädt, C. (2007). Acidifiers in animal nutrition: a guide for feed preservation and acidification to promote animal performance. *Acidifiers in Animal Nutrition: A Guide for Feed Preservation and Acidification to Promote Animal Performance.*

Lückstädt, C. (2008). The use of acidifiers in fish nutrition. *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources*, *3*. https://doi.org/10.1079/PAVSNNR20083044

Luna, G. M., Corinaldesi, C., Dell'Anno, A., Pusceddu, A., & Danovaro, R. (2013). Impact of aquaculture on benthic virus–prokaryote interactions in the Mediterranean Sea. *Water Research*, *47*(3), 1156–1168. https://doi.org/10.1016/J.WATRES.2012.11.036

Luna, M., Llorente, I., Cobo, · Angel, & Es, M. L. (2019). Determination of feeding strategies in aquaculture farms using a multiple-criteria approach and genetic algorithms. *Annals of Operations Research*. https://doi.org/10.1007/s10479-019-03227-w

Martinez-Porchas, M., & Martinez-Cordova, L. R. (2012). World aquaculture: Environmental impacts and troubleshooting alternatives. *The Scientific World Journal*, *2012*. https://doi.org/10.1100/2012/389623

Mateus, A. P., Costa, R., Gisbert, E., Pinto, P. I. S., Andree, K. B., Estévez, A., & Power, D. M. (2017). Thermal imprinting modifies bone homeostasis in cold-challenged sea bream (Sparus aurata). *Journal of Experimental Biology*, *220*(19), 3442–3454. https://doi.org/10.1242/jeb.156174

Matias, A. C., Ribeiro, L., Araujo, R. L., & Pousão-Ferreira, P. (2018). Preliminary studies on haematological and plasmatic parameters in gilthead sea bream (Sparus aurata) held under day/night temperature variations. *Fish Physiology and Biochemistry*, *44*(1), 273–282. https://doi.org/10.1007/s10695-017-0432-6

Melis, R., Sanna, R., Braca, A., Bonaglini, E., Cappuccinelli, R., Slawski, H., Roggio, T., Uzzau, S., & Anedda, R. (2017). Molecular details on gilthead sea bream (Sparus aurata) sensitivity to low water temperatures from 1H NMR metabolomics. *Comparative Biochemistry and Physiology - Part A : Molecular and Integrative Physiology*, *204*, 129–136. https://doi.org/10.1016/j.cbpa.2016.11.010

Mininni, A. N., Milan, M., Ferraresso, S., Petochi, T., Di Marco, P., Marino, G., Livi, S., Romualdi, C., Bargelloni, L., & Patarnello, T. (2014). Liver transcriptome analysis in gilthead sea bream upon exposure to low temperature. *BMC Genomics*, *15*(1). https://doi.org/10.1186/1471-2164-15-765

Moncada, C., Hassenrück, C., Gärdes, A., & Conaco, C. (2019). Microbial community composition of sediments influenced by intensive mariculture activity. *FEMS Microbiology Ecology*, *95*(2), 6. https://doi.org/10.1093/FEMSEC/FIZ006

Mongile, F., Bonaldo, A., Fontanillas, R., Mariani, L., Badiani, A., Bonvini, E., & Parma, L. (2014). Effects of Dietary Lipid Level on Growth and Feed Utilisation of Gilthead Seabream (*Sparus Aurata* L.) Reared at Mediterranean Summer Temperature. *Italian Journal of Animal Science*, *13*(1), 2999. https://doi.org/10.4081/ijas.2014.2999

Mongile, F., Bonaldo, A., Fontanillas, R., Mariani, L., Badiani, A., Bonvini, E., Parma, L., Mediche, S., Bologna, U., & Bo, E. (2014). *utilisation of gilthead reared at Mediterranean on co m er ci al us e on l Lipid level seabream at summer temperature on co m m er al*. *13*. https://doi.org/10.4081/ijas.2014.2999

Montoya, A., López-Olmeda, J. F., Garayzar, A. B. S., & Sánchez-Vázquez, F. J. (2010). Synchronization of daily rhythms of locomotor activity and plasma glucose, cortisol and thyroid hormones to feeding in Gilthead seabream (Sparus aurata). *Physiol Behav*, *101*, 101–107.

Mortezaei, F., Royan, M., Noveirian, H. A., Babakhani, A., Kordghashlaghi, H. A., & Balc Azar, J. L. (2020). In vitro assessment of potential probiotic characteristics of indigenous Lactococcus lactis and Weissella oryzae isolates from rainbow trout (Oncorhynchus mykiss Walbaum). *Journal of Applied Microbiology*, *129*(4), 1004–1019. https://doi.org/10.1111/jam.14652

Navarro-Barró, E., Herná Ndez, C., Llera-Herrera, R. L., García-Gasca, A., & Gó Mez-Gil, B. (2019). Overfeeding a High-Fat Diet Promotes Sex-Specific Alterations on the Gut Microbiota of the Zebrafish (Danio rerio). *Zebrafish*, *16*(3), 268–279. https://doi.org/10.1089/zeb.2018.1648

Nayak, S. K. (2010). Role of gastrointestinal microbiota in fish. *Aquaculture Research*, *41*(11), 1553–1573. https://doi.org/10.1111/J.1365-2109.2010.02546.X

Ng, W. K., & Koh, C. B. (2017). The utilization and mode of action of organic acids in the feeds of cultured aquatic animals. *Reviews in Aquaculture*, *9*(4), 342–368. https://doi.org/10.1111/RAQ.12141

Nguyen, M. Van, Espe, M., Conceição, L. E. C., Le, H. M., Yúfera, M., Engrola, S. A. D., Jordal, A. E. O., & Rønnestad, I. (2019). The role of dietary methionine concentrations on growth, metabolism and N-retention in cobia (Rachycentron canadum) at elevated water temperatures. *Aquaculture Nutrition*, *25*(2), 495–507. https://doi.org/10.1111/anu.12875

Nicholson, J. K., Holmes, E., Kinross, J., Burcelin, R., Gibson, G., Jia, W., & Pettersson, S. (2012). Host-Gut Microbiota Metabolic Interactions. *Science*, *336*(6086), 1262–1267. https://doi.org/10.1126/SCIENCE.1223813

Okuda, T. (2005). Systematics and health effects of chemically distinct tannins in medicinal plants. *Phytochemistry*, *66*(17), 2012–2031. https://doi.org/10.1016/J.PHYTOCHEM.2005.04.023

Ottinger, M., Clauss, K., & Kuenzer, C. (2016). Aquaculture: Relevance, distribution, impacts and spatial assessments – A review. *Ocean & Coastal Management*, *119*, 244–266. https://doi.org/10.1016/J.OCECOAMAN.2015.10.015

Palladino, G., Rampelli, S., Scicchitano, D., Musella, M., Quero, G. M., Prada, F., Mancuso, A., Seyfarth, A. M., Turroni, S., Candela, M., & Biagi, E. (2021). Impact of marine aquaculture on the microbiome associated with nearby holobionts: The case of patella caerulea living in proximity of sea bream aquaculture cages. *Microorganisms*, *9*(2), 1–17. https://doi.org/10.3390/microorganisms9020455

Parma, L., Candela, M., Soverini, M., Turroni, S., Consolandi, C., Brigidi, P., Mandrioli, L., Sirri, R., Fontanillas, R., Gatta, P. P., & Bonaldo, A. (2016). Next-generation sequencing characterization of the gut bacterial community of gilthead sea bream (Sparus aurata, L.) fed low fishmeal based diets with increasing soybean meal levels. *Animal Feed Science and Technology*, *222*, 204–216. https://doi.org/10.1016/j.anifeedsci.2016.10.022

Parma, L., Pelusio, N. F., Gisbert, E., Esteban, M. A., D'Amico, F., Soverini, M., Candela, M., Dondi, F., Gatta, P. P., & Bonaldo, A. (2020). Effects of rearing density on growth, digestive conditions, welfare indicators and gut bacterial community of gilthead sea bream (Sparus aurata, L. 1758) fed different fishmeal and fish oil dietary levels. *Aquaculture*, *518*, 734854. https://doi.org/10.1016/j.aquaculture.2019.734854

Pelusio, N. F., Rossi, B., Parma, L., Volpe, E., Ciulli, S., Piva, A., D'Amico, F., Scicchitano, D., Candela, M., Gatta, P. P., Bonaldo, A., & Grilli, E. (2020). Effects of increasing dietary level of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (Oncorhynchus mykiss) reared at normal and high temperature. *Fish & Shellfish Immunology*, *107*, 324–335. https://doi.org/10.1016/J.FSI.2020.10.021

Pereira, L. F., Peixoto, M. J., Carvalho, P., Sansuwan, K., Santos, G. A., Gonçalves, J. F. M., & Ozório, R. O. A. (2018). Cross-effects of dietary probiotic supplementation and rearing temperature on growth performance, digestive enzyme activities, cumulative mortality and innate immune response in seabass (Dicentrarchus labrax). *Aquaculture Nutrition*, *24*(1), 453–460. https://doi.org/10.1111/anu.12578

Peres, H., Santos, S., & Oliva-Teles, A. (2013). Selected plasma biochemistry parameters in gilthead seabream (Sparus aurata) juveniles. *Journal of Applied Ichthyology*, *29*(3), 630–636. https://doi.org/10.1111/j.1439-0426.2012.02049.x

Quast, C., Klindworth, A., Pruesse, E., Schweer, T., Horn, M., & Glo, F. O. (2013). *Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies*. *41*(1), 1–11. https://doi.org/10.1093/nar/gks808

Refstie, S., Sahlström, S., Bråthen, E., Baeverfjord, G., & Krogedal, P. (2005). Lactic acid fermentation eliminates indigestible carbohydrates and antinutritional factors in soybean meal for Atlantic salmon (Salmo salar). *Aquaculture*, *246*(1–4), 331–345. https://doi.org/10.1016/J.AQUACULTURE.2005.01.001

Reguera, P., Couceiro, L., & Fernández, N. (2018). A review of the empirical literature on the use of limpets Patella spp. (Mollusca: Gastropoda) as bioindicators of environmental quality. *Ecotoxicology and Environmental Safety*, *148*, 593–600. https://doi.org/10.1016/J.ECOENV.2017.11.004

Richard, N., Silva, T., Wulff, T., Schrama, D., Dias, J., P, R., & L, C. (2016). Nutritional mitigation of winter thermal stress in gilthead seabream: associated metabolic pathways and potential indicators of nutritional state. *Journal of Proteomics*, *142*, 1–14. https://doi.org/10.1016/j.jprot.2016.04.037

Ringø, E., Hoseinifar, S. H., Ghosh, K., Doan, H. Van, Beck, B. R., & Song, S. K. (2018). Lactic acid bacteria in finfish-An update. In *Frontiers in Microbiology* (Vol. 9, Issue AUG, p. 1818). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2018.01818

Ringø, E., Van Doan, H., Lee, S. H., Soltani, M., Hoseinifar, S. H., Harikrishnan, R., & Song, S. K. (2020). Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture. *Journal of Applied Microbiology*, *129*(1), 116–136. https://doi.org/10.1111/jam.14628

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ*, *2016*(10), e2584. https://doi.org/10.7717/peerj.2584

Roques, S., Deborde, C., Richard, N., Skiba-Cassy, S., Moing, A., & Fauconneau, B. (2020). Metabolomics and fish nutrition: a review in the context of sustainable feed development. *Reviews in Aquaculture*, *12*(1), 261–282. https://doi.org/10.1111/RAQ.12316

Rotllant, J., Balm, P. H. M., & Pe, J. (2001). *Pituitary and Interrenal Function in Gilthead Sea Bream (Sparus aurata L ., Teleostei) after Handling and Confinement Stress*. *342*, 333–342. https://doi.org/10.1006/gcen.2001.7604

Sala-Rabanal, M., Sánchez, J., Ibarz, A., Fernández-Borràs, J., Blasco, J., & Gallardo, M. A. (2003). Effects of low temperatures and fasting on hematology and plasma composition of gilthead sea bream (Sparus aurata). In *Fish Physiology and Biochemistry* (Vol. 29).

San Diego-McGlone, M. L., Azanza, R. V., Villanoy, C. L., & Jacinto, G. S. (2008). Eutrophic waters, algal bloom and fish kill in fish farming areas in Bolinao, Pangasinan, Philippines. *Marine Pollution Bulletin*, *57*(6–12), 295–301. https://doi.org/10.1016/J.MARPOLBUL.2008.03.028

Sánchez-Nuño, S., Eroldogan, O. T., Sanahuja, I., özşahinoğlu, I., Blasco, J., Fernández-Borràs, J., Fontanillas, R., Guerreiro, P. M., & Ibarz, A. (2018). Cold-induced growth arrest in gilthead

sea bream Sparus aurata: Metabolic reorganisation and recovery. *Aquaculture Environment Interactions*, *10*, 511–528. https://doi.org/10.3354/AEI00286

Sánchez-Nuño, S., Sanahuja, I., Fernández-Alacid, L., Ordóñez-Grande, B., Fontanillas, R., Fernández-Borràs, J., Blasco, J., Carbonell, T., & Ibarz, A. (2018). Redox challenge in a cultured temperate marine species during low temperature and temperature recovery. *Frontiers in Physiology*, *9*(JUL). https://doi.org/10.3389/fphys.2018.00923

Sepulveda, J., & Moeller, A. H. (2020a). The Effects of Temperature on Animal Gut Microbiomes. In *Frontiers in Microbiology* (Vol. 11). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2020.00384

Sepulveda, J., & Moeller, A. H. (2020b). The Effects of Temperature on Animal Gut Microbiomes. *Frontiers in Microbiology*, *11*, 384. https://doi.org/10.3389/FMICB.2020.00384/BIBTEX

Serra, C. R., Oliva-Teles, A., Enes, P., & Tavares, F. (2021). Gut microbiota dynamics in carnivorous European seabass (Dicentrarchus labrax) fed plant-based diets. *Scientific Reports 2021 11:1*, *11*(1), 1–13. https://doi.org/10.1038/s41598-020-80138-y

Silva, T., da Costa, A., Conceição, L., Dias, J., Rodrigues, P., & Richard, N. (2014). Metabolic fingerprinting of gilthead seabream (Sparus aurata) liver to track interactions between dietary factors and seasonal temperature variations. *PeerJ*, *2*, e527. https://doi.org/10.7717/peerj.527

Smith, T. B., Wahl, D. H., & Mackie, R. I. (1996). Volatile fatty acids and anaerobic fermentation in temperate piscivorous and omnivorous freshwater fish. *Journal of Fish Biology*, *48*(5), 829–841. https://doi.org/10.1111/J.1095-8649.1996.TB01479.X

Soriano, E. L., Ramírez, D. T., Araujo, D. R., Gómez-Gil, B., Castro, L. I., & Sánchez, C. G. (2018). Effect of temperature and dietary lipid proportion on gut microbiota in yellowtail kingfish Seriola lalandi juveniles. *Aquaculture*, *497*, 269–277. https://doi.org/10.1016/j.aquaculture.2018.07.065

Vargas-Albores, F., Martínez-Córdova, L. R., Hernández-Mendoza, A., Cicala, F., Lago-Lestón, A., & Martínez-Porchas, M. (2021). Therapeutic modulation of fish gut microbiota, a feasible strategy for aquaculture? *Aquaculture*, *544*, 737050. https://doi.org/10.1016/J.AQUACULTURE.2021.737050

Vargas-Chacoff, L., Arjona, F. J., Polakof, S., del Río, M. P. M., Soengas, J. L., & Mancera, J. M. (2009). Interactive effects of environmental salinity and temperature on metabolic responses of gilthead sea bream Sparus aurata. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *154*(3), 417–424.

Vasconi, M., Caprino, F., Bellagamba, F., & Moretti, V. M. (2017). Fatty acid composition of gilthead sea bream (Sparus aurata) fillets as affected by current changes in aquafeed formulation. *Turkish Journal of Fisheries and Aquatic Sciences*, *17*(3), 51–459. https://doi.org/10.4194/1303-2712-v17_3_01

Vatsos, I. N. (2017). Standardizing the microbiota of fish used in research. *Laboratory Animals*, *51*(4), 353–364. https://doi.org/10.1177/0023677216678825/ASSET/IMAGES/LARGE/10.1177_0023677216678825-FIG2.JPEG

Velázquez, M., Zamora, S., & Martínez, F. J. (2006). Effect of dietary energy content on gilthead sea bream (Sparus aurata) feeding behaviour and nutritional use of the diet. *Aquaculture Nutrition*, *12*(2), 127–133. https://doi.org/10.1111/j.1365-2095.2006.00391.x

Viñas, L., Pérez-Fernández, B., Soriano, J. A., López, M., Bargiela, J., & Alves, I. (2018). Limpet (Patella sp) as a biomonitor for organic pollutants. A proxy for mussel? *Marine Pollution Bulletin*, *133*, 271–280. https://doi.org/10.1016/J.MARPOLBUL.2018.05.046

Yúfera, M., Nguyen, M. V., Navarro-Guillén, C., Moyano, F. J., Jordal, A. E. O., Espe, M., Conceição, L. E. C., Engrola, S., Le, M. H., & Rønnestad, I. (2019). Effect of increased rearing temperature on digestive function in cobia early juvenile. *Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology*, *230*, 71–80. https://doi.org/10.1016/j.cbpa.2019.01.007

Yukgehnaish, K., Kumar, P., Sivachandran, P., Marimuthu, K., Arshad, A., Paray, B. A., & Arockiaraj, J. (2020). Gut microbiota metagenomics in aquaculture: factors influencing gut microbiome and its physiological role in fish. *Reviews in Aquaculture*, *12*(3), 1903–1927. https://doi.org/10.1111/RAQ.12416

Zarkasi, K. Z., Taylor, R. S., Abell, G. C. J., Tamplin, M. L., Glencross, B. D., & Bowman, J. P. (2016). Atlantic Salmon (Salmo salar L.) Gastrointestinal Microbial Community Dynamics in Relation to Digesta Properties and Diet. *Microbial Ecology*, *71*(3), 589–603. https://doi.org/10.1007/s00248-015-0728-y

Zhang, K., Zheng, X., He, Z., Yang, T., Shu, L., Xiao, F., Wu, Y., Wang, B., Li, Z., Chen, P., & Yan, Q. (2020). Fish growth enhances microbial sulfur cycling in aquaculture pond sediments. *Microbial Biotechnology*, *13*(5), 1597–1610. https://doi.org/10.1111/1751-7915.13622

Zheng, Y., Wu, W., Hu, G., Qiu, L., Meng, S., Song, C., Fan, L., Zhao, Z., Bing, X., & Chen, J. (2018). Gut microbiota analysis of juvenile genetically improved farmed tilapia (Oreochromis niloticus) by dietary supplementation of different resveratrol. *Fish and Shellfish Immunology*, *77*, 200–207.

2.3 Study I - Effects of increasing dietary level of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (Oncorhynchus mykiss) reared at normal and high temperature

Pelusio, N. F., Rossi, B., Parma, L., Volpe, E., Ciulli, S., Piva, A., ... & Grilli, E. (2020). Effects of increasing dietary level of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (Oncorhynchus mykiss) reared at normal and high temperature. *Fish & Shellfish Immunology*, *107*, 324-335.

Introduction

To secure optimal growth, gut health and function in farmed fish, there is now particular focus on various feed additives used for their nutritional value, and also for their health-promoting and disease preventing properties. Functional feeds are typically supplied to ensure good health and to help the animal ward off pathogens during both normal and challenging farming conditions [1]. Organic acids (OA) are any organic carboxylic acids with the general structure R–COOH. These OA (and their salts) are manufactured by chemical synthesis or fermentation systems [2], and their utilization as feed additives is well known to improve performance and health in terrestrial livestock, especially in swine and poultry [3,4]. However, knowledge of their potential as growth promoters in aquaculture is more recent and less well-known due to limited research but is expected to significantly increase in coming years [5]. Many advantages are reported in connection with lowered pH of feed and digesta owing to diet OA addition: (i) increase in feed hygiene by inhibiting growth of microbial acid-intolerant species, (ii) increase in digestive enzyme activity, leading to higher nutrient digestibility and feed utilization, (iii) modulate host gut microbiota and animal health [5,6]. Among the large variety of OA, dietary inclusion of citric acid was found to improve growth, feed intake, specific growth rate (SGR) and feed conversion rate (FCR) of various aquaculture species such as red drum (*Sciaenops cellatus*), rainbow trout (*Oncohrynchus mykiss*), beluga sturgeon (*Huso huso*), yellowtail (*Seriola quinqueradiata*), tilapia (*Oreochromis niloticus*), and red sea bream (*Pagrus major*) [7–14]. Concerning rainbow trout, while some studies on dietary citric acid displayed a positive effect on growth performance [9,12], a reduction in feed intake and no weight gain were found by other authors [15,16]. Another important OA is sorbic acid, a long chain unsaturated fatty acid known for its antimicrobial activity by inhibiting the microbial enzymatic apparatus and nutrient transport system [3]. Its growth-promoting effect was explored as combined in dietary blends within formic and benzoic acids and their respective salts, resulting in a significant increase in weight gain in rainbow trout [17]. During the past decade, botanicals and nature-identical compounds (NIC) have also gained great interest as novel animal feed additives for the positive effects reported on feed palatability and control of gut microbiota pathogens and for a possible direct effect on the immune system as recently reported in some fish species *i.e.* red hybrid tilapia (*Oreochromis niloticus* ♀ X *Oreochromis aureus* ♂) [18], rainbow trout [19–21], tilapia [22] and zebrafish (*Danio rerio*) [18]. Among the most studied NIC, thymol is a monoterpene proposed as a growth promoter, antimicrobial and anti-inflammatory agent in mammals [23,24], whereas few studies have been conducted on fish species [18,25]. Similarly, vanillin, known as food and feed flavouring, also has potential antimicrobial activity by causing loss of membrane function and inhibiting cell respiration in several sensitive bacteria [26,27]. Using OA and NIC blends in aquafeeds could be an optimal strategy to test their potential synergistic effects on growth, nutrient utilization and gut health. In addition, the encapsulation process capable of protecting the compounds against interactions with the host, food ingredients, and environment may increase the reliability and predictability of their beneficial actions [5,28]. Moreover, to the best of our knowledge the combination of citric and sorbic acids, vanillin and thymol has only been tested in sea bass (*Dicentrarchus labrax*) [29], while no data in rainbow trout are available. For this reason, the aims of the present study of rainbow trout were: 1) to evaluate the effects of dietary increasing level of a blend of citric acid, sorbic acid, thymol and vanillin on growth, feed utilization, intestinal cytokine gene expression and gut bacterial community; 2) to explore the effects of this

blend on intestinal cytokine gene expression and gut bacterial community after one week exposure to high water temperature conditions.

Materials and methods

Experimental diets

An extruded commercial diet (Ecofish 4, Veronesi SpA, Verona, Italy) (5 mm diameter pellet size - 42.1 ± 0.2% crude protein, 20.1 ± 0.1% crude lipid) was coated with increasing dose (D) levels (D0, D250, D500 and D1000; 0, 250, 500 and 1000 ppm respectively) of a blend of OA and NIC (providing 25% citric acid, 16.7% sorbic acid, 1.7% thymol and 1% vanillin) microencapsulated in a matrix of hydrogenated fats (AviPlus®Aqua - Vetagro SpA, Reggio Emilia, Italy; US patent # 7,258,880; EU patent # 1-391-155B1; CA patent # 2,433,484).

Fish and feeding trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. Rainbow trout specimens were obtained from an Italian fish farm (Pescicoltura Brenta snc, Vicenza, Italy). Animals were adapted to the facilities for 1 week before the start of the experiment. At the beginning of the trial, sixty fish (initial weight average 100.5 ± 0.4 g) were randomly distributed into each of twelve 800 L squared fibreglass tanks with a conical base. Each diet was randomly allocated and administered to triplicate groups over a period of 82 days. During the experiment, tanks were provided with tap freshwater and connected to a closed recirculation system (overall water volume: 15 m^3). The rearing system consisted of a mechanical sand filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25 mJ/cm^2: 32 m^3 h^{-1}, Blaufish, Barcelona, Spain), a biofilter (PTK 1200, Astralpool, Barcelona, Spain) and an active carbon filter. The water exchange rate within each tank was 100% every hour, while the overall water renewal amount in the system was 5% daily. During the trial, the temperature was kept at 15.0 ± 1.0 °C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. The oxygen level was kept constant (10.0 ± 1.0 mg L^{-1}) by a liquid oxygen system regulated by a software programme (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen ≤ 0.1 mg L^{-1}) and nitrite (≤0.2 mg L^{-1}) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate (NaOHCO3) was added on a daily basis to keep pH constant at 7.1–7.5. Feed was provided to satiation by oversupplying the feed via automatic feeders, twice a day (8:30, 16:30) for six days a week, while one meal was supplied on Sundays. Each meal lasted 1 h, after which the uneaten pellets of each tank were gathered, dried overnight at 105 °C, and their weight was deducted for overall calculation.

Suboptimal rearing temperature

After the end of the feeding trial, fish belonging to D0 and D1000 groups were exposed to high rearing temperature of 23 °C for 7 days. The highest inclusion level (D1000) was chosen for comparison according to the results of growth and feed utilization achieved during the feeding trial. To this purpose water temperature was gradually increased (4-degree day^{-1}) up to 23 °C and then maintained over one week. During this period, the feed and feeding procedures were provided as previously reported.

Sampling

At the beginning (day 0), in the middle (day 40) and at the end (day 82) of the feeding trial, all the fish were individually weighed. Before each sampling procedures, fish were anaesthetised (100 mg L^{-1}) or euthanized (300 mg L^{-1}) by MS222. Specific growth rate (SGR), feed intake (FI) and feed conversion rate (FCR) were calculated. The proximate composition

of the carcasses was determined at the beginning of the trial on a pooled sample of 15 fish and on a pooled sample of 5 fish per tank at the end of the trial. Protein efficiency rate (PER), gross protein efficiency (GPE), lipid efficiency rate (LER) and gross lipid efficiency (GLE) were calculated. Furthermore, total body length, wet weight, viscera and liver weight were individually recorded for 5 fish per tank to determine condition factor (CF), viscerosomatic index (VSI) and hepatosomatic index (HSI) at the end of the feeding trial. At the beginning (day 0, 15 fish in total), at the end of the feeding trial (day 82, 5 fish per tank^{-1}), and after the high rearing temperature period (5 fish per tank), fish were sampled for pro-inflammatory and anti-inflammatory cytokine gene expression from the distal intestine. At the same time, 5 fish per tank were also sampled for gut bacterial community analysis. All experimental procedures were evaluated by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

Analytical methods

Diets and whole body were analysed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105 C overnight. Crude protein was determined as total nitrogen (N*6.25) after performing the Kjeldahl method. Total lipids were determined according to Bligh and Dyer's [30] extraction method. Ash content was estimated by incineration in a muffle oven at 450 ◦C overnight. Gross energy was determined by a calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, U.S.A).

Calculations

The formulae employed for growth performances were as follows: Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW- ln IBW)/days (where FBW and IBW represent the final and the initial body weights). Feed conversion rate (FCR) = feed intake/weight gain. Viscerosomatic index (VSI) (%) = 100 * (viscera weight/body weight). Hepatosomatic index (HSI) (%) = 100 * (liver weight/body weight). Condition factor (CF) = 100*(FBW/length^3). Protein efficiency rate (PER) = (FBW–IBW)/protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)]/total protein intake fish. Lipid efficiency rate (LER) = (FBW–IBW)/lipid intake. Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)]/total lipid intake fish. Feed intake (FI) (% ABW^{-1} day^{-1})=((100 * total ingestion)/(ABW))/days)) (where average body weight, ABW=(IBW + FBW)/2.

Cytokine gene expression analyses by real-time polymerase chain reaction

Total RNA was isolated from 50 mg of distal intestine samples stored in RNA Later (Sigma) using the NucleoSpin RNA extraction kit following the manufacturer's instructions. The RNA extraction protocol includes a treatment with DNAse I in order to remove genomic DNA. The first strand of cDNA was synthesized by reverse transcription using the GoScript® Reverse Transcriptase (Promega). cDNA concentration was quantified using a Qubit Fluorometer (ThermoFisher). Real-time PCR was performed with an ABI PRISM 7300 instrument (Applied Biosystems) using BRYT Green® GoTaq® qPCR (Promega). 10 ng of each cDNA sample was added to a reaction mix containing 2 x GoTaq® qPCR Master Mix (Promega), 300 nM of CXR and 200 nM of each primer. The primers used for β-actin (β-act), Interleukin 1β (IL-1β), 6 (IL-6), 8 (IL-8), 10 (IL-10), Tumor necrosis factor α (TNF-α), Transforming growth factor β (TGF-β), are shown in **Table 1**.

Table 1. Primer sequences used for gene expression analyses.

Gene	Abbreviation	GenBank ID	Primer sequence (5'- 3')	Amplicon (bp)	References
β-actin	β-act	EZ908974	GCCGGCCGCGACCTCACAGACTAC CGGCCGTGGTGGTGAAGCTGTAAC	73	(Caipang et al., 2008)
Interleukin 1 β	IL-1β	AJ223954	CTCTACCTGTCCTGCTCCAAA ATGTCCGTGCTGATGAACC	194	(Caipang et al., 2008)
Interleukin 6	IL-6	DQ866150	CAATCAACCCTACTCCCCTCT CCTCCACTACCTCAGCAACC	91	(Caipang et al., 2008)
Interleukin 8	IL-8	AJ279069	AGAATGTCAGCCAGCCTTGT TCTCAGACTCATCCCCTCAGT	69	(Caipang et al., 2008)
Interleukin 10	IL-10	AB118099	CGACTTTAAATCTCCCATCGAC GCATTGGACGATCTCTTTCTTC	70	(Caipang et al., 2008)
Tumor necrosis factor α	TNF-α	AJ277604 AJ401377	CCACACACTGGGCTCTTCTT GTCCGAATAGCGGGAAATAA	128	(Caipang et al., 2008)
Transforming growth factor β	TGF-β	X99303	TCCGCTTCAAAATATCAGGG TGATGGCATTTTCATGGCTA	71	(Caipang et al., 2008)

Reaction mixtures were incubated for 2 min at 95 °C, followed by 50 cycles of 10 s at 95 °C, 30 s at 60 °C. Before the experiments, the specificity of each primer pair was studied using positive and negative samples. A melting curve analysis of the amplified products validated the primers for specificity. After these verifications, all cDNA samples were analysed in triplicate. Negative controls with no template were always included in the reactions. For each sample, gene expression was normalized against beta-actin (β-actin) gene and expressed as $2^{-\Delta\Delta Ct}$, where ΔCt is determined by subtracting the β-actin Ct value from the target Ct. Gene expression of untreated and treated samples collected at time one (T1, day 82) and two (T2, day 89) were expressed as "fold changes" relative to untreated controls sampled at time zero (T0, day 0).

Gut bacterial community DNA extraction and sequencing

At the end of feeding trial, total bacterial DNA was extracted from pools of hindgut content obtained from 5 fish per tank (100 mg of hindgut content per fish) for a total of 12 (at day 82) and 6 (at day 89) samples, as previously reported in Parma [31]. Afterwards, the V3–V4 hypervariable region of the 16S rRNA gene was amplified, purificated and sequenced as describe in paragraph 2.2. Sequencing was carried out on Illumina MiSeq platform using a 2 × 250 bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA). After the sequencing process reads were processed using a pipeline combining QIIME2 [35] and PANDAseq [36]. Then, using DADA2 [37] and VSEARCH [38], reads were cleaned and clustered into OTUs at a 0.99 similarity threshold. Assignment was carried out by using the RDP classifier against Greengenes database (May 2013 release). During the bioinformatic analysis, one of the samples of D500 diet was excluded from the following analysis because of the low number of high-quality sequences obtained. Alpha-diversity analysis were performed using OTU species count (observed_otus), Chao1 index for microbial richness and Shannon index for biodiversity. Beta-diversity was estimated by Bray-Curtis distances, which were used as input for principal coordinates analysis (PCoA).

Statistical analysis

All data are represented as mean ± standard deviation (SD). A tank was used as the experimental unit for analysing growth performance, and a pool of five fish was considered as the experimental unit for analysing carcass composition, whereas fifteen individual fish per treatment were used for analysing immunity response expression. Results on growth, nutritional

indices and cytokine expression at the end of the feeding trial (day 82) were analysed by applying linear regression model in order to measure the effect of the increasing doses of dietary OA and NIC on considered data, with a significance attributed for $p \leq 0.05$. Cytokine expression data in fish treated with D0 and D1000 on day 82 and 89 were analysed by two-way analysis of variance (ANOVA) considering diet and time as independent factors, and in case of significance ($p \leq 0.05$) Tukey's post hoc test was performed. Gut microbial statistical analysis of gut bacterial community was carried out as described in paragraph 2.2. The rest of the statistical analyses were performed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA, USA).

Results

Growth and proximate composition

Growth performances are reported in **Table 2**. No significant dose effect was found concerning FBW, weight gain, FCR, SGR, FI and mortality during the first period of the trial (days 0–40). Regarding the second period (days 40–82), SGR ($p = 0.0294$) increased at increasing OA and NIC level while a decreasing effect was observed on FCR ($p = 0.0439$). Results on biometric indices, nutritional indices and proximate whole-body composition are summarized in **Table 3**. No significant dose effects were found on VSI, HSI, CF and whole proximate composition. Concerning nutritional indices, PER ($p = 0.0202$) and LER ($p = 0.0156$) increased significantly at increasing dietary OA-NIC dose level. No significant dose effects were found in GPE and GLE.

Table 2. Growth performance and feed intake of rainbow trout fed increasing dietary level of organic acids and nature-identical compounds

	Experimental diets				
	D0	D250	D500	D1000	*P*-value
Time range day 0 – 40					
IBW (g)	100.1 ± 3.5	100.8 ± 4.0	100.1 ± 3.1	100.9 ± 2.4	n.s.
FBW (g)	193.9 ± 5.0	200.6 ± 8.4	193.4 ± 12.6	193.6 ± 10.2	n.s.
Weight gain (g)	93.7 ± 7.9	99.8 ± 4.4	93.3 ± 12.1	92.7 ± 11.4	n.s.
SGR (% day^{-1})	1.65 ± 0.14	1.72 ± 0.01	1.64 ± 0.16	1.63 ± 0.17	n.s.
FCR	1.06 ± 0.07	1.06 ± 0.01	1.08 ± 0.05	1.09 ± 0.05	n.s.
FI	1.67 ± 0.03	1.73 ± 0.03	1.71 ± 0.07	1.69 ± 0.05	n.s.
Survival (%)	100.0 ± 0.0	100.0 ± 0.0	99.4 ± 1.0	99.4 ± 1.0	n.s.
Time range day 40 – 82					
IBW (g)	190.8 ± 3.4	199.2 ± 9.7	194.4 ± 11.4	193.7 ± 11.0	n.s
FBW (g)	296.9 ± 6.6	319.7 ± 8.0	309.8 ± 16.0	320.1 ± 13.7	n.s
Weight gain (g)	106.0 ± 7.7	120.5 ± 10.0	115.4 ± 5.0	126.4 ± 2.9	n.s
SGR (% day^{-1})	1.05 ± 0.07	1.13 ± 0.11	1.11 ± 0.03	1.20 ± 0.04	*0.029*
FCR	1.24 ± 0.09	1.15 ± 0.06	1.18 ± 0.01	1.12 ± 0.03	*0.043*
FI	1.27 ± 0.07	1.26 ± 0.05	1.27 ± 0.01	1.27 ± 0.02	n.s
Survival (%)	97.2 ± 0.96	99.4 ± 0.1	99.0 ± 1.0	98.3 ± 0.6	n.s

Data are given as the tanks mean (n=3) ± SD. n.s.: non-significant ($p > 0.05$).
D= dose blend (citric acid 25%, sorbic acid 16.7%, thymol 1.7%, vanillin 1%, matrix of hydrogenated fats) inclusion in diet; D0 = 0 ppm, D250 = 250 ppm; D500 = 500 ppm; D1000= 1000 ppm).
IBW = Initial body weight.
FBW = Final body weight.
FI = Feed intake (FI) (% ABW^{-1} day^{-1})=((100 ∗ total ingestion)/(ABW))/days)) (where average body weight, ABW=(IBW+FBW)/2;
FCR = Feed conversion rate = feed intake / weight gain.
SGR = Specific growth rate (% day-1) = 100 * (ln FBW- ln IBW) / days.

Table 3. Biometric indices, body composition and nutritional indices of rainbow trout fed increasing dietary level of organic acids and nature-identical compounds over 82 days

	Experimental diets				
	D0	D250	D500	D1000	*P-value*
Biometric indices					
VSI	13.23 ± 2.53	13.77 ± 1.74	13.11 ± 2.84	14.23 ± 1.75	n.s.
HSI	1.27 ± 0.28	1.42 ± 0.33	1.41 ± 0.28	1.41 ± 0.41	n.s.
CF	1.36 ± 0.48	1.28 ± 0.11	1.25 ± 0.12	1.24 ± 0.12	n.s.
Whole body composition, %					
Protein	16.48 ± 0.25	16.32 ± 0.23	15.94 ± 0.54	16.30 ± 0.24	n.s.
Lipid	15.74 ± 1.34	18.21 ± 0.54	17.70 ± 0.35	16.19 ± 0.85	n.s.
Ash	1.87 ± 0.27	2.10 ± 0.30	1.98 ± 0.30	1.94 ± 0.19	n.s.
Moisture	63.59 ± 1.98	60.69 ± 1.98	62.15 ± 0.35	64.44 ± 1.01	n.s.
Nutritional indices					
PER	2.05 ± 0.07	2.13 ± 0.04	2.11 ± 0.03	2.18 ± 0.07	*0.020*
LER	4.28 ± 0.14	4.45 ± 0.09	4.42 ± 0.07	4.56 ± 0.14	*0.015*
GPE	32.98 ± 0.66	33.92 ± 0.53	32.43 ± 2.14	34.68 ± 1.02	n.s.
GLE	81.21 ± 7.11	99.29 ± 5.25	95.94 ± 2.42	88.32 ± 6.19	n.s.

Data are given as the mean (n=15 for VSI, HIS, CF) ± SD. n.s.: not significant ($p > 0.05$).
D= dose blend (citric acid 25%, sorbic acid 16.7%, thymol 1.7%, vanillin 1%, matrix of hydrogenated fats) inclusion in diet; D0 = 0 ppm, D250 = 250 ppm; D500 = 500 ppm; D1000= 1000 ppm).
VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
CF = Condition factor = $100*(FBW/length^3)$.
PER = Protein efficiency rate = ((FBW-IBW)/protein intake).
LER= Lipid efficiency rate = ((FBW-IBW)/lipid intake).
GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.
GLE = Gross lipid efficiency = 100*[(%final body lipid*FBW) - (%initial body lipid*IBW)]/total lipid intake fish.

Immune and inflammatory gene expression in intestinal mucosa

The gene expression of six genes involved in the immune and inflammatory response are presented in **Figs. 1 and 2**. The comparison of gene expression in the intestine of fish fed different diets and sampled at the end of the feeding trial (day 82, time 1, T1) showed slight variations of some target genes analyzed **(Fig. 1)**, but no significant dose effect was observed. The comparison of gene expression between D0 and D1000 groups before (T1) and after (day 89, time 2, T2) the exposure to high water temperature showed an upregulation of some pro-inflammatory genes analyzed (**Fig. 2**). Particularly, IL-8 was significantly upregulated ($p < 0.001$) after the exposure to a high water temperature in both D0 and D1000 groups, while TNF-α was significantly upregulated in D1000 group ($p < 0.05$). *3.3. Gut bacterial community profiles* Twelve pools (at day 82) and six pools (at day 89) of the content of fish distal intestine were analyzed to determine the gut microbial community of fish fed with increasing inclusion levels of the dietary blend (OA + NIC).

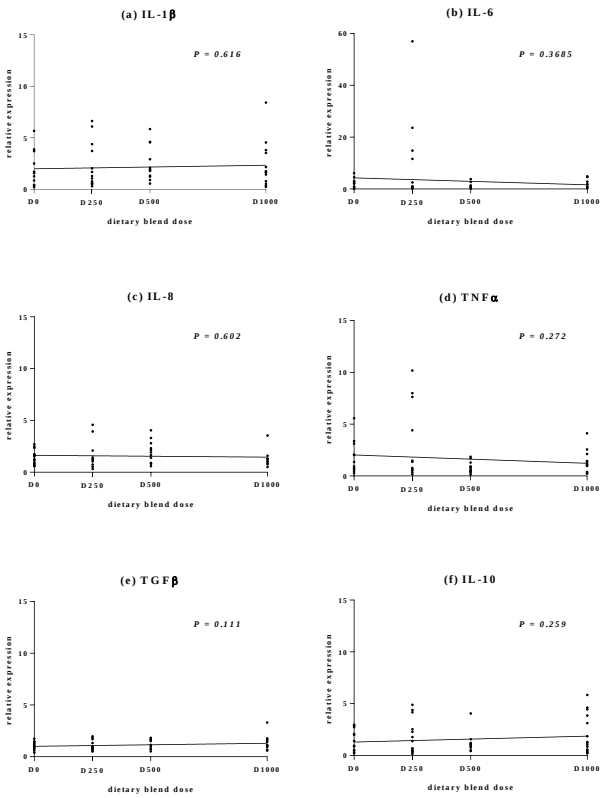

Figure 1. Immune and inflammatory cytokine gene expression in intestinal mucosa of rainbow trout fed increasing dietary blend (organic acids and natural identical compounds) levels over 82 days. Data are given as 15 individuals per diet. In each graph, significance is attributed to $P \leq 0.05$. D= dose blend (citric acid 25%, sorbic acid 16.7%, thymol 1.7%, vanillin 1%, matrix of hydrogenated fats) inclusion in diet; D0 = 0 ppm, D250 = 250 ppm; D500 = 500 ppm; D1000= 1000 ppm. IL-1β = Interleukin 1β; IL-6 = Interleukin 6; IL-8 = Interleukin 8; TNFα = Tumor necrosis factor α; TGFβ = Transforming growth factor β; IL-10 = Interleukin 10.

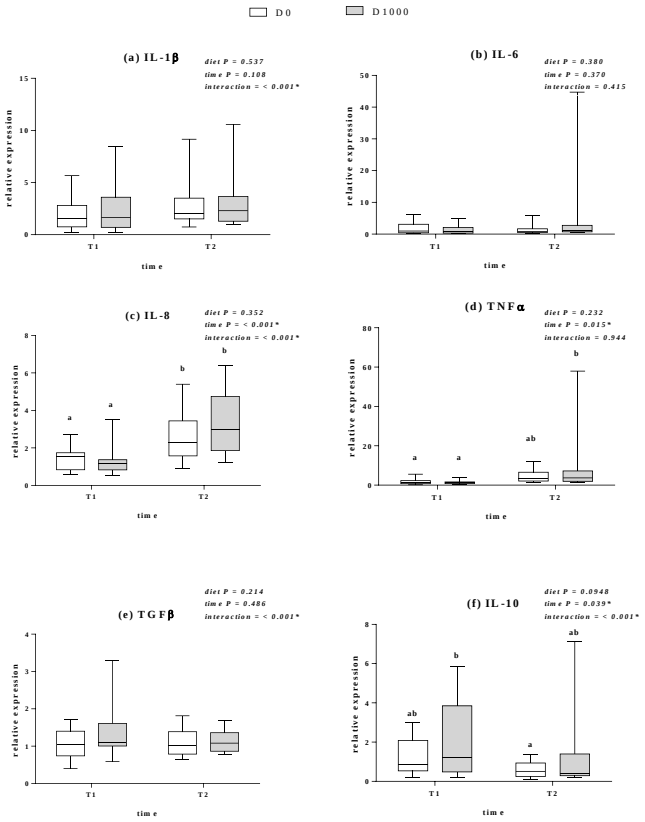

Figure 2. Immune and inflammatory cytokine gene expression in intestinal mucosa of rainbow trout fed dietary blend (organic acids and natural identical compounds) before (day 82, time 1, T1) and after (day 89, tme 2, T2) exposure to high water rearing temperature of 23°C for 7 days. Data are given as 15 individuals per diet. In each graph, different superscript letters indicate significant differences among treatments ($P \leq 0.05$). D= dose blend (citric acid 25%, sorbic acid 16.7%, thymol 1.7%, vanillin 1%, matrix of hydrogenated fats) inclusion in diet; D0 = 0 ppm, D1000= 1000 ppm. IL-1β = Interleukin 1β; IL-6 = Interleukin 6; IL-8 = Interleukin 8; TNFα = Tumor necrosis factor α; TGFβ = Transforming growth factor β; IL-10 = Interleukin 10.

In order to assess whether the different diets can influence fish gut bacterial community at day 82, the beta-diversity of the GM ecosystem was evaluated by performing a Principal Coordinates Analysis (PCoA) based on the Bray-Curtis distances among the gut bacterial community profiles (**Fig. 3A**). Even though no significant differences among dietary groups were detected (permutation test with pseudo-F ratios (Adonis); $p > 0.05$), PCoA showed a tendency of sample separation based on different diets, the D0 group clustered separately from the other dietary groups (i.e. D250, D500, D1000). More specifically, it was possible to identify a tendency of separation between D0 group and D500 group (pairwise Adonis permutation test; $p < 0.1$). In addition, compared to the other groups, D250 group showed better clustering, indicating a greater uniformity of this group. Different metrics were used to estimate α-diversity of each sample, and no significant differences, according to the different diets, were detected (Kruskal-Wallis test; $p > 0.05$) (**Fig. 3B**). In order to analyse further the GM composition of fish fed diets with different OA + NIC inclusions (i.e. D0, D250, D500, D1000), phylogenetic characterisation was highlighted in **Fig. 3C and D**. The GM of each group showed a similar profile in terms of phylum and genus taxonomic levels. In particular, at the phylum level, the most abundant taxa observed were *Firmicutes*, *Proteobacteria* and *Actinobacteria*, which represented about 98% of the whole intestinal bacterial ecosystem (**Fig. 3C**). On the other hand, the genera most represented, all belonging to *Firmicutes* phylum, were *Lactobacillus* (mean relative abundance ± SD; D0: 29.0% ± 2.5%; D250: 29.3% ± 1.6%; D500: 27.7% ± 0.4%; D1000: 31.3% ± 2.2%), *Leuconostoc* (D0: 18.2% ± 4.8%; D250: 20.1% ± 1.4%; D500: 19.7% ± 4.7%; D1000: 18.7% ± 4.7%) and *Streptococcus* (D0: 16.5% ± 0.7%; D250: 13.6% ± 0.9%; D500: 13.8% ± 0.1%; D1000: 13.6% ± 0.7%) (**Fig. 3D**).

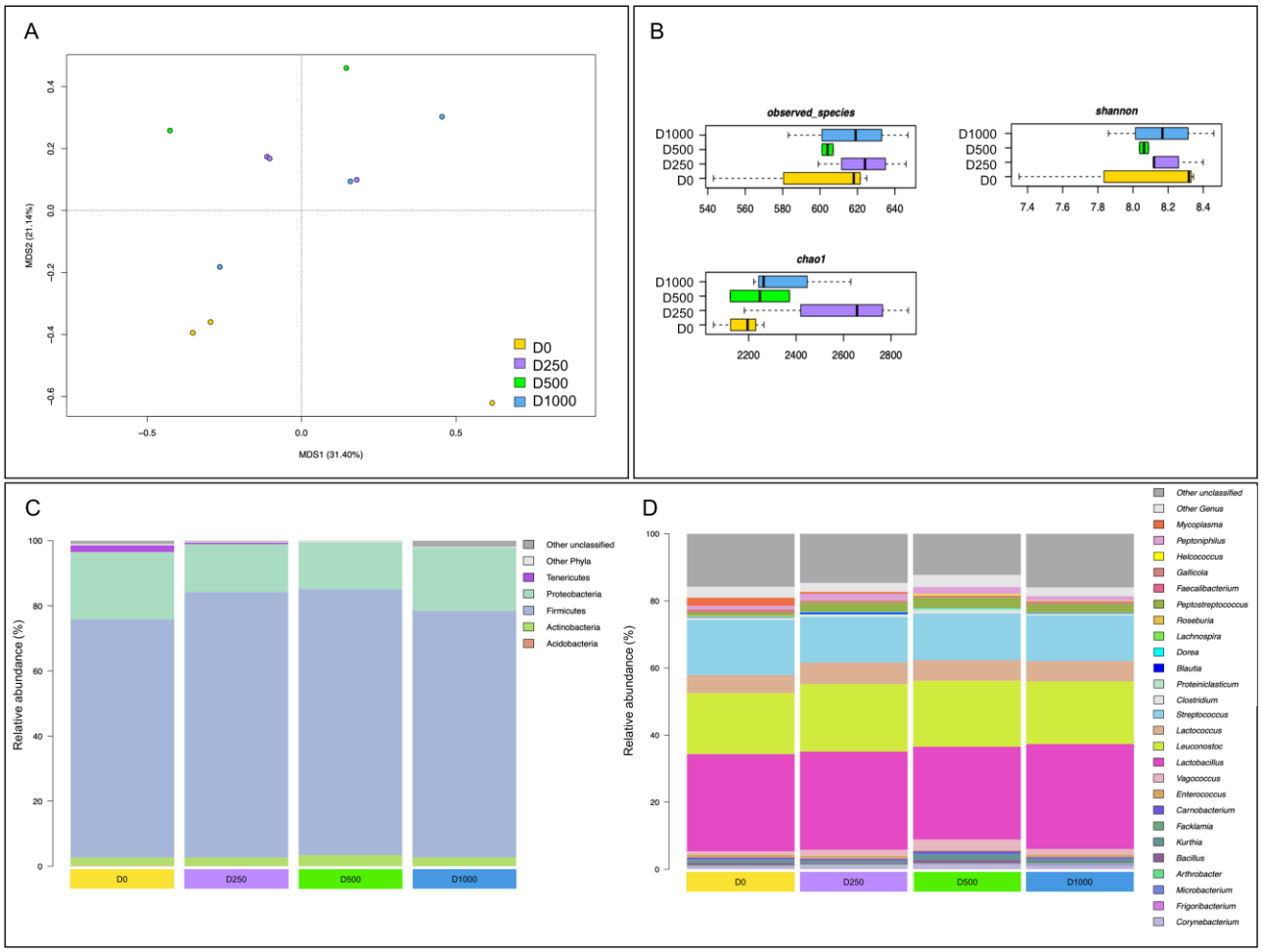

Figure 3 Gut microbiota diversity and composition of rainbow trout fed for 82 days with increasing dietary blend levels (organic acids and natural identical compounds) (i.e. D0, D250, D500, D1000). Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances between samples taken from each diet groups is highlighted in panel A. No significant separation was observed among groups (permutation test with pseudo-F ratios (Adonis); $p > 0.05$). (B) Boxplots showing alpha diversity values for each dietary group, measured by OTU species count (observed_otus), Chao1 index and Shannon index. No significant differences were observed among groups for all the metrics (Kruskal-Wallis test; $p > 0.05$). Barplots showing the GM composition at phylum and genus taxonomic level for all the different dietary interventions are displayed in panel C and D respectively. Only phyla with a relative abundance $\geq 0.5\%$ in at least 9% of samples, and genera with relative abundance $\geq 0.5\%$ in at least 9% of samples are represented.

However, no significant differences (Wilcoxon rank-sum test; $p > 0.05$) among groups at phylum and genus level were detected between different diets (**Supplementary Fig. 1A**). Despite these primary results, a paired statistical analysis against each dietary group revealed several tendencies at the genus taxonomic level. Both *Vagococcus* and *Peptoniphilus* genera appeared to be most abundant in D250 group compared to D0 group (Wilcoxon rank-sum test; $p < 0.1$). On the other hand, for the genera *Streptococcus* and *Faecalibacterium* a reduction trend was observed in the D250 and D1000 groups compared to D0 group (Wilcoxon rank-sum test; $p < 0.1$). In addition, the *Clostridium* genus also seemed to be less abundant in the D250 compared to D0 group (Wilcoxon rank-sum test; $p < 0.1$) (**Supplementary Fig. 1**). Subsequently, in order to understand whether the exposure to high water temperature can influence the gut bacterial ecosystem of fish, beta diversity analysis based on the Bray-Curtis distances among both D0 and D1000 groups in the two timepoints was performed (**Fig. 4A**). In this case, the PCoA showed a significant separation between different groups in the two-dimensional space (permutation test with pseudo-F ratios (Adonis); $p = 0.003$), principally driven by the separation between D0 (T2) vs D0 (T1) (pairwise Adonis permutation test; $p = 0.001$), D0 (T2) vs D1000 (T1) ($p = 0.01$), D1000 (T2) vs D0 (T1) ($p = 0.001$), D1000 (T2) vs D1000 (T1) ($p = 0.007$) and D0 (T1) vs D1000 (T1) ($p = 0.01$). Concerning the α-diversity of each sample, it was possible to observe a significant difference among groups before and

after the exposure to high water temperature (Kruskall-Wallis test; $p < 0.05$) (**Fig. 4B**). All measures showed significantly less GM diversity under high water temperature conditions (T2) compared to normal conditions (T1), for both types of diets (Kruskall-Wallis test; $p < 0.05$). The overall composition of fish gut bacterial community from D0 and D1000 groups before (day 82, T1) and after (day 89, T2) the exposure to high water temperature is represented in **Fig. 4C and D**. In particular, *Firmicutes*, *Proteobacteria* and *Actinobacteria* phyla dominated the intestinal bacterial community in all conditions and represented about 97% of the whole sample set (**Fig. 4C**). At the genus level, the most represented taxa all belonging to *Firmicutes* phylum, were *Lactobacillus, Leuconostoc, Streptococcus and Lactococcus*, below indicated as mean relative abundance ± SD. *Lactobacillus*: D0 (T2), 30.9% ± 0.6%; D1000 (T2), 29.0% ± 0.4%; D0 (T1), 29.0% ± 2.5%; D1000 (T1), 31.3% ± 2.2%). *Leuconostoc*: (D0 (T2), 7.4% ± 1.1%; D1000 (T2), 6.4% ± 1.2%; D0 (T1), 18.2% ± 4.8%; D1000 (T1), 18.7% ± 4.7%. Streptococcus: D0 (T2), 9.6% ± 2.5%; D1000 (T2), 9.7% ± 2.8%; D0 (T1), 16.5% ± 0.7%; D1000 (T1), 13.6% ± 0.7%. *Lactococcus*: D0 (T2), 5.2% ± 1.0%; D1000 (T2), 5.7% ± 0.4%; D0 (T1), 5.4% ± 0.9%; D1000 (T1), 6.1% ± 1.4% (**Fig. 4D**).

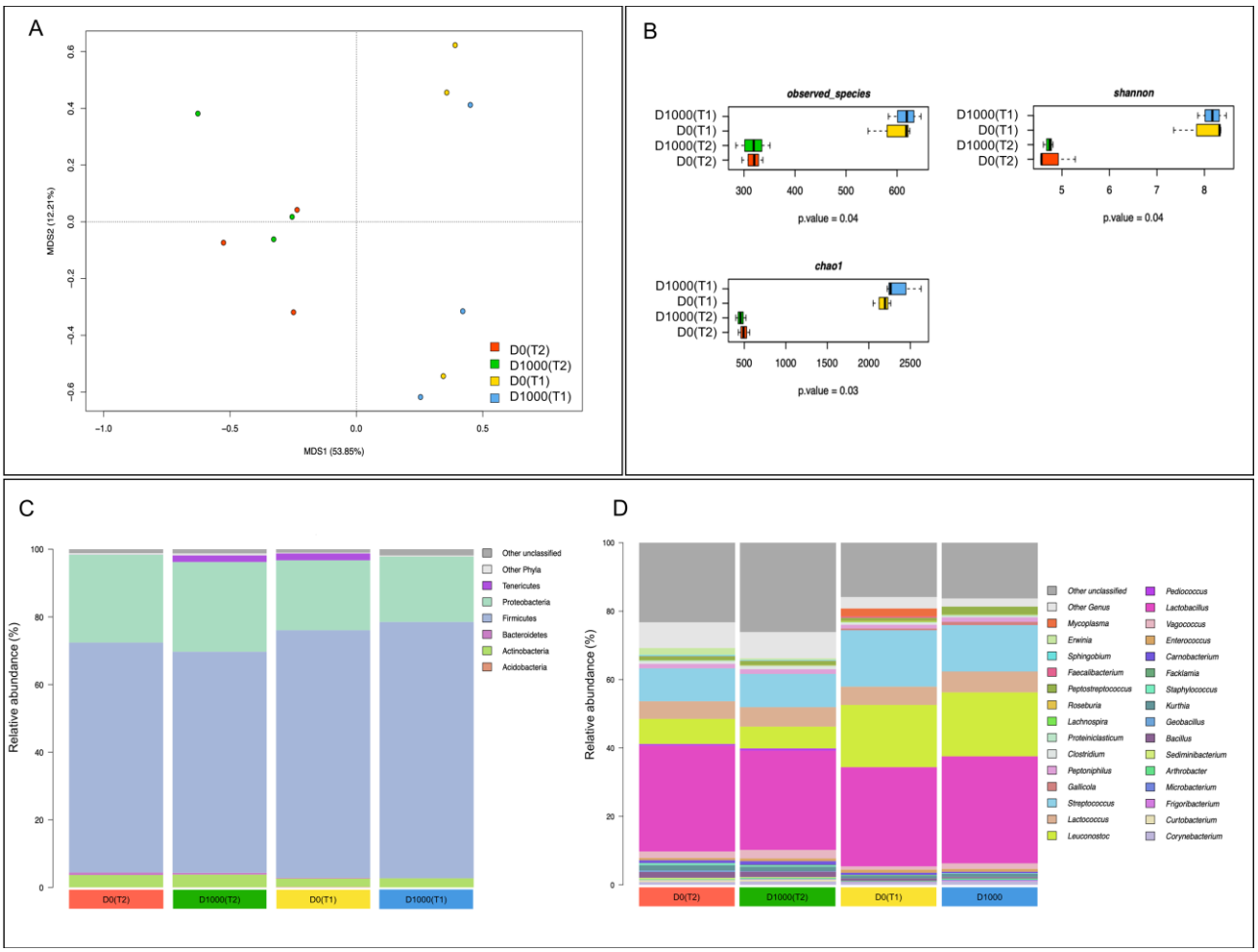

Figure 4 Gut microbiota diversity and composition of rainbow trout fed with D0 and D1000 diets before (day 82, T1) and after (day 89, T2) exposure to highwater rearing temperature of 23°C for 7 days. Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances between samples taken from each group is displayed in panel A. A significant separation among groups was observed (permutation test with pseudo-F ratios (Adonis); $p = 0.003$). (B,) Boxplots showing alpha diversity values, measured by OTU species count (observed_otus), Chao1 index and Shannon index. All metrics showed lower GM diversity in groups exposed to a sub-optimal condition (T2) compared to groups in a standard condition (T1), for both type of diets (D0 vs. D1000) (Kruskal-Wallis test; $p < 0.05$). Barplots highlighting the GM composition at phylum-and genus level of different conditions are showed in panel C and D respectively. Only phyla with a relative abundance ≥ 0.5% in at least 8% of samples, and genera with relative abundance ≥ 0.5% in at least 8% of samples are represented.

A paired statistical analysis among groups did not highlight any significant difference at either phylum or genus level (Wilcoxon rank-sum test; $p > 0.05$) (**Supplementary Fig. 2**). However, at genus phylogenetic level it was possible to

identify certain tendencies: both *Leuconostoc* and *Streptococcus* genera were less abundant in D0 (T2) and D1000 (T2) groups, i.e. in a sub-optimal condition, compared to D0 (T1) and D1000 (T1) groups (Wilcoxon rank-sum test; $p < 0.1$). On the other hand, the *Erwinia* genus was found to be more abundant in the D0 (T2) group compared to D0 (T1) and D1000 (T1) groups (Wilcoxon rank-sum test $p < 0.1$) (**Supplementary Fig. 2**).

Discussion

Feeding strategies addressing the use of organic acid and botanicals as potential growth and health promoters have attracted increasing interest in fish production [5,28]. In the present study, feeding rainbow trout with increasing dietary blend OA and NIC did not show a significant dose effect on growth performance in the first period of the trial. Similar outcomes were found in other studies conducted on salmonids species. Gao's study [39] did not report significant growth improvement of rainbow trout (233.32 g initial body weight) fed fishmeal or plant protein-based diets added with 10 g acid moiety/kg OA salt blends (mixture of sodium formate and butyrate, ratio 2:1) before and after feed extrusion. Another study by Bjerkeng [40] on OA salts blend (sodium acetate, sodium propionate and sodium butyrate, weight (w) concentration 5:5:2 w/w/w) at 0, 5 and 20 g kg^{-1} dietary dose supplied to Atlantic salmon (*Salmo salar*) for 175 days displayed no significant effects on growth or apparent digestibility of macronutrients. In the present work, even though there was no significant evidence of growth changes in the first period of the feeding trial, a significant improvement in performance was found in the second half of the study (days 40–82): Dietary OA and NIC inclusion led to a significant improvement of SGR and FCR. These results are also reinforced by the significant increasing of protein and lipid utilization as stated by PER and LER at the end of the trial. In agreement with the present findings, De Wet [17] found an improvement in growth when rainbow trout fingerlings (40 g initial body weight) were fed diets supplemented with 10 or 15 g kg^{-1} OA blends, compared to the non-supplemented treatment in a four-month trial. Pandey [41] reported consistently better growth and mineral utilization in rainbow trout fed for 12 weeks with low fishmeal-based (15%) diets supplemented with different OA at 1% (citric, lactic, fumaric, formic and acetic), especially for citric and fumaric acid. More recently, Villumsen [42] found that the dietary supplementation of organic acids and β-glucan showed improved FCR and LER in rainbow trout juveniles after 37 days of feeding. The work mentioned suggested that improved performance and digestibility of OA supplementation may be explained by lowered pH resulting in a higher dissociation of mineral compounds, reduced rate of gastric emptying and formation of chelated mineral complexes that can be easily absorbed. In addition, an improvement in digestive enzyme activities was also reported when 0.6% humic acid sodium salt supplementation was provided [43]. In Tran-Ngoc [44], Nile tilapias were fed with 2 g kg^{-1} potassium diformate (KDF), 2 g kg^{-1} calcium butyrate (CAB) and 4 g kg^{-1} KDF and CAB mixture ration 1:1 dietary level inclusion for five weeks in normoxic state (6 mg L^{-1}), followed by another period of exposure to hypoxic (3 mg L^{-1}) condition. Outputs found significant differences only in the last 5 weeks under challenging conditions, where singular OAs feed inclusions enhanced growth and nutrient digestibility by improving intestinal morphology. Interestingly, the same OA and NIC blend of the present study showed induced significant higher average daily gain during the second half of a two-week trial compared to a control diet also in pigs [45]. Organic acid and botanicals are known to modulate gut microbial community in fish species as reviewed by Ng [5] and Sutili [28] with several beneficial properties including prebiotic-like effect, direct action towards pathogens, reduced pathogen motility and invasion, interference with quorum sensing communication processes operating in different signal components of the bacterial cells, reduction of biofilm formation, and inhibition of extracellular protease activity and expression. Gut microbiota of rainbow trout using NGS technique have been extensively studied in relation to vegetal or animal ingredients to replace FM [46–49], while no data concerning

the application of the employed OA and NIC are available. Our finding displayed *Firmicutes, Proteobacteria*, and *Actinobacteria* as the most abundant phyla while *Lactobacillus, Leuconostoc* and *Streptococcus* were the genus most represented. These results are consistent with previous finding in rainbow trout, indicating that the inclusion of plant ingredients favoured the presence of *Firmicutes* over *Proteobateria*, which were more abundant in marine-derived diets [46,47]. In addition, different genera of lactic acid bacteria such as *Lactobacillus and Leuconostoc*, all belonging to *Firmicutes*, are generally considered beneficial microorganisms associated with a healthy intestinal epithelium in trout and other fish species [31,50,51]. Our finding showed only a moderate impact for the different OA + NIC inclusion levels on the gut bacterial composition of rainbow trout as shown by PCoA analysis. However, even though no significant differences among dietary groups were detected, PCoA showed a tendency to cluster in D0 group separately from the other dietary groups. Although there were no statistically significant differences affecting the relative abundance of specific taxa, a reduction trend for *Streptococcus* spp. was observed in the D250 and D1000 groups. Up to date, several bacterial species within the genus *Streptococcus* spp. have been reported as important pathogens of fish species including salmonids [52,53]. Previous *in vivo* studies have found that dietary essential oils/plant extracts such as rosemary (*Rosmarinus officinalis*), mangrove plant (*Excoecaria agallocha*), Aloe (*Aloe vera*), and Shirazi thyme (*Zataria multiflora*) are able to counteract *Streptococcus* pathogens in fish species via inhibition of bacterial growth, repressing of cytotoxin production, or by enhancing the non-specific immunity and disease resistance [54–56]. Interestingly, Soltani [57] showed that Shirazi thyme was able to reduce growth of *Streptococcus iniae* from a rainbow trout disease outbreak and had a stronger effect on down-regulating streptolysin S-related gene compared to rosemary. The authors attributed this effect as being due to the higher content of monoterpenoid phenols such as thymol from the essential oil of thyme. Temperature plays a key role in determining microbial diversity globally [58,59], and therefore may directly alter gut microbiomes in animals, especially in those that are unable to thermoregulate. Although the effects of temperature-induced changes in the gut microbiota on host colonization resistance have not been established yet, recent studies suggest that disruption of animal gut microbiota by temperature may reduce the resistance of hosts to invasion and colonization by pathogenic microorganisms [59]. In the present study, a significant reduction in the gut microbial diversity was obtained in both dietary treatments after one week's exposure to high water temperature conditions at 23 ◦C. A few studies investigated the temperature effects and diet–temperature interactions on the diversity of gut bacteria in rainbow trout using high-throughput sequencing. Among these, Huyben [60] evaluating the effects of dietary substitution of fishmeal (FM) with live yeast, and increasing water temperature, reported a lower gut microbial diversity in trout reared at warm temperatures (18 ◦C), compared to those reared in cold conditions (11 ◦C), while no effect on diet was obtained. In contrast, in yellowtail kingfish *Seriola lalandi* (a warm water marine fish species) the temperature of 20 ◦C was associated with a decrease in the richness and relative abundance of microbiota compared to fish kept at 26 ◦C [61]. The decreasing of alpha-diversity indices in fish species may lead to reduced competition for opportunistic or invading pathogens which may enter the gastrointestinal tract of fish via stressful conditions such as rearing density [51] and feeding competition [63] and also characterized the GM of unhealthy rainbow trout experiencing bacterial kidney disease (BKD) [64]. The intestine has an important immunological role and constitutes a physical barrier against pathogens [65]. The gastrointestinal mucosal surface is a natural interface where the intestinal microbiota and antigens cross-talk with the host fish [66]. The gut mucosa is rich in immune cells such as lymphocytes, plasma cells, eosinophilic granulocytes, and macrophages which can elicit local responses [67]. Regulatory systems involved in acquired and innate immune systems is brought about by direct cell-to-cell contact involving adhesion molecules and by the production of chemical messengers. Chief among these chemical messengers are proteins called cytokines, which can induce a broad range of

activities via multiple target cell types and through their redundancy, indicated by the overlap in activities among different cytokines [68]. Cytokines released by activated phagocytes are key factors in the inflammation process, particularly IL-1β, an important pro-inflammatory cytokine, interferons, tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β) and several chemokines. When an inflammatory response is induced, the cascade of cytokine secretion begins with the release of TNF-α; this stimulates the release of IL-1β, which is then followed by the release of IL-6. The initiation of inflammation leads to the release of a myriad of other cytokines, which include chemoattractants that signal neutrophils and macrophages to migrate to the site of infection (e.g. chemokines) [69]. In our study, during the feed trial (82 days) no significant differences were recorded in animals fed with increasing dose of OA and NIC. This finding proves that OA and NIC do not have inflammatory activities, pointing out the lack of the cytokine secretion cascade activation characteristic of the inflammation process. Accordingly, the growth performance and the gut microbiota composition recorded during the feeding trial did not show differences among tested diets. Thus, the establishment of a healthy microbiota plays an important role in the generation of immunophysiologic regulation in the host by providing crucial signals for the development and maintenance of the immune system [70]. The groups exposed to high water temperature showed no significantly different expression levels among the immune genes studied, except for IL-8 and TNF-α. Particularly, IL-8 is a potent inflammatory cytokine, which is known as neutrophil chemotactic factor and is produced by various immunocytes during oxidative stress and infections [71]. In addition, TNF-α is another inflammatory multifunctional cytokine synthesized by various kinds of cells and involved in the proliferation of immunocytes and in their migration, apoptosis, and phagocytic activity as well as in the expression of other pro-inflammatory cytokines [72]. The upregulation of IL-8 in D0 and D1000 groups and of the TNF-α in D1000 suggests the activation of a stress-condition triggered by the high temperature condition. In D0 and D1000 groups slight modifications in gut microbiota after the exposure to high temperature condition were also observed and these changes may have led to the upregulation of these cytokines. In this context, the concomitant reduction of the lactic acid bacteria (LAB) *Leuconostoc* should be mentioned. As single-strain probiotic, this genus showed to exert positive effects on growth performance in fish [73]. LAB are known to be involved in the modulation of topical and systemic immune systems being able to suppress the production of pro-inflammatory cytokines [74,75]. Contrary to our findings, rainbow trout exposed to stress conditions such as high stocking densities for 30 days showed different expression levels of pro-inflammatory cytokines. Particularly, the fish reared at high stocking densities (40 and 80 kg m^{-3}), showed a significant density-dependent downregulation in the expression of IL-1β and IL-8 and the lowest level was observed in the highest stocking density group [76]. The differences in the results between the present study with the findings of Yarahmadi [76] are supported by the fact that short-term stress (acute) and long-term stress (chronic) have different effects on fish immune system [77]. However, in agreement with our findings, Castillo [78] reported that adrenaline at a concentration of 1 mM caused increased expression of TNF-α gene but suppressed the expression of IL-1β in gilthead sea bream (*Sparus aurata*) head kidney cells. Moreover, Caipang [79] reported that short-term overcrowding up-regulated several immune-related genes such as IL-1β, IL-8 and g-type lysozyme in the blood of Atlantic cod (*Gadus morhua*). In the present study temperature seems to exert a somewhat effect on gut health status as supported by both reduced diversity of GM and increased intestinal inflammatory cytokine gene expression. However, the up-regulation of IL-8 and TNF-α, and the absence of regulation of other pro- or anti-inflammatory genes suggest the lack of a substantial inflammation process able to compromise the functional activity of the intestine. The cytokine gene expression pattern obtained in this study may have been affected by the time course and the persistence of the stressor as previously suggested [80]. Furthermore, available literature suggests that feed additives

may regulate inflammatory effects in an inconsistent pattern, possibly depending on the differences of composition, dosage, quality, route, and exposure time [81].

Conclusions

In conclusion, the dietary microencapsulated blend of OA and NIC employed at the tested inclusions improved growth and feed utilization of rainbow trout. Significant dose effects on the improvement of SGR and FCR were evident during the second half of the trial (days 40–82), probably indicating that the duration of feed administration plays a role in inducing an improvement of digestive conditions or microbiome modulation. Gut microbiota (GM) diversity and composition and cytokine gene expression analysis showed no significant differences in fish fed with increasing doses of OA and NIC after 82 days, pointing out the lack of inflammatory activity in the intestinal mucosa of rainbow trout fed increasing dietary blend. After exposure to high water temperature, a lower GM diversity and an increased intestinal inflammatory cytokine gene expression were observed for both types of diets (D0 vs. D1000) compared to groups in a standard condition. Although further studies should be conducted to fully clarify this mechanism, cytokine up-regulation seems to be concomitant to the reduction of GM diversity and particularly to the reduction of specific bacterial genera such as *Leuconostoc*. The feeding of this microencapsulated blend of OA + NIC at the tested doses can be a useful strategy to improve growth and feed utilization in rainbow trout under normal temperature conditions. According to the results, organic acids and nature-identical compounds did not revert the effects triggered by the increased temperature of water.

References

[1] S. Satoh, V. Kiron, Supplementation of citric acid and amino acid chelated trace elements in low-fish meal diet for rainbow trout affect, Growth and Phosphorus Utilization 43 (2012) 688–696.

[2] FEFANA, Organic Acids in Animal Nutrition, FEFANA Publication, 2014. http ://fefana.org/wp-content/uploads/2017/08/2014_08_20-BOOKLET-OA.pdf. accessed June 10, 2020.

[3] J.J. Dibner, P. Buttin, Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism, J. Appl. Poultry Res. 11 (2002) 453–463, https://doi.org/10.1093/JAPR/11.4.453.

[4] C. Lückst¨adt, Acidifiers in Animal Nutrition A Guide for Feed Preservation and Acidification to Promote Animal Performance, Nottingham University Press, Nottingham, UK, 2007. www.nup.com (accessed June 10, 2020).

[5] W.-K. Ng, C.-B. Koh, The utilization and mode of action of organic acids in the feeds of cultured aquatic animals, Rev. Aquacult. 9 (2017) 342–368, https://doi.org/ 10.1111/raq.12141.

[6] C. Lückst¨adt, The use of acidifiers in fish nutrition, CAB Rev. Perspect. Agric. Vet. Sci. Nutr. Nat. Resour. 3 (2008) 1–8, https://doi.org/10.1079/ PAVSNNR20083044.

[7] M.S.A. Sarker, S. Satoh, V. Kiron, Inclusion of citric acid and/or amino acid-chelated trace elements in alternate plant protein source diets affects growth and excretion of nitrogen and phosphorus in red sea bream Pagrus major, Aquaculture 262 (2007) 436–443, https://doi.org/10.1016/j.aquaculture.2006.10.007.

[8] M.A. Hossain, A. Pandey, S. Satoh, Effects of organic acids on growth and phosphorus utilization in red sea bream Pagrus major, Fish. Sci. 73 (2007) 1309–1317, https://doi.org/10.1111/j.1444-2906.2007.01469.x.

[9] A. Pandey, G. Angad, D. Veterinary, S. Satoh, Effects of Organic Acids on Growth and Phosphorus Utilization in Rainbow Trout Oncorhynchus mykiss, 2008, https:// doi.org/10.1111/j.1444-2906.2008.01601.x.

[10] J.S. Li, J.L. Li, T.T. Wu, Effects of non-starch polysaccharides enzyme, phytase and citric acid on activities of endogenous digestive enzymes of tilapia (Oreochromis niloticus × Oreochromis aureus), Aquacult. Nutr. 15 (2009) 415–420, https://doi. org/10.1111/j.1365-2095.2008.00606.x.

[11] F. Khajepour, S.A. Hosseini, Citric acid improves growth performance and phosphorus digestibility in Beluga (Huso huso) fed diets where soybean meal partly replaced fish meal, Anim. Feed Sci. Technol. 171 (2012) 68–73, https://doi.org/ 10.1016/j.anifeedsci.2011.10.001.

[12] A.J. Hern´andez, S. Satoh, V. Kiron, Supplementation of citric acid and amino acid chelated trace elements in low-fish meal diet for rainbow trout affect growth and phosphorus utilization, J. World Aquacult. Soc. 43 (2012) 688–696, https://doi. org/10.1111/j.1749-7345.2012.00589.x.

[13] M.S.A. Sarker, S. Satoh, K. Kamata, Y. Haga, Y. Yamamoto, Partial replacement of fish meal with plant protein sources using organic acids to practical diets for juvenile yellowtail, Seriola quinqueradiata, Aquacult. Nutr. 18 (2012) 81–89, https://doi.org/10.1111/j.1365-2095.2011.00880.x.

[14] S. Castillo, M. Rosales, C. Pohlenz, D.M.G. Iii, Effects of organic acids on growth performance and digestive enzyme activities of juvenile red drum Sciaenops ocellatus, Aquaculture 433 (2014) 6–12, https://doi.org/10.1016/j. aquaculture.2014.05.038.

[15] S.H. Sugiura, J. Gabaudan, F.M. Dong, R.W. Hardy, Dietary microbial phytase supplementation and the utilization of phosphorus, trace minerals and protein by rainbow trout [Oncorhynchus mykiss (Walbaum)] fed soybean meal-based diets, Aquacult. Res. 32 (2001) 583–592, https://doi.org/10.1046/j.1365- 2109.2001.00581.x.

[16] J. Vielma, K. Ruohonen, S.P. Lall, Supplemental citric acid and particle size of fish bone-meal influence the availability of minerals in rainbow trout Oncorhynchus mykiss (Walbaum), Aquacult. Nutr. 5 (1999) 65–71, https://doi.org/10.1046/ j.1365-2095.1999.00092.x.

[17] L. De Wet, Can organic acid effectively replace antibiotic growth promotants in diets for rainbow trout, Oncorhynchus mykiss raised under suboptimal water temperatures, in: Conf WAS, Indones Bali (Eds.), World Aquaculture Society: World Aquaculture 2005 - Presentation Abstract, 2005. http://www.was.org/meetingabstracts/ShowAbstract.aspx?Id=8529. accessed June 10, 2020.

[18] C. Ran, J. Hu, W. Liu, Z. Liu, S. He, B.C.T. Dan, N.N. Diem, E.L. Ooi, Z. Zhou, Thymol and carvacrol affect hybrid Tilapia through the combination of direct stimulation and an intestinal microbiota-mediated effect: insights from a germ-free zebrafish model, J. Nutr. 146 (2016) 1132–1140, https://doi.org/10.3945/ jn.115.229377.

[19] P. Navarrete, I. Toledo, P. Mardones, R. Opazo, R. Espejo, J. Romero, Effect of Thymus vulgaris essential oil on intestinal bacterial microbiota of rainbow trout, Oncorhynchus mykiss (Walbaum) and

bacterial isolates, Aquacult. Res. 41 (2010), https://doi.org/10.1111/j.1365-2109.2010.02590.x.
[20] I. Giannenas, E. Trianta, S. Stavrakakis, M. Margaroni, S. Mavridis, T. Steiner, E. Karagouni, Assessment of dietary supplementation with carvacrol or thymol containing feed additives on performance, intestinal microbiota and antioxidant status of rainbow trout, Oncorhynchus mykiss 353 (2012) 26–32, https://doi.org/ 10.1016/j.aquaculture.2012.04.027.
[21] R.M. Jaafar, P.W. Kania, A.H. Larsen, D.S. Nielsen, B. Fouz, C. Browdy, K. Buchmann, Gut microbiota changes in rainbow trout, Oncorhynchus mykiss (Walbaum), during organic acid feed supplementation and Yersinia ruckeri infection, J. Fish. Dis. 36 (2013) 599–606, https://doi.org/10.1111/jfd.12047.
[22] C.-B. Koh, N. Romano, A.S. Zahrah, W.-K. Ng, Effects of a dietary organic acids blend and oxytetracycline on the growth, nutrient utilization and total cultivable gut microbiota of the red hybrid tilapia, *Oreochromis* sp., and resistance to *Streptococcus agalactiae*, Aquacult. Res. 47 (2016) 357–369, https://doi.org/ 10.1111/are.12492.
[23] M.F. Nagoor Meeran, G.S. Jagadeesh, P. Selvaraj, Thymol attenuates inflammation in isoproterenol induced myocardial infarcted rats by inhibiting the release of lysosomal enzymes and downregulating the expressions of proinflammatory cytokines, Eur. J. Pharmacol. 754 (2015) 153–161, https://doi.org/10.1016/j. ejphar.2015.02.028.
[24] M.F. Costa, A.O. Durço, T.K. Rabelo, R.S.S. de Barreto, A.G. Guimar˜aes, Effects of Carvacrol, Thymol and essential oils containing such monoterpenes on wound healing: a systematic review, J. Pharm. Pharmacol. 71 (2019) 141–155, https:// doi.org/10.1111/jphp.13054.
[25] I. Giannenas, E. Triantafillou, S. Stavrakakis, M. Margaroni, S. Mavridis, T. Steiner, E. Karagouni, Assessment of dietary supplementation with carvacrol or thymol containing feed additives on performance, intestinal microbiota and antioxidant status of rainbow trout (Oncorhynchus mykiss), Aquaculture 350–353 (2012) 26–32, https://doi.org/10.1016/j.aquaculture.2012.04.027.
[26] D.J. Fitzgerald, M. Stratford, M.J. Gasson, J. Ueckert, A. Bos, A. Narbad, Mode of antimicrobial of vanillin against Escherichia coli, Lactobacillus plantarum and Listeria innocua, J. Appl. Microbiol. 97 (2004) 104–113, https://doi.org/10.1111/ j.1365-2672.2004.02275.x.
[27] I. Giannenas, E. Bonos, E. Christaki, P. Florou-Paneri, Essential oils and their applications in animal nutrition, Med. Aromatic Plants 2 (2013) 1–12, https://doi. org/10.4172/2167-0412.1000140.
[28] F.J. Sutili, D.M. Gatlin, B.M. Heinzmann, B. Baldisserotto, Plant essential oils as fish diet additives: benefits on fish health and stability in feed, Rev. Aquacult. 10 (2018) 716–726, https://doi.org/10.1111/raq.12197.
[29] A. Bonaldo, L. Parma, S. Ciulli, E. Volpe, B. Rossi, A. Piva, M. Soverini, F. D'Amico, M. Candela, P.P. Gatta, E. Grilli, Effects of increasing level of dietary organic acids and nature-identical compounds on growth, intestinal immune parameters and gut bacterial communities of european sea bass Dicentrarchus labrax juveniles, in: EAS AQUA Conf. Montpellier, Fr., World Aquaculture Society, 2018. AQUA 2018 - Presentation abstract, https://www.was.org/Meetings/ShowAbstract.aspx? Id=104103. accessed June 10, 2020.
[30] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917, https://doi.org/10.1139/o59-099.
[31] L. Parma, M. Yúfera, C. Navarro-Guill´en, F.J. Moyano, M. Soverini, F. D'Amico, M. Candela, R. Fontanillas, P.P. Gatta, A. Bonaldo, Effects of calcium carbonate inclusion in low fishmeal diets on growth, gastrointestinal pH, digestive enzyme activity and gut bacterial community of European sea bass (Dicentrarchus labrax L.) juveniles, Aquaculture 510 (2019) 283–292, https://doi.org/10.1016/j. aquaculture.2019.05.064.
[32] E. Bolyen, J.R. Rideout, M.R. Dillon, N.A. Bokulich, C.C. Abnet, G.A. Al-Ghalith, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2, Nat. Biotechnol. 37 (2019) 852–857, https://doi.org/10.1038/s41587- 019-0209-9.
[33] A.P. Masella, A.K. Bartram, J.M. Truszkowski, D.G. Brown, J.D. Neufeld, PANDAseq: paired-end assembler for illumina sequences, BMC Bioinf. 13 (2012) 1–7, https://doi.org/10.1186/1471-2105-13-31.
[34] B.J. Callahan, P.J. McMurdie, M.J. Rosen, A.W. Han, A.J.A. Johnson, S.P. Holmes, DADA2: high-resolution sample inference from Illumina amplicon data, Nat. Methods 13 (2016) 581–583, https://doi.org/10.1038/nmeth.3869.
[35] T. Rognes, T. Flouri, B. Nichols, C. Quince, F. Mah´e, VSEARCH: a versatile open source tool for metagenomics, PeerJ 2016 (2016), e2584, https://doi.org/ 10.7717/peerj.2584.
[36] Y. Gao, T. Storebakken, K.D. Shearer, M. Penn, M. Øverland, Supplementation of fishmeal and plant protein-based diets for rainbow trout with a mixture of sodium formate and butyrate, Aquaculture 311 (2011) 233–240, https://doi.org/10.1016/ j.aquaculture.2010.11.048.
[37] B. Bjerkeng, T. Storebakken, E. Wathne, Cholesterol and short-chain fatty acids in diets for Atlantic salmon Salmo salar (L.): effects on growth, organ indices, macronutrient digestibility, and fatty acid composition, Aquacult. Nutr. 5 (1999) 181–191, https://doi.org/10.1046/j.1365-2095.1999.00103.x.
[38] A. Pandey, S. Satoh, Effects of organic acids on growth and phosphorus utilization in rainbow trout Oncorhynchus mykiss, Fish. Sci. 74 (2008) 867–874, https://doi. org/10.1111/j.1444-2906.2008.01601.x.
[39] K.R. Villumsen, M. Ohtani, T. Forberg, J. Tinsley, M. Boye, A.M. Bojesen, Citrus flavonoids, β-Glucan and organic acid feed additives decrease relative risk during Yersinia ruckeri O1 biotype 2 infection of rainbow trout (Oncorhynchus mykiss), PeerJ 2020 (2020), e8706, https://doi.org/10.7717/peerj.8706.
[40] S. Yılmaz, S. Ergun, E.S¸. Çelik, M. Yigit, Effects of dietary humic acid on growth performance, haemato-immunological and physiological responses and resistance of Rainbow trout, *Oncorhynchus mykiss* to *Yersinia ruckeri*, Aquacult. Res. 49 (2018) 3338–3349, https://doi.org/10.1111/are.13798.
[41] K.T. Tran-Ngoc, S.T. Huynh, J. Send˜ao, T.H. Nguyen, A.J. Roem, J.A.J. Verreth, J. W. Schrama, Environmental conditions alter the effect of organic acid salts on digestibility and intestinal morphology in Nile tilapia (*Oreochromis niloticus*), Aquacult. Nutr. 25 (2019) 134–144, https://doi.org/10.1111/anu.12837.
[42] E. Grilli, B. Tugnoli, J.L. Passey, C.H. Stahl, A. Piva, A.J. Moeser, Impact of dietary organic acids and botanicals on intestinal integrity and inflammation in weaned pigs, BMC Vet. Res. (2015) 1–10, https://doi.org/10.1186/s12917-015-0410-0.
[43] A.R. Desai, M.G. Links, S.A. Collins, G.S. Mansfield, M.D. Drew, A.G. Van Kessel, J. E. Hill, Effects of plant-based diets on the distal gut microbiome of rainbow trout (Oncorhynchus mykiss), Aquaculture 350–353 (2012) 134–142, https://doi.org/ 10.1016/j.aquaculture.2012.04.005.
[44] H.C. Ingerslev, L. von Gersdorff Jørgensen, M. Lenz Strube, N. Larsen, I. Dalsgaard, M. Boye, L. Madsen, The development of the gut microbiota in rainbow trout (Oncorhynchus mykiss) is affected by first feeding and diet type, Aquaculture 424–425 (2014) 24–34, https://doi.org/10.1016/j.aquaculture.2013.12.032.
[45] S.C. Michl, J.M. Ratten, M. Beyer, M. Hasler, J. La Roche, C. Schulz, The malleable gut microbiome of juvenile rainbow trout (Oncorhynchus mykiss): dietdependent shifts of bacterial community structures, PloS One 12 (2017), https://doi.org/ 10.1371/journal.pone.0177735.
[46] P.C. Blaufuss, J.W. Bledsoe, T.G. Gaylord, W.M. Sealey, K.E. Overturf, M.S. Powell, Selection on a plant-based diet reveals changes in oral tolerance, microbiota and growth in rainbow trout (Oncorhynchus mykiss) when fed a high soy diet, Aquaculture 525 (2020) 735287, https://doi.org/10.1016/j. aquaculture.2020.735287.
[47] S. Rimoldi, G. Terova, C. Ascione, R. Giannico, F. Brambilla, Next generation sequencing for gut microbiome characterization in rainbow trout (Oncorhynchus mykiss) fed animal by-product meals as an alternative to fishmeal protein sources, PloS One 13 (2018), https://doi.org/10.1371/journal.pone.0193652.
[48] L. Parma, N.F. Pelusio, E. Gisbert, M.A. Esteban, F. D'Amico, M. Soverini, M. Candela, F. Dondi, P.P. Gatta, A. Bonaldo, Effects of rearing density on growth, digestive conditions, welfare indicators and gut bacterial community of gilthead sea bream (Sparus aurata, L. 1758) fed different fishmeal and fish oil dietary levels, Aquaculture 518 (2020) 734854, https://doi.org/10.1016/j. aquaculture.2019.734854.
[49] E. Ringø, S.H. Hoseinifar, K. Ghosh, H. Van Doan, B.R. Beck, S.K. Song, Lactic acid bacteria in finfish-An update, Front. Microbiol. 9 (2018) 1818, https://doi.org/ 10.3389/fmicb.2018.01818.
[50] M. Halimi, M. Alishahi, M.R. Abbaspour, M. Ghorbanpoor, M.R. Tabandeh, High efficacy and economical procedure of oral vaccination against Lactococcus garvieae/Streptococcus iniae in rainbow trout (Oncorhynchus mykiss), Fish Shellfish Immunol. 99 (2020) 505–513, https://doi.org/10.1016/j. fsi.2020.02.033.

[51] A.A. Laith, A.G. Mazlan, A.W. Effendy, M.A. Ambak, W.W.I. Nurhafizah, A.S. Alia, A. Jabar, M. Najiah, Effect of Excoecaria agallocha on non-specific immune responses and disease resistance of Oreochromis niloticus against Streptococcus agalactiae, Res. Vet. Sci. 112 (2017) 192–200, https://doi.org/10.1016/j. rvsc.2017.04.020.
[52] S. Abutbul, A. Golan-Goldhirsh, O. Barazani, D. Zilberg, Use of Rosmarinus officinalis as a treatment against Streptococcus iniae in tilapia (Oreochromis sp.), Aquaculture 238 (2004) 97–105, https://doi.org/10.1016/j. aquaculture.2004.05.016.
[53] N.N. Gabriel, J. Qiang, J. He, X.Y. Ma, M.D. Kpundeh, P. Xu, Dietary Aloe vera supplementation on growth performance, some haemato-biochemical parameters and disease resistance against Streptococcus iniae in tilapia (GIFT), Fish Shellfish Immunol. 44 (2015) 504–514, https://doi.org/10.1016/j.fsi.2015.03.002.
[54] M. Soltani, M. Ghodratnama, H.A. Ebrahimzadeh-Mosavi, G. Nikbakht-Brujeni, S. Mohamadian, M. Ghasemian, Shirazi thyme (Zataria multiflora Boiss) and Rosemary (Rosmarinus officinalis) essential oils repress expression of sagA, a streptolysin S-related gene in Streptococcus iniae, Aquaculture 430 (2014) 248–252, https://doi.org/10.1016/j.aquaculture.2014.04.012.
[55] L.R. Thompson, J.G. Sanders, D. McDonald, A. Amir, J. Ladau, K.J. Locey, et al. A communal catalogue reveals Earth's multiscale microbial diversity, Nature 551 (2017) 457–463, https://doi.org/ 10.1038/nature24621.
[56] J. Sepulveda, A.H. Moeller, The effects of temperature on animal gut microbiomes, Front. Microbiol. 11 (2020), https://doi.org/10.3389/fmicb.2020.00384.
[57] D. Huyben, L. Sun, R. Moccia, A. Kiessling, J. Dicksved, T. Lundh, Dietary live yeast and increased water temperature influence the gut microbiota of rainbow trout, J. Appl. Microbiol. 124 (2018) 1377–1392, https://doi.org/10.1111/jam.13738.
[58] E.L. Soriano, D.T. Ramírez, D.R. Araujo, B. G´omez-Gil, L.I. Castro, C.G. S´anchez, Effect of temperature and dietary lipid proportion on gut microbiota in yellowtail kingfish Seriola lalandi juveniles, Aquaculture 497 (2018) 269–277, https://doi. org/10.1016/j.aquaculture.2018.07.065.
[59] E. Apper, D. Weissman, F. Respondek, A. Guyonvarch, F. Baron, P. Boisot, A. Rodiles, D.L. Merrifield, Hydrolysed wheat gluten as part of a diet based on animal and plant proteins supports good growth performance of Asian seabass (Lates calcarifer), without impairing intestinal morphology or microbiota, Aquaculture 453 (2016) 40–48, https://doi.org/10.1016/j. aquaculture.2015.11.018. [60] A. Singh, J.J. Faber Hammond, C.F. O'Rourke, S.C.P. Renn, Gut microbial diversity increases with social rank in the African cichlid fish, Astatotilapia burtoni, Anim. Behav. 152 (2019) 79–91, https://doi.org/10.1016/j.anbehav.2019.04.003.
[61] A.N. Parshukov, E.N. Kashinskaya, E.P. Simonov, O.V. Hlunov, G.I. Izvekova, K. B. Andree, M.M. Solovyev, Variations of the intestinal gut microbiota of farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), depending on the infection status of the fish, J. Appl. Microbiol. 127 (2019) 379–395, https://doi.org/10.1111/ jam.14302.
[62] M. Grosell, A. Farrell, C. Brauner, Fish Physiology: the Multifunctional Gut of Fish - Google Libri, Academic Press, 2010. https://books.google.it/books?hl=it&lr=&id=eddWw_nyRlYC&oi=fnd&pg=PP1&dq=Fish+Physiology:+The+Multifunctional+Gut+of+Fish&ots=cbMvbYxx9Y&sig=g8bQzcyYUKcuJqrf5IMzzQZzKXw&redir_esc=y#v=onepage&q=Fish Physiology%3A The Multifu nctional Gut of Fish&f=false (accessed June 10, 2020).
[63] M. Montalto, F. D'Onofrio, A. Gallo, A. Cazzato, G. Gasbarrini, Intestinal microbiota and its functions, Dig. Liver Dis. Suppl. 3 (2009) 30–34, https://doi. org/10.1016/S1594-5804(09)60016-4.
[64] C.M.L. Press, Ø. Evensen, The morphology of the immune system in teleost fishes, Fish Shellfish Immunol. 9 (1999) 309–318, https://doi.org/10.1006/ fsim.1998.0181.
[65] M.Y. Engelsma, M.O. Huising, W.B. Van Muiswinkel, G. Flik, J. Kwang, H.F. J. Savelkoul, B.M.L. Verburg-Van Kemenade, Neuroendocrine-immune interactions in fish: a role for interleukin-1, in: Vet. Immunol. Immunopathol., Elsevier, 2002, pp. 467–479, https://doi.org/10.1016/S0165-2427(02)00077-6.
[66] G.D. G´omez, J.L. Balc´azar, A review on the interactions between gut microbiota and innate immunity of fish, FEMS Immunol. Med. Microbiol. 52 (2008) 145–154, https://doi.org/10.1111/j.1574-695X.2007.00343.x.
[67] S.J. Salminen, M. Gueimonde, E. Isolauri, Probiotics that modify disease risk, J. Nutr. 135 (2005) 1294–1298, https://doi.org/10.1093/jn/135.5.1294.
[68] N. Jimenez, J. Coll, F.J. Salguero, C. Tafalla, Co-injection of interleukin 8 with the glycoprotein gene from viral haemorrhagic septicemia virus (VHSV) modulates the cytokine response in rainbow trout (Oncorhynchus mykiss), Vaccine 24 (2006) 5615–5626, https://doi.org/10.1016/j.vaccine.2006.04.061.
[69] C. Yuan, X. Pan, Y. Gong, A. Xia, G. Wu, J. Tang, X. Han, Effects of Astragalus polysaccharides (APS) on the expression of immune response genes in head kidney, gill and spleen of the common carp, Cyprinus carpio L, Int. Immunopharm. 8 (2008) 51–58, https://doi.org/10.1016/j.intimp.2007.10.009.
[70] S.K. Allameh, F.M. Yusoff, E. Ringø, H.M. Daud, C.R. Saad, A. Ideris, Effects of dietary mono- and multiprobiotic strains on growth performance, gut bacteria and body composition of Javanese carp (*Puntius gonionotus* , Bleeker 1850), Aquacult. Nutr. 22 (2016) 367–373, https://doi.org/10.1111/anu.12265.
[71] C. Matsuzaki, A. Hayakawa, K. Matsumoto, T. Katoh, K. Yamamoto, K. Hisa, Exopolysaccharides produced by Leuconostoc mesenteroides strain NTM048 as an immunostimulant to enhance the mucosal barrier and influence the systemic immune response, J. Agric. Food Chem. 63 (2015) 7009–7015, https://doi.org/ 10.1021/acs.jafc.5b01960.
[72] B. Yousefi, M. Eslami, A. Ghasemian, P. Kokhaei, A. Salek Farrokhi, N. Darabi, Probiotics importance and their immunomodulatory properties, J. Cell. Physiol. 234 (2019) 8008–8018, https://doi.org/10.1002/jcp.27559.
[73] P. Yarahmadi, H.K. Miandare, S. Fayaz, C.M.A. Caipang, Increased stocking density causes changes in expression of selected stress- and immune-related genes, humoral innate immune parameters and stress responses of rainbow trout (Oncorhynchus mykiss), Fish Shellfish Immunol. 48 (2016) 43–53, https://doi.org/ 10.1016/j.fsi.2015.11.007.
[74] G. Nardocci, C. Navarro, P.P. Cort´es, M. Imarai, M. Montoya, B. Valenzuela, P. Jara, C. Acu˜na-Castillo, R. Fern´andez, Neuroendocrine mechanisms for immune system regulation during stress in fish, Fish Shellfish Immunol. 40 (2014) 531–538, https://doi.org/10.1016/j.fsi.2014.08.001.
[75] J. Castillo, M. Teles, S. Mackenzie, L. Tort, Stress-related hormones modulate cytokine expression in the head kidney of gilthead seabream (Sparus aurata), Fish Shellfish Immunol. 27 (2009) 493–499, https://doi.org/10.1016/j. fsi.2009.06.021.
[76] C.M.A. Caipang, M.F. Brinchmann, I. Berg, M. Iversen, R. Eliassen, V. Kiron, Changes in selected stress and immune-related genes in Atlantic cod, *Gadus morhua* , following overcrowding, Aquacult. Res. 39 (2008) 1533–1540, https://doi.org/ 10.1111/j.1365-2109.2008.02026.x.
[77] L. Tort, Stress and immune modulation in fish, Dev. Comp. Immunol. 35 (2011) 1366–1375, https://doi.org/10.1016/j.dci.2011.07.002. [81] J. Wang, P. Lei, A.A.A. Gamil, L. Lagos, Y. Yue, K. Schirmer, L.T. Mydland, M. Øverland, Å. Krogdahl, T.M. Kortner, Rainbow trout (Oncorhynchus mykiss) intestinal epithelial cells as a model for studying gut immune function and effects of functional feed ingredients, Front. Immunol. 10 (2019) 152, https://doi.org/ 10.3389/fimmu.2019.00152.
[78] J.P. Evenhuis, B.M. Cleveland, Modulation of rainbow trout (Oncorhynchus mykiss) intestinal immune gene expression following bacterial challenge. Vet. Immunol. Immunopathol., 146(1), 8-17, doi: 10.1016/j.vetimm.2012.01.008.
[79] F. Gagn´e, A. Bruneau, P. Turcotte, C. Gagnon, E. Lacaze, An investigation of the immunotoxicity of oil sands processed water and leachates in trout leukocytes Ecotox, Environ. Safe. 141 (2017) 43–51.
[80] M.K. Raida, L. Holten-Andersen, K. Buchmann, Association between Yersinia ruckeri infection, cytokine expression and survival in rainbow trout (Oncorhynchus mykiss), Fish Shellfish Immunol. 30 (2011) 1257–1264, https:// doi.org/10.1016/j.fsi.2011.03.022.
[81] E. Chaves-Pozo, J. Zou, C.J. Secombes, A. Cuesta, C. Tafalla, The rainbow trout (Oncorhynchus mykiss) interferon response in the ovary, Mol. Immunol. 47 (2010) 1757–1764, https://doi.org/10.1016/j.molimm.2010.02.030.

2.4 Study II - Interaction between dietary lipid level and seasonal temperature changes in gilthead sea bream Sparus aurata: effects on growth, fat deposition, plasma biochemistry, digestive enzyme activity and gut bacterial community

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Introduction

Today, feeding strategy optimization related to environmental conditions is extremely necessary to pursue more intensive and more efficient aquaculture production in the Mediterranean basin. Feed production is the greatest cost in the aquaculture sector and can account for up to 60-80% of overall expenses (Hasan, 2007; Luna et al., 2019). Dietary lipid supplementation has been largely developed to reduce feed cost and reduce the need for limited and expensive protein ingredients in many farmed fish species (Bell & Koppe, 2010; Bonaldo et al., 2010; Leaver et al., 2008) including gilthead sea bream (*Sparus aurata*) which is one of the most important marine finfish species farmed in the Mediterranean area (Guillén, 2019). Currently, commercial diet composition for the grow-out phase of this species consists on average of 43% protein and 20% fat (Arantzamendi et al., 2019; Koven, 2002; Vasconi et al., 2017). Among abiotic factors, water temperature is the key environmental factor, playing a crucial role on metabolism, nutrient utilization, fat deposition and welfare, in particular for this species which is highly susceptible to thermal seasonal changes and fluctuation (Ibarz et al., 2010; Sánchez-Nuño, Eroldogan, et al., 2018). Although it is known that the optimal temperature range is between 18 °C and 26 °C (Davis, 1988; Jobling & Peruzzi, 2010), this species is yearly subjected to large temperature fluctuations (from 11 °C to 26 °C) in most farming conditions. Previous works found that increasing dietary lipids from 16 to 24% produced no significant differences on final body weight and specific growth rate at summer temperatures between 24 °C and 27 °C (Bonaldo et al., 2010; Mongile, Bonaldo, Fontanillas, Mariani, Badiani, Bonvini, & Parma, 2014; Velázquez et al., 2006). On the other hand, several studies have also been devoted to developing winter feeds for overcoming metabolic alterations, immune suppression and nutritional disorders (Richard et al., 2016; Silva et al., 2014). While most of these diets were formulated in order to test the effectiveness of functional ingredients such as immunostimulants and antioxidants, their lipid content ranged from 17 to 19.7%. In addition, especially at temperatures below 13 °C, if there is an excess of dietary lipid it can be accumulated as a fat depot in perivisceral tissue due to low metabolic activity (Ibarz et al., 2010; Ibarz, Beltrán, et al., 2007a). To the best of our knowledge, few studies have been carried out to assess optimal lipid composition during water temperature seasonal changes. (Sánchez-Nuño, Eroldogan, et al., 2018) found that dietary lipid content 18 vs 14% did not affect growth in fish subjected to temperature fluctuations from 22 to 14 °C; however the authors suggested adopting lower lipid levels to avoid excessive fat deposition and putative oxidative stress during recovery. Environmental temperature fluctuation during seasonal changes may also affect fish metabolism, digestive enzyme activity and gut bacterial community, which may influence performance, tissue composition and fish health (Couto et al., 2012; García-Meilán et al., 2013; Guerreiro et al., 2016; Sepulveda & Moeller, 2020; Zarkasi et al., 2016). To date, very limited studies have investigated how seasonal changes of water

temperature interact with diet in shaping the gut microbiome structure in teleost species, and none of them deals with sea bream. The aim of the present study was to explore the effects of dietary lipid level and seasonal temperature changes on growth, digestive enzyme activity, plasma biochemistry and gut microbiome structure during the on-growing of gilthead sea bream.

Materials and methods

Experimental diets

Ingredients and proximate composition of the experimental diets are represented in **Table 1**. Two isonitrogenous (43.7 %) extruded diets (sinking pellet size diameter 4.00 mm) were produced to contain a low 16% (L16) and high 21% (L21) dietary lipid level. Diets were formulated with fish meal and with a mixture of vegetable ingredients currently used for sea bream in aquafeed (Parma et al., 2016). Diets were produced by Sparos Lda (Olhão, Portugal).

Table 1. Ingredients and proximate composition of the experimental diets

	L16	L21
Ingredients, % of the diet		
Fishmeal Super Prime	15.00	15.00
Soy protein concentrate	16.00	16.00
Wheat gluten	7.45	8.00
Corn gluten	9.00	9.00
Soybean meal 44	20.00	20.00
Wheat meal	16.65	12.00
Fish oil	6.50	8.55
Rapeseed oil	6.50	8.55
Vitamin and Mineral Premix INVIVO 1%	1.00	1.00
Antioxidant	0.20	0.20
Sodium propionate	0.10	0.10
MAP (Monoammonium phosphate)	1.00	1.00
L-Lysine	0.25	0.25
DL-Methionine	0.35	0.35
Proximate composition, % on a wet weight basis		
Moisture	5.69	5.84
Protein	43.59	43.75
Lipid	16.30	20.81
Ash	6.29	6.24
Gross energy cal g^{-1}	4819.12	5051.63

Vitamins and mineral premix (iu or mg kg-1 diet; invivo nsa,: portugal); dl-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 10 mg; retinyl acetate, 16,650 iu; dl-cholecalciferol, 2000 iu; thiamine, 25 mg; riboflavin, 25 mg; pyridoxine, 25 mg; cyanocobalamin, 0.1 mg; niacin, 150 mg; folic acid, 15 mg; l-ascorbic acid monophosphate, 750 mg; inositol, 500 mg; biotin, 0.75 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg; copper sulphate heptahydrate, 25 mg; ferric sulphate monohydrate, 100 mg; potassium iodide, 2 mg; manganese sulphate monohydrate, 100 mg; sodium selenite, 0.05 mg; zinc sulphate monohydrate, 200 mg; yttrium oxide, 100 mg.

Seasonal temperature changes

Before the beginning of the trial, fish were adapted to the laboratory facilities at the constant water rearing temperature of 20 °C for ten days. At the beginning of the trial, triplicate tanks were randomly divided into two groups: one at high (H) temperature 23.17 ± 1.11 °C and one at low (L) temperature 17.34 ± 0.92 °C respectively and maintained at these constant temperatures for 58 days.

On day 58 fish were exposed to a switch in temperature (fish kept at H were transferred to L , HL and the fish kept at L were transferred to H (LH) while continuing to receive the same diet in each group. Thus, fish which

were brought from 23 °C to 17 °C (HL) were subjected to summer-autumn variation, while fish brought from 17 °C to 23 °C (LH) underwent spring-summer changes.

Fish and rearing conditions

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. Gilthead sea bream juveniles were obtained from Panittica Pugliese (Torre Canne di Fasano, Brindisi, Italy). At the beginning of the trial 30 fish (initial average weight: 67.50 ± 1.66 g) per tank were randomly distributed into twelve 450 L square tanks. Experimental diets (L16 and L21) were administered to triplicate groups to visual satiation twice a day (h 8.30 and h 16.00) for six days a week. While temperatures were switched after intermediate day sampling, each tank continued to receive the same dietary treatment until the end of the trial. Tanks were provided with natural seawater and connected to a closed recirculating aquaculture system (RAS) with an overall water volume capacity of 6000 L. The rearing system consisted of a mechanical sand filter (0.4 m^3 of silica sand, 0.4–0.8 mm. PTK 1200, Astral Pool, Servaqua S.A. Barsareny, Spain), ultraviolet lights (SH-63, BLUGEO S.r.l., Parma, Italy) and a biofilter (PTK 1200, Astral Pool, Servaqua S.A. Barsareny, Spain). During the trial, photoperiod was maintained at 12 h light and 12 h dark through artificial light (light intensity on the water surface 400 lux). The oxygen level was kept constant (8.0 ± 1.0 mg L^{-1}) by a liquid oxygen system regulated by a software program (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen, TAN ≤ 0.1 mg L^{-1}), nitrite (NO_2 ≤ 0.2 mg L^{-1}), nitrate (NO_3 ≤ 50 mg L^{-1}) and salinity (25-30 g L^{-1}) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate was added daily to keep pH constant at 7.8–8.0. The feeding trial lasted a total of 121 days. In the RAS water temperature was maintained warmer in six tanks by a heater (H03609-00.B-2012/01, Zodiac Pool Care, Saint-Barthélemy-d'Anjou, France), while the water in the remaining tanks was kept cooler (AWP 16 SP R407C, GENCOLD S.r.l., Cesena, Italy) for the whole experiment.

Sampling

At the beginning, half-way through and at the end of the experiment, all the fish in each tank were anaesthetised by Tricaine Methanesulfonate at 100 mg L^{-1} and individually weighed. The proximate composition of the carcasses was determined on pooled samples at the beginning (10 fish per tank), at the half-way stage before the temperature switch (3 fish per tank), and at the end of the trial (5 fish per tank).

Furthermore, wet weight of viscera, liver and perivisceral fat were individually recorded for intermediate (6 fish per tank) and final (5 fish per tank) pools to determine viscerosomatic index (VSI) hepatosomatic index (HSI) and mesenteric fat index (MFI). Moreover, liver pooled samples (from 6 individuals per tank) were taken out at the end of the trial and stored at -20 °C until analysed to access the fat liver content in animals subjected to seasonal temperature change. At 5 hours post meal (hpm), 3 fish per tank (n=9/treatment) on day 58 (before temperature changes) and 5 fish per tank (n=15/treatment) on day 121 were sampled and dissected to obtain their whole gastrointestinal tract, then they were first stored at −80 °C and subsequently freeze-dried until digestive enzyme activity analysis according to (Busti et al., 2020).

Digesta content (n=3 fish per tank on intermediate sampling day 58, n=9 fish per diet treatment; n=3 fish per tank on final sampling day 121st, n=9 fish per diet treatment) from posterior intestine was also individually sampled and immediately stored at -80°C for gut microbiota investigation according to (Parma et al., 2016).
Blood was collected from the caudal vein in 3 fish per tank on intermediate sampling (n=9 fish per treatment), and in 5 fish per tank (n=15 fish per treatment) on final sampling. Samples were then centrifuged (3000 g for 10 min at 4°C) and plasma aliquots were stored at -80°C until analysis according to (Bonvini et al., 2018).
All experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

Calculations

The formulae employed to calculate growth performance, somatic indices, nutritional indices and relative variations were as follows:

Specific growth rate (SGR) (% day-1) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights).
FI = Feed intake (g kg ABW−1 day−1) = ((1000*total ingestion)/(ABW))/days)).
Feed conversion ratio (FCR) = feed intake / weight gain.
Viscerosomatic index (VSI) (%) = 100 * (viscera weight / body weight).
Hepatosomatic index (HSI) (%) = 100 * (liver weight / body weight).
Mesenteric fat index (MFI) (%) = 100 * (mesenteric fat weight / body weight).
Protein efficiency rate (PER) = (FBW – IBW) / protein intake.
Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish.
Lipid efficiency rate (LER) = (FBW – IBW) / lipid intake.
Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake fish.
Relative variation = (final considered value – initial considered value) / initial considered value.

Proximate composition analysis

Diets and whole bodies were analysed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105 °C overnight. Crude protein was determined as total nitrogen (N*6.25) after performing Kjeldahl's method. Total lipids were determined according to (Bligh & Dyer, 1959) extraction method. The same method was performed also on final liver pools samples in order to estimate their fat content. Ash content was estimated by incineration in a muffle oven at 450 °C overnight. Gross energy was determined by a calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, U.S.A).

Digestive enzyme activity analysis

Stomach and proximal intestine, including the pyloric caeca, of each individual were separately homogenised in distilled water (1:3 w/v), and were centrifuged at 4 °C, 13,000 g, for 10 min. Supernatants were stored at –20 °C until being processed. Using the stomach homogenate, pepsin activity was measured according to the methodology described in (Anson, 1938). In brief, 10 μL of the enzyme extract was diluted in 1 mL of 0.1 M HCl-glycine buffer (pH 2.0) containing 0.5 % bovine haemoglobin. The mixture was incubated for 20 min at

room temperature (approximately 25°C). The reaction was terminated by adding 0.5 mL of 20 % trichloroacetic acid (TCA) and was cooled at 4 °C for 15 min to facilitate precipitation. After centrifuging at 13,000 g for 15 min at 4 ºC, 200 µL of the supernatant was used to measure absorbance at 280 nm. One unit of enzyme activity was defined as 1 µg tyrosine released per minute using a specific absorptivity of 0.008 ug^{-1} cm^{-1} at 280 nm. In the proximal intestine homogenate, trypsin and chymotrypsin activity were measured using Nα-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) and N-Glutaryl-L-phenylalanine p-nitroanilide (GAPNA) as substrates, according to Erlanger et al., (1961) and (1966) respectively. For each of these enzymes, substrate stock (0.5 mM of BAPNA or GAPNA in dimethyl sulfoxide) was brought to the working concentration by 1/10th dilutions using 50 mM Tris-HCl and 20 mM $CaCl_2$ buffer (pH 8.5). The change in absorbance at 405 nm was measured over 10 min at room temperature, for 10 to 15 µL of the enzyme extract and 200 µL of substrate per each microplate well. For these enzymes, one unit of activity was defined as 1 µmol p-nitroaniline released per minute using coefficients of molar extinction of 8270 M^{-1} cm^{-1} at 405 nm. Amylase activity was measured following the 3,5-di-nitrosalicylic acid (DNSA) method (Bernfeld, 1955). In brief, 30 µL of enzyme extract and 300 µL of substrate (2% soluble starch in 100 mM phosphate and 20 mM $NaCl_2$ buffer (pH 7.5) were incubated at 37 °C for 30 min. The reaction was stopped by the addition of 150 µL DNSA and was heated in boiling water for 5 min. After cooling on ice, 1.5 mL of distilled water was added to the mixture and the absorbance was measured at 530 nm. One unit of amylase activity was defined as the amount of enzyme needed to catalyse the formation of 1 µg of maltose equivalent per minute. Lipase activity was measured using 4-Nitrophenyl myristate as substrate, according to Albro et al., (1985). Briefly, 10 µL of enzyme extract was added to 50 µL Sodium taurocholate (0.4 mg mL^{-1}) and 130 µL of 100 mM Tris-HCL buffer (pH 8.0) per each microplate well. The change in the absorbance at 405 nm was measured over 10 min at room temperature. One unit of amylase activity was defined as the amount of enzyme needed to catalyse the production of 1 µg of p-nitrophenol per minute. All the activities were expressed in units per g of wet weight of fish, considering both the total amount of tissue used for enzyme determination and the live weight of each sampled fish.

Metabolic parameters in plasma

The levels of glucose (GLU), urea, creatinine (CREA), uric acid (Uric Ac), total bilirubin (Tot Bil), bile acid (Bil Ac), cholesterol (CHOL), triglycerides (TRIG), high density lipoprotein (HDL), total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH), calcium (Ca^{+2}), inorganic phosphorus (P), potassium (K^{+}) sodium (Na^{+}), iron (Fe), chloride (Cl), magnesium (Mg) and cortisol (CORT) were determined in the plasma using samples of 500µL on an automated analyser (AU 400; Beckman Coulter) according to the manufacturer's instructions. The albumin/globulin (ALB/GLO), Ca x P, and Na/K ratio were calculated.

Gut bacterial community DNA extraction and sequencing

Total DNA was extracted and analysed from individual distal intestine content obtained from 3 fish per tank (300 mg per fish) on day 58 and day 121, as previously reported in (Parma et al., 2020). Amplification. Purification and sequencing of V3-V4 hypervariable region of the 16S rRNA bacterial gene was carried out as described in paragraph 2.2. Sequencing was performed on Illumina MiSeq platform using a 2 × 250 bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA). Raw sequences were processed as described in paragraph 2.2.

Statistical analysis

All data are represented as mean ± standard deviation (SD). A tank was used as the experimental unit for analysing growth performance, and a pool of three (on intermediate sampling, day 58) and five (on final sampling, day 121) fish was considered as the experimental unit for analysing carcass composition, liver fat content and nutritional indices, whereas nine (on intermediate sampling, day 58) and fifteen (on final sampling, day 121) individual fish per treatment were used for analysing somatic indices, digestive enzyme activity, blood biochemistry and gut bacterial community profiles. Data of growth performance, nutritional indices, somatic indices, fat liver content, enzyme activity and plasma parameters were analysed by a two-way analysis of variance (ANOVA) with Tukey's post hoc test. In order to assess the amplitude of variations occurring before and after the temperature change, relative variations on growth parameters, morphometric indices, nutritional indices, digestive enzyme activity and plasma biochemistry were calculated and analysed by a two-way analysis of variance (ANOVA) with Tukey's post hoc test. All gut microbiota statistical analyses were performed using R project (https://www.r-project.org/) as described in paragraph 2.2. PCoA plots were generated using the "vegan" (http://www.cran.r-project.org/package-vegan/) and "Made4" packages (Culhane et al., 2005), and for all PCoA betadisper and permutest functions were used to assess homogeneity of dispersion of our data (in all tests p-value was > 0.05). A p-value ≤ 0.05 was considered statistically significant, while a p-value between 0.05 and 0.1 was seen as a trend.

Results

Growth

Results on growth performance parameters and nutritional indices are summarised in **Table 2**. Concerning the seasonal temperature change occurring in the overall experimental period (day 0-121 period), no significant effects of diet nor temperature ($p > 0.05$) on growth (FBW, WG, SGR), FI and survival were detected, while FCR was significantly influenced by temperature ($p < 0.05$) with lower values in LH (animals first exposed to L temperature and then switched to H). At the same time diet and temperature had a significant effect on LER and GLE ($p < 0.05$), both of which were found to be higher in animals fed L16, while among groups fed the same dietary treatment, they were slightly lower in HL. Over day 0-58 period, no dietary effect was detected on FBW, WG, SGR, FCR, FI and survival ($p > 0.05$) while temperature had a significant effect ($p < 0.05$) on

Table 2. Growth performance and nutritional indices of gilthead seabream fed experimental diets and exposed to seasonal water temperature changes.

	Overall period day 0 – 121						
	L16		L21		*P-value*		
	HL	LH	HL	LH	*Inte*	*Temp*	*Diet*
Growth performances							
IBW	66.83 ± 1.07	68.13 ± 0.64	67.27 ± 2.05	67.80 ± 2.77	*0.727*	*0.412*	*0.964*
FBW	175.07 ± 9.91	181.01 ± 10.81	170.84 ± 19.28	169.65 ± 20.71	*0.708*	*0.803*	*0.421*
WG	108.24 ± 8.89	112.88 ± 10.79	103.57 ± 17.83	101.85 ± 19.33	*0.721*	*0.870*	*0.388*
SGR	0.80 ± 0.03	0.81 ± 0.05	0.77 ± 0.07	0.75 ± 0.08	*0.728*	*>0.999*	*0.311*
FCR	1.34 ± 0.01	1.30 ± 0.04	1.32 ± 0.01	1.29 ± 0.03	*0.750*	*0.036*	*0.352*
FI	1.01 ± 0.04	0.99 ± 0.05	0.96 ± 0.09	0.92 ± 0.05	*0.930*	*0.391*	*0.162*
Survival	78.89 ± 1.92	78.89 ± 1.92	76.67 ± 5.77	77.78 ± 3.85	*0.805*	*0.805*	*0.457*
Nutritional indices							
PER	1.67 ± 0.01	1.74 ± 0.02	1.70 ± 0.03	1.74 ± 0.08	*0.624*	*0.062*	*0.466*
GPE	29.5 ± 1.34	30.8 ± 0.41	29.9 ± 0.88	29.6 ± 1.53	*0.232*	*0.489*	*0.552*
GLE	66.9 ± 1.40 ^{b}	76.5 ± 1.91 ^{b}	54.4 ± 5.93 ^{a}	56.0 ± 5.02 ^{a}	*0.128*	*0.044*	*0.000*
LER	4.47 ± 0.03 ^{b}	4.64 ± 0.05 ^{b}	3.58 ± 0.05 ^{a}	3.67 ± 0.17 ^{a}	*0.425*	*0.036*	*0.000*
	Before seasonal change day 0 – 58						
	H	L	H	L	*Inte*	*Temp*	*Diet*
Growth performances							
IBW	66.83 ± 1.07	68.13 ± 0.64	67.27 ± 2.05	67.80 ± 2.77	*0.727*	*0.412*	*0.964*
FBW	134.74 ± 13.45 ^{b}	95.16 ± 4.13 ^{a}	136.12 ± 13.74 ^{b}	99.44 ± 8.42 ^{a}	*0.821*	*0.000*	*0.659*
WG	67.91 ± 12.53 ^{b}	27.03 ± 3.96 ^{a}	68.86 ± 12.22 ^{b}	31.64 ± 5.66 ^{a}	*0.745*	*0.000*	*0.622*
SGR	1.20 ± 0.15 ^{b}	0.57 ± 0.07 ^{a}	1.21 ± 0.13 ^{b}	0.66 ± 0.08 ^{a}	*0.558*	*0.000*	*0.527*
FCR	1.25 ± 0.04	1.35 ± 0.09	1.23 ± 0.05	1.28 ± 0.01	*0.410*	*0.051*	*0.205*
FI	1.45 ± 0.16 ^{b}	0.77 ± 0.05 ^{a}	1.44 ± 0.18 ^{b}	0.84 ± 0.10 ^{a}	*0.622*	*0.000*	*0.715*
Survival	100.00 ± 0.00	100.00 ± 0.00	98.89 ± 1.92	98.89 ± 1.92	*>0.999*	*>0.999*	*0.195*
Nutritional indices							
PER	1.83 ± 0.07	1.69 ± 0.11	1.85 ± 0.06	1.78 ± 0.01	*0.461*	*0.043*	*0.250*
GPE	33.0 ± 1.16 ^{b}	28.1 ± 0.47 ^{a}	30.8 ± 1.17 ^{ab}	29.6 ± 2.55 ^{ab}	*0.070*	*0.008*	*0.718*
GLE	71.3 ± 10.36	77.5 ± 11.72	65.4 ± 2.35	52.0 ± 15.36	*0.163*	*0.586*	*0.039*
LER	4.89 ± 0.19 ^{b}	4.52 ± 0.30 ^{b}	3.89 ± 0.13 ^{a}	3.73 ± 0.02 ^{a}	*0.360*	*0.044*	*0.000*
	After seasonal change day 59 – 121						
	L	H	L	H	*Inte*	*Temp*	*Diet*
Growth performances							
IBW	134.74 ± 13.45 ^{b}	95.16 ± 4.13 ^{a}	136.12 ± 13.74 ^{b}	99.44 ± 8.42 ^{a}	*0.821*	*0.000*	*0.659*
FBW	175.07 ± 9.91	181.01 ± 10.81	170.84 ± 19.28	169.65 ± 20.71	*0.708*	*0.803*	*0.421*
WG	40.33 ± 4.50 ^{a}	85.85 ± 6.94 ^{b}	34.71 ± 5.63 ^{a}	70.21 ± 17.12 ^{b}	*0.407*	*0.000*	*0.100*
SGR	0.42 ± 0.08 ^{a}	1.02 ± 0.04 ^{b}	0.36 ± 0.02 ^{a}	0.84 ± 0.15 ^{b}	*0.288*	*0.000*	*0.045*
FCR	1.55 ± 0.12 ^{b}	1.30 ± 0.02 ^{a}	1.53 ± 0.12 ^{ab}	1.29 ± 0.05 ^{a}	*0.900*	*0.002*	*0.802*
FI	0.66 ± 0.08 ^{a}	1.29 ± 0.07 ^{c}	0.56 ± 0.04 ^{a}	1.07 ± 0.11 ^{b}	*0.193*	*0.000*	*0.008*
Survival	98.6 ± 2.4	98.6 ± 2.4	97.1 ± 5.0	98.6 ± 2.5	*0.709*	*0.709*	*0.697*
Nutritional indices							
PER	1.45 ± 0.09 ^{a}	1.75 ± 0.04 ^{b}	1.47 ± 0.11 ^{a}	1.75 ± 0.15 ^{b}	*0.850*	*0.001*	*0.892*
GPE	24.5 ± 4.88	31.6 ± 0.41	28.6 ± 3.78	30.0 ± 2.63	*0.169*	*0.059*	*0.532*
GLE	59.1 ± 11.72 ^{ab}	76.2 ± 5.94 ^{b}	37.0 ± 19.49 ^{a}	57.8 ± 14.35 ^{ab}	*0.822*	*0.044*	*0.034*
LER	3.88 ± 0.24 ^{bc}	4.68 ± 0.11 ^{c}	3.10 ± 0.22 ^{a}	3.68 ± 0.32 ^{ab}	*0.445*	*0.001*	*0.000*

Data are given as the tanks mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$). L16 = low lipid 16% diet ; L21 = high lipid 21% diet ; HL = constant temperature exposure to high (H) 23°C until seasonal thermic switch (day 0-58th), then to constant low (L) 17°C until end of trial (day 59th -121st) ; LH =. constant temperature exposure to low (L) 17°C until seasonal thermic switch (day 0-58th), then to constant high (H) 23°C until end of trial (day 59th -121st) ; H = constant temperature exposure to high (H) 23°C ; L = constant temperature exposure to low (L) 17°C.

FBW, WG, SGR and FI with higher values in fish reared at 23 °C (H groups) compared to those at 17 °C (L groups). FCR was moderately lower ($p = 0.0505$) at high temperature. A significant dietary effect was recorded on LER and GLE ($p < 0.05$) but not on PER and GPE ($p > 0.05$). At the same time, significant temperature effect ($p < 0.05$) was found on PER, GPE and LER, with lower levels in animals held at 17 °C (L).

In the period following the seasonal change (day 59-121), diet and temperature had a significant effect ($p < 0.05$) on SGR and FI, with higher values in animals fed diet L16 and maintained at H temperature. Significant temperature effects ($p < 0.05$) were also found on WG and FCR showing higher WG and lower FCR in H

temperature. At the same time significant ($p < 0.05$) dietary and temperature effects were found on GLE and LER, with the highest values in fish fed L16 and kept at H and the lowest levels in individuals fed L21 and reared at L (17 °C). No significant dietary effect ($p > 0.05$) was found on PER and GPE. A significant temperature effect ($p < 0.05$) was noticed on PER, which showed higher in animals reared at H temperature. No significant temperature effect was detected on GPE ($p > 0.05$). Relative variations of growth performance and nutritional indices calculated between day 58 and day 121 (before-after seasonal change) are represented in **Supplementary Table 1**. WG, SGR and FI showed a higher increment/lower reduction in L16 compared to L21 (diet effect $p \leq 0.05$). A significant temperature effect was found on the relative variation of FBW, WG, SGR, FCR and FI with lower increment in HL compared to LH. In addition, the relative variation of FI displayed significant interaction effect ($p < 0.05$). Diet had no significant effect on the relative variations of nutritional indices ($p > 0.05$) while temperature affected PER, GPE and LER relative variations showing decreasing values in HL compared to LH (temperature effect < 0.05). No significant dietary nor temperature effect were found on GLE relative variation ($p > 0.05$). Proximate body composition and somatic indices data are shown in **Table 3**. Before seasonal change (day 58) protein displayed significant effects of diet, temperature, and interaction ($p < 0.05$) with higher amounts in L16 and H temperature. For lipid content there were significant effects of temperature and interaction ($p < 0.05$), where groups fed L21 retained both the highest and the lowest percentages for H and L, respectively. Ash body percentage was significantly affected ($p > 0.05$) only by diet, with higher values in groups fed L16. Significant dietary and temperature effects ($p < 0.05$) were also found for moisture. At the same time no significant dietary effect ($p > 0.05$) was found on HSI, MFI and VSI while a significant temperature effect occurred on HSI ($p < 0.05$) with higher levels in fish kept at low temperature (L 17 °C).

Table 3. Body composition and somatic indices of gilthead sea bream fed experimental diets and exposed to seasonal water temperature changes

	Before seasonal change – day 58						
	L16		L21		*P-value*		
	H	L	H	L	*Inte*	*Temp*	*Diet*
Whole body composition, %							
Protein	17.54 ± 0.05 [b]	16.90 ± 0.37 [ab]	16.83 ± 0.07 [a]	16.89 ± 0.46 [a]	*0.008*	*0.022*	*0.007*
Lipid	13.13 ± 1.11 [ab]	13.11 ± 0.75 [ab]	14.21 ± 0.39 [b]	12.39 ± 1.29 [a]	*0.011*	*0.035*	*0.436*
Ash	3.94 ± 0.27	3.92 ± 0.08	3.64 ± 0.51	3.55 ± 0.14	*0.796*	*0.687*	*0.022*
Moisture	64.37 ± 1.06 [a]	64.84 ± 0.28 [ab]	64.67 ± 0.48 [ab]	65.70 ± 0.57 [b]	*0.316*	*0.013*	*0.044*
Somatic indices							
HSI	1.98 ± 0.10 [a]	2.40 ± 0.16 [b]	1.90 ± 0.07 [a]	2.33 ± 0.11 [b]	*0.934*	*0.000*	*0.396*
MFI	1.42 ± 0.34	1.45 ± 0.19	1.53 ± 0.28	1.64 ± 0.81	*0.846*	*0.673*	*0.421*
VSI	9.05 ± 0.62	9.62 ± 0.94	10.04 ± 1.54	9.13 ± 0.21	*0.236*	*0.309*	*0.222*
	After seasonal change – day 121						
	L	H	L	H	*Int.*	*Temp*	*Diet*
Whole body composition, %							
Protein	17.41 ± 0.55	17.46 ± 0.12	17.36 ± 0.30	16.99 ± 0.40	*0.161*	*0.287*	*0.102*
Lipid	13.68 ± 0.28	14.63 ± 0.41	13.81 ± 1.26	13.78 ± 0.51	*0.119*	*0.145*	*0.249*
Ash	3.87 ± 0.13	3.84 ± 0.11	3.72 ± 0.21	3.70 ± 0.20	*0.977*	*0.782*	*0.099*
Moisture	64.05 ± 0.32	63.46 ± 0.56	64.52 ± 1.25	64.61 ± 0.70	*0.255*	*0.405*	*0.012*
Somatic indices							
HSI	2.42 ± 0.54 [b]	1.74 ± 0.26 [a]	2.56 ± 0.38 [b]	1.60 ± 0.22 [a]	*0.141*	*0.000*	*0.993*
MFI	1.11 ± 0.39	1.23 ± 0.55	1.38 ± 0.49	1.52 ± 0.41	*0.924*	*0.301*	*0.022*
VSI	8.38 ± 1.26 [a]	9.46 ± 1.19 [ab]	9.92 ± 1.23 [b]	8.84 ± 2.60 [ab]	*0.015*	*0.299*	*0.012*
Lipid liver	10.80 ± 0.66	13.25 ± 4.32	11.81 ± 1.68	12.76 ± 0.77	*0.598*	*0.249*	*0.853*

Data are given as the mean (n=9 diet^{-1} on day 58th ; n=15 diet^{-1} on day 121st) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$). L16 = low lipid 16% diet ; L21 = high lipid 21% diet ; H = constant temperature exposure to high (H) 23°C ; L = constant temperature exposure to low (L) 17°C.
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
MFI = Mesenteric Fat Index (%) = 100*(mesenteric fat weight/FBW).
VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).
SD = Standard deviation.

At the end of the trial (day 121), no significant dietary and temperature effects were found in body protein, lipid, and ash percentages ($p > 0.05$). Moisture showed a significant dietary effect ($p < 0.05$), displaying a higher level in L21. Fat liver content was not significantly affected by diet nor temperature ($p > 0.05$). Concerning somatic indices, diet showed a significant effect on MFI and VSI ($p < 0.05$) both higher in L21 than L16. An additional interaction effect in VSI was also observed. HSI was significantly affected by temperature with higher values observed in the L groups for both dietary regimes. Relative variations of proximate body composition and somatic indices calculated between day 58 and day 121 (before-after seasonal change) are represented in **Supplementary Table 2**. No significant dietary or temperature effects were found on relative variations of protein, lipid, ash, and moisture ($p > 0.05$). Relative variation of HSI was not significantly affected by diet ($p > 0.05$); however, HL groups displayed a relative increment while LH groups showed decreasing values (temperature effect $p < 0.05$). MFI and VSI relative variations were not significantly affected by diet nor temperature ($p > 0.05$).

Digestive enzyme activity

Digestive enzymes activities measured before and after water temperature change are shown in **Figure 1**. Before seasonal change, pepsin activity was significantly ($p < 0.05$) higher in fish fed on diet L21, but no

significant differences were observed between groups maintained at low or high temperature. After seasonal change, the only significant differences observed were associated with interactions between the effects of dietary lipid level and low temperature; the highest and lowest activities were measured in fish fed on low lipid and high lipid diets, respectively, maintained at low temperature. On the other hand, no significant effect of temperature change was evidenced, irrespective of diet composition.

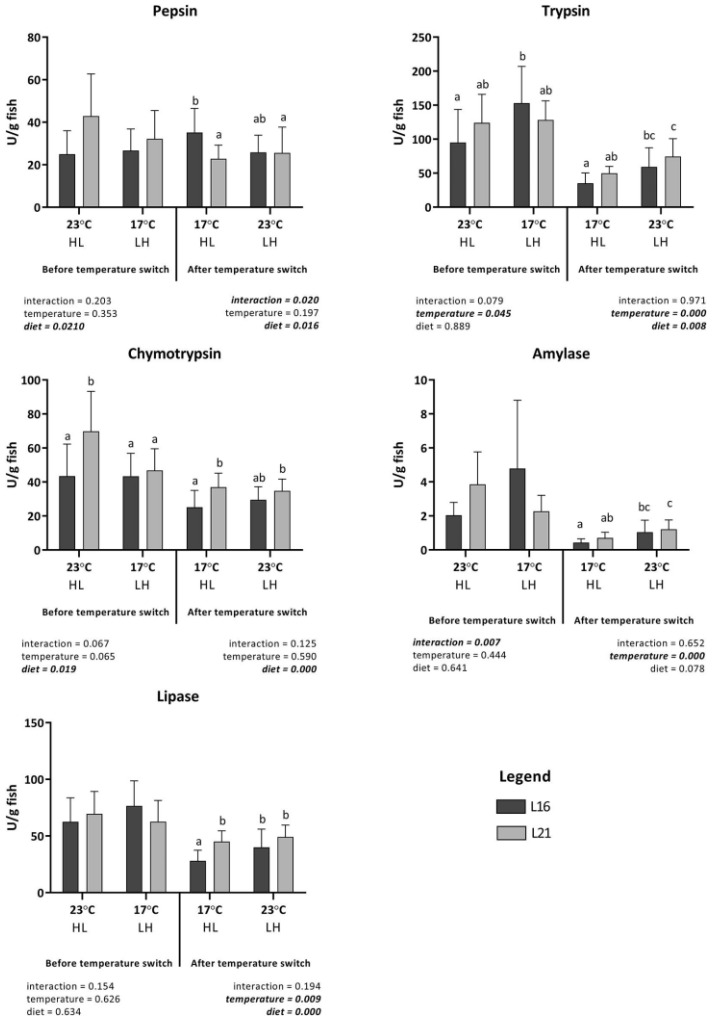

Figure 1 Digestive gut enzymes activity (expressed as U g fish body weight-1) of gilthead seabream fed the experimental diets and exposed to seasonal temperature changes over 121 days. Data are given as the mean of triplicate tank individual samples (n=3 per tank before seasonal change, n=5 per tank after seasonal change) ± SD. Different letters indicate significant difference (Two-way ANOVA $P \leq 0.05$) between treatments. L16 = low lipid 16% diet ; L21 = high lipid 21% diet. HL = constant temperature exposure to high (H) 23°C until seasonal thermic switch (day 0-58th), then to constant low (L) 17°C until end of trial (day 59th -121st); LH =. constant temperature exposure to low (L) 17°C until seasonal thermic switch (day 0-58th), then to constant high (H) 23°C until end of trial (day 59th -121st).

In the case of trypsin, a significant effect of rearing temperature and none of dietary lipid level was evidenced during the first part of the assay, with higher values measured in fish maintained at 17 °C when compared to those at 23 °C. After temperature inversion, significantly higher values were measured in fish fed on high lipids and maintained at high temperature when compared to those fed on low lipids and maintained at low temperature. On the other hand, significantly higher values of chymotrypsin activity were measured in fish fed on high dietary lipids, both before and after seasonal change. Amylase activity was not significantly affected either by diet or temperature during the initial period of the experiment, but a significant interaction diet x temperature occurred ($p < 0.05$). After temperature inversion, no significant effect of dietary lipid level was evidenced on amylase activity, while significantly higher values of this enzyme were measured in fish maintained at high temperature.

Relating to lipase activity, while no significant effect of diet or temperature were measured during the initial period, these were evidenced after seasonal change. Significantly lower activity was linked to the consumption of low lipid diet and, within the same dietary treatment a higher activity was detected in fish kept at 23 °C compared to those at 17 °C. Relative variations of digestive enzyme activity calculated between day 58 and day 121 are represented in **Supplementary Table 3**. Relative variations of digestive enzymes were not significantly affected by diet nor temperature ($p > 0.05$), except for amylase, which displayed a significant effect of temperature and interaction.

In particular, a lower reduction was observed for LH treatment and this reduction was less evident under L21 then under L16.

Plasma biochemistry

The results of plasma parameters are shown in **Table 4** and **Table 5**. Before seasonal temperature change, significant dietary effect ($p < 0.05$) was displayed in Tot Bil, Ca^{2+}, P, Na^{+}, CORT Ca x P, Cur Ca^{2+}, having higher levels in animals fed L21, except for CORT that appeared to be more elevated in groups treated with L16. At the same time a significant temperature effect was noticed on TRIG, TP, AST, LDH, HDL, ALP and Fe where all subjects reared at L temperature tended to have higher values than those at H temperature, except for ALP and Fe. Significant dietary and temperature effects ($p < 0.05$) were observed on ALB/GLO before seasonal change (day 58). Moreover, significant interaction effect on CREA was found in the same period of time ($p < 0.05$). Before seasonal change, no significant dietary and temperature effects ($p > 0.05$) were found in GLU, urea, Uric Ac, CHOL, ALB, CK, K^{+}, Cl, Mg and Na/K.

Table 4. Plasma biochemistry values for gilthead seabream fed the experimental diets and exposed two different temperatures before seasonal temperature changes over 121 days.

	Before seasonal change – day 58						
	L16		L21		*P - value*		
	H	L	H	L	*Inte*	*Temp*	*Diet*
GLU	90.56 ± 7.02	98.63 ± 26.72	82.78 ± 11.63	86.89 ± 8.64	*0.701*	*0.243*	*0.066*
Urea	6.11 ± 1.09	7.20 ± 2.93	6.56 ± 0.76	6.82 ± 1.50	*0.484*	*0.259*	*0.947*
CREA	0.20 ± 0.03	0.22 ± 0.03	0.23 ± 0.03	0.20 ± 0.03	*0.022*	*0.449*	*0.747*
Uric Ac	0.03 ± 0.04	0.03 ± 0.03	0.01 ± 0.00	0.01 ± 0.00	*0.987*	*0.987*	*0.053*
Tot Bil	0.05 ± 0.03 ^{ab}	0.04 ± 0.02 ^{a}	0.07 ± 0.01 ^{b}	0.06 ± 0.01 ^{ab}	*0.800*	*0.211*	*0.006*
Bil Ac	36.10 ± 20.78	31.03 ± 6.01	50.68 ± 15.98	38.38 ± 17.74	*0.686*	*0.340*	*0.234*
CHOL	231.00 ± 43.91	248.25 ± 28.00	240.78 ± 41.48	253.88 ± 58.17	*0.892*	*0.325*	*0.615*
TRIG	811.11 ± 524.56	1116.13 ± 584.34	823.11 ± 231.47	1205.25 ± 541.40	*0.819*	*0.048*	*0.764*
HDL	48.44 ± 13.36 ^{a}	66.25 ± 8.89 ^{b}	52.11 ± 8.25 ^{ab}	61.00 ± 12.60 ^{ab}	*0.248*	*0.001*	*0.836*
TP	3.56 ± 0.27	3.79 ± 0.50	3.51 ± 0.22	3.74 ± 0.23	*0.989*	*0.043*	*0.627*
ALB	0.95 ± 0.08	0.96 ± 0.14	0.89 ± 0.05	0.92 ± 0.05	*0.801*	*0.607*	*0.096*
AST	22.11 ± 15.98	51.13 ± 46.11	17.89 ± 9.57	43.50 ± 26.50	*0.858*	*0.007*	*0.534*
ALP	691.44 ± 316.46 ^{bc}	295.38 ± 178.67 ^{a}	936.33 ± 425.29 ^{c}	418.22 ± 129.45 ^{ab}	*0.539*	*0.000*	*0.070*
CK	92.89 ± 84.00	498.13 ± 833.58	28.00 ± 14.17	153.00 ± 131.14	*0.314*	*0.062*	*0.144*
LDH	455.56 ± 416.36 ^{ab}	1195.75 ± 1089.30 ^{b}	226.00 ± 131.44 ^{a}	1013.88 ± 837.50 ^{ab}	*0.922*	*0.004*	*0.400*
Ca^{+2}	12.14 ± 0.65	12.00 ± 0.85	12.70 ± 0.43	12.38 ± 0.61	*0.687*	*0.298*	*0.044*
P	11.18 ± 0.88 ^{ab}	10.55 ± 1.62 ^{a}	12.50 ± 1.73 ^{b}	12.54 ± 1.03 ^{b}	*0.485*	*0.526*	*0.001*
K^{+}	5.03 ± 1.07	4.84 ± 0.87	4.29 ± 0.40	5.00 ± 0.54	*0.090*	*0.327*	*0.270*
Na^{+}	184.44 ± 6.71	182.75 ± 3.54	187.67 ± 4.95	188.78 ± 9.02	*0.526*	*0.895*	*0.043*
Fe	129.89 ± 37.03 ^{ab}	97.75 ± 27.44 ^{a}	147.78 ± 32.20 ^{b}	115.50 ± 31.03 ^{ab}	*0.995*	*0.007*	*0.119*
Cl	162.91 ± 4.18	162.08 ± 3.25	164.77 ± 5.01	167.41 ± 8.34	*0.366*	*0.637*	*0.067*
Mg	2.62 ± 0.16	2.77 ± 0.17	2.70 ± 0.16	2.67 ± 0.15	*0.114*	*0.284*	*0.849*
CORT	20.83 ± 9.08	28.24 ± 15.17	14.42 ± 9.87	10.68 ± 14.70	*0.211*	*0.677*	*0.010*
ALB/GLO	0.37 ± 0.02 ^{b}	0.34 ± 0.01 ^{a}	0.34 ± 0.01 ^{a}	0.33 ± 0.02 ^{a}	*0.498*	*0.001*	*0.004*
CaxP	136.11 ± 16.11 ^{ab}	127.50 ± 26.00 ^{a}	159.22 ± 27.46 ^{b}	155.50 ± 19.73 ^{ab}	*0.757*	*0.437*	*0.003*
Na/K	38.56 ± 9.53	39.00 ± 8.70	44.00 ± 4.12	38.22 ± 3.77	*0.197*	*0.267*	*0.330*

Data are given as the mean (n=9 diet^{-1} on day 58th ; n=15 diet^{-1} on day 121st) ± SD. Different letters indicate significant difference (Two-way ANOVA $P \leq 0.05$) between treatments. L16 = low lipid 16% diet ; L21 = high lipid 21% diet ; H = constant temperature exposure to high (H) 23°C ; L = constant temperature exposure to low (L) 17°C.

GLU, glucose , (mg dL^{-1}) ; Urea , (mg dL^{-1}) ; CREA, creatinine , (mg dL^{-1}) ; Uric Ac, uric acid , (mg dL^{-1}) ; Tot Bil, total bilirubin , (mg dL^{-1}) ; Bil Ac, bile acid , (µmol dL^{-1}) ; CHOL, cholesterol , (mg dL^{-1}) ; TRIG, triglycerides , (mg dL^{-1}) ; HDL, high density lipoprotein ; TP, total protein , (mg dL^{-1}) ; Alb, albumin , (g dL^{-1}) ; Ast, aspartate aminotransferase , (U L^{-1}); Alp, alkaline phosphatase , (U L^{-1}) ; CK, creatine kinase , (U L^{-1}) ; LDH, lactate dehydrogenase , (U L^{-1}) ; Ca^{+2} , calcium , (mg dL^{-1}) ; P, inorganic phosphorus , (mg dL^{-1}) ; K^{+}, potassium , (mEq L^{-1}) ; Na^{+}, sodium , (mEq L^{-1}) ; Fe, iron , (µg dL^{-1}) ; Cl, chloride , (mEq L^{-1}) ; Mg, magnesium , (mg dL^{-1}) ; CORT, cortisol , (µg dL^{-1}) ; ALB/GLO, albumin/globulin; CaxP, calcium*phosphorus ; Na/K, sodium/potassium

At the end of the experiment (day 121) diet significantly affected TP, ALB, AST, LDH, Ca^{2+}, K^{+}, Na^{+}, Cl and Na/K on day 121 ($p < 0.05$). Plasma AST, LDH, K^{+}, Na^{+} and Cl were higher in L21 supplied individuals, and contrariwise for TP, ALB, Ca^{2+} and Na/K. At the same time temperature significantly affected GLU, CREA, Bil Ac and CK ($p < 0.05$). Among them, GLU, CREA and Bil Ac presented higher levels in individuals maintained at H temperature, while plasma CK concentration showed the opposite trend. Significant dietary and temperature effects ($p < 0.05$) were observed on Tot Bil, ALP and ALB/GLO. Uric Ac was significantly affected by diet, temperature, and interaction ($p < 0.05$). Moreover, a significant interaction effect on TRIG was found at the same time ($p < 0.05$); no significant dietary and temperature effect ($p > 0.05$) were observed

Table 5. Plasma biochemistry values for gilthead seabream fed the experimental diets and exposed two different temperatures after seasonal temperature changes over 121 days.

	After seasonal change – day 121						
	L	H	L	H	*Inte*	*Temp*	*Diet*
GLU	74.57 ± 15.51 ^{a}	99.73 ± 13.99 ^{b}	73.67 ± 15.40 ^{a}	106.14 ± 31.67 ^{b}	*0.737*	*0.000*	*0.878*
Urea	8.05 ± 1.80	8.89 ± 0.95	8.16 ± 1.80	8.60 ± 1.67	*0.677*	*0.187*	*0.839*
CREA	0.17 ± 0.02 ^{ab}	0.19 ± 0.03 ^{b}	0.16 ± 0.02 ^{a}	0.18 ± 0.03 ^{ab}	*0.558*	*0.002*	*0.299*
Uric Ac	0.03 ± 0.03 ^{a}	0.03 ± 0.03 ^{a}	0.03 ± 0.02 ^{a}	0.08 ± 0.06 ^{b}	*0.034*	*0.014*	*0.014*
Tot Bil	0.03 ± 0.02 ^{a}	0.04 ± 0.02 ^{b}	0.02 ± 0.02 ^{a}	0.03 ± 0.01 ^{a}	*0.081*	*0.036*	*0.011*
Bil Ac	11.02 ± 2.40 ^{a}	35.77 ± 25.98 ^{bc}	13.87 ± 6.61 ^{ab}	38.81 ± 34.62 ^{c}	*0.987*	*0.000*	*0.633*
CHOL	214.15 ± 26.58	219.53 ± 26.11	216.57 ± 42.01	213.83 ± 34.33	*0.653*	*0.884*	*0.856*
TRIG	316.07 ± 118.68	473.40 ± 249.43	477.50 ± 265.41	353.58 ± 172.86	*0.018*	*0.772*	*0.719*
HDL	75.43 ± 10.66	70.73 ± 9.51	67.93 ± 14.49	70.67 ± 10.66	*0.236*	*0.753*	*0.228*
TP	3.85 ± 0.35 ^{b}	3.64 ± 0.27 ^{ab}	3.51 ± 0.33 ^{a}	3.40 ± 0.36 ^{a}	*0.577*	*0.078*	*0.002*
ALB	0.98 ± 0.10 ^{c}	0.96 ± 0.08 ^{bc}	0.85 ± 0.07 ^{a}	0.87 ± 0.11 ^{ab}	*0.391*	*0.918*	*0.000*
AST	19.08 ± 11.47 ^{ab}	14.93 ± 9.86 ^{a}	35.00 ± 29.77 ^{b}	32.33 ± 17.51 ^{ab}	*0.887*	*0.515*	*0.002*
ALP	83.71 ± 33.37 ^{a}	216.47 ± 117.78 ^{b}	168.87 ± 70.95 ^{ab}	256.42 ± 126.14 ^{b}	*0.372*	*0.000*	*0.016*
CK	135.50 ± 163.01	54.40 ± 45.02	81.20 ± 74.99	49.50 ± 39.93	*0.338*	*0.032*	*0.251*
LDH	411.38 ± 280.24	266.13 ± 250.62	751.00 ± 788.72	657.50 ± 568.17	*0.857*	*0.406*	*0.013*
Ca^{+2}	12.48 ± 0.58 ^{b}	12.43 ± 0.69 ^{ab}	11.79 ± 0.63 ^{a}	12.35 ± 0.78 ^{ab}	*0.091*	*0.159*	*0.036*
P	10.90 ± 1.09	11.03 ± 0.80	11.02 ± 1.38	11.17 ± 1.55	*0.981*	*0.660*	*0.691*
K^{+}	5.11 ± 0.60 ^{ab}	4.83 ± 0.66 ^{a}	5.60 ± 0.54 ^{b}	5.54 ± 0.51 ^{b}	*0.480*	*0.262*	*0.000*
Na^{+}	187.57 ± 4.65	187.40 ± 3.91	189.27 ± 4.70	191.79 ± 7.04	*0.327*	*0.392*	*0.030*
Fe	102.00 ± 21.49	99.80 ± 23.04	95.93 ± 18.20	89.00 ± 17.85	*0.666*	*0.406*	*0.128*
Cl	164.20 ± 4.68	162.99 ± 2.91	166.83 ± 4.58	166.37 ± 5.21	*0.747*	*0.473*	*0.012*
Mg	2.70 ± 0.23	2.76 ± 0.18	2.69 ± 0.23	2.81 ± 0.20	*0.617*	*0.107*	*0.732*
CORT	21.18 ± 16.40	16.89 ± 9.17	13.97 ± 11.42	17.65 ± 7.99	*0.196*	*0.921*	*0.295*
ALB/GLO	0.34 ± 0.01 ^{b}	0.36 ± 0.01 ^{b}	0.32 ± 0.02 ^{a}	0.34 ± 0.02 ^{b}	*0.624*	*0.000*	*0.000*
CaxP	136.29 ± 17.88	137.53 ± 17.32	130.40 ± 21.54	138.67 ± 25.71	*0.529*	*0.394*	*0.670*
Na/K	37.14 ± 4.42 ^{ab}	39.67 ± 6.15 ^{b}	34.13 ± 3.76 ^{a}	34.86 ± 3.53 ^{a}	*0.460*	*0.185*	*0.002*

Data are given as the mean (n=9 diet^{-1} on day 58th ; n=15 diet^{-1} on day 121st) ± SD. Different letters indicate significant difference (Two-way ANOVA $P \leq 0.05$) between treatments. L16 = low lipid 16% diet ; L21 = high lipid 21% diet ; H = constant temperature exposure to high (H) 23°C ; L = constant temperature exposure to low (L) 17°C.

GLU, glucose , (mg dL^{-1}) ; Urea , (mg dL^{-1}) ; CREA, creatinine , (mg dL^{-1}) ; Uric Ac, uric acid , (mg dL^{-1}) ; Tot Bil, total bilirubin , (mg dL^{-1}) ; Bil Ac, bile acid , (µmol dL^{-1}) ; CHOL, cholesterol , (mg dL^{-1}) ; TRIG, triglycerides , (mg dL^{-1}) ; HDL, high density lipoprotein, ; TP, total protein , (mg dL^{-1}) ; Alb, albumin , (g dL^{-1}) ; Ast, aspartate aminotransferase , (U L^{-1}); Alp, alkaline phosphatase , (U L^{-1}) ; CK, creatine kinase , (U L^{-1}) ; LDH, lactate dehydrogenase , (U L^{-1}) ; Ca^{+2} , calcium , (mg dL^{-1}) ; P, inorganic phosphorus , (mg dL^{-1}) ; K^{+}, potassium , (mEq L^{-1}) ; Na^{+}, sodium , (mEq L^{-1}) ; Fe, iron , (µg dL^{-1}) ; Cl, chloride , (mEq L^{-1}) ; Mg, magnesium , (mg dL^{-1}) ; CORT, cortisol , (µg dL^{-1}) ; ALB/GLO, albumin/globulin; CaxP, calcium*phosphorus ; Na/K, sodium/potassium ; SD, standard deviation.

on urea, CHOL, P, Fe, Mg, Ca x P, Cur Ca^{2+} and HDL.

Relative variations of plasma parameters calculated between day 58 and day 121 are represented in **Supplementary Table 4**. Uric Ac, AST and CK showed a higher relative increment in L16 compared to L21, while Ca^{2+} and Cur Ca^{2+} were significantly reduced in fish fed L21 compared to those fed L16 (dietary effect $p \leq 0.05$). HL animals displayed higher TP, AST, CK, LDH and HDL relative increments compared to LH ones, while GLU, Bil Ac, ALP and ALB/GLO relative increments were higher in LH animals than HL ones

(temperature effect $p < 0.05$). In addition, significant interaction was found on relative variation of CK ($p < 0.05$). No significant dietary or temperature effect was found on relative variations of Urea, CREA, Tot Bil, CHOL, TRIG, ALB, P, K, Na^+, Fe, Cl, Mg, CORT and CaxP, Na/K ($p > 0.05$).

Faecal bacterial community profiles before and after water temperature changes

The 16S rRNA gene sequencing was performed on a total of 71 distal intestine content samples, yielding 1,724,306 high-quality reads (mean ± SD, 24,286 ± 6,505) and clustered into a total of 2,726 ASVs, of which 2,434 assigned at family level and 2,002 assigned at genus level. In order to assess whether the different diets (L16 and L21) result in a specific gut microbiome (GM) response to water temperature changes, for each dietary regime the gut microbiome was sampled before and after the HL (autumn shift) and LH (summer shift) temperature transitions. The correspondent variations in the gut microbiome profiles were assessed by the PCoA of the unweighted UniFrac distances between samples collected at the different temperatures, taxa most explaining samples segregation are superimposed on the bidimensional space. Finally, for each diet and temperature transition, changes in the gut microbiome internal diversity are shown according to 3 different metrics: PD_whole_tree, Chao1 and observed_ASVs. According to our findings (**Figure 2A and B**), under the L16 dietary regime only the HL transition (autumn shift) resulted in a significant variation of the overall gut microbiome composition, both in terms of overall compositional structure ("Adonis", $p = 0.001$) and in terms of reduction in the internal ecosystem diversity (Kruskal-Wallis test $p = 0.002$; $p = 0.002$; $p = 0.006$). Particularly, the transition to low temperature brings about the reduction of *Bacillus* in the fish gut microbiome. Conversely, in fishes fed with the L21 diet both the LH (summer shift) and HL (autumn shift) resulted in significant gut microbiome compositional changes (**Figure 2C and D**, "Adonis", $p < 0.01$). However, in these conditions, no significant variations in the gut microbiome compositional diversity were observed.

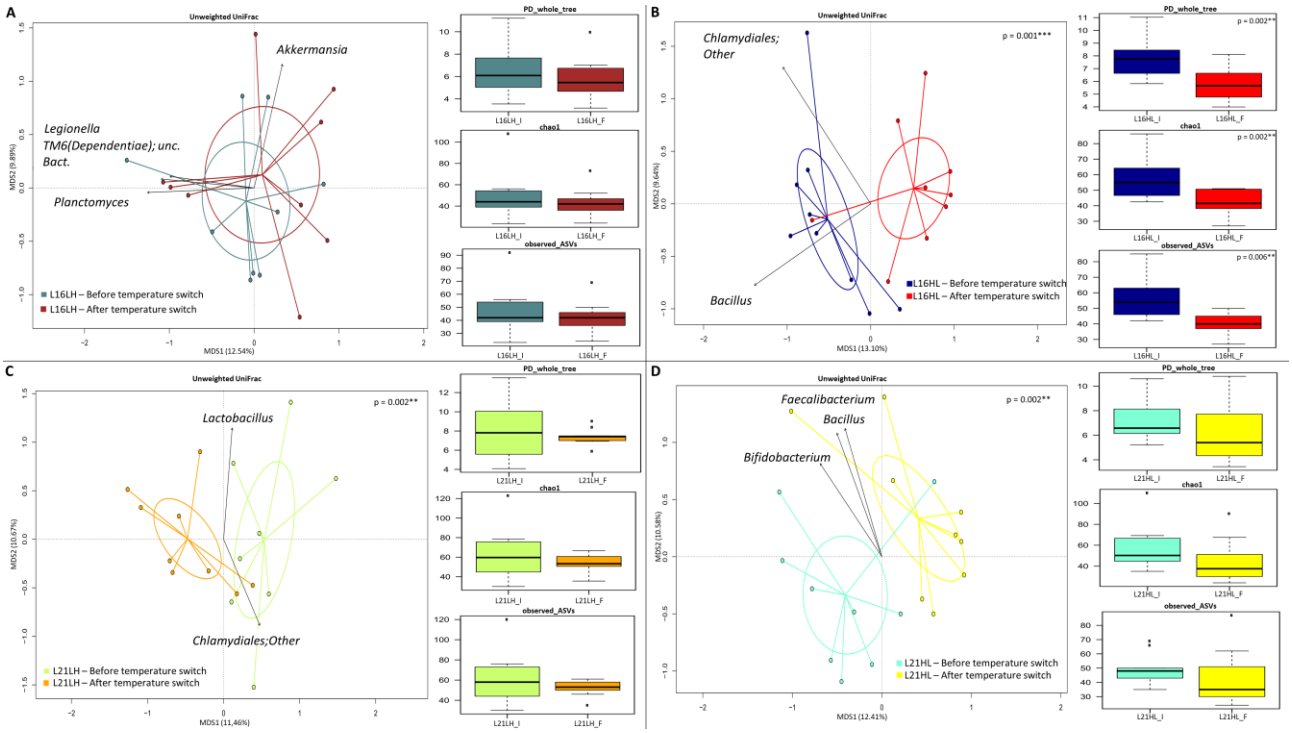

Figure 2. Beta diversity and alpha diversity of gut microbiota of gilthead sea bream fed with the experimental diets and exposed to temperature switch over 121 days. **(A,B)** PCoA based on unweighted UniFrac distances between gut microbiota structure of animals fed with L16 diet and exposed, respectively, to summer shift (LH transition) and autumn shift (HL transition). Samples are significantly separated, only in the autumn shift condition (permutation test with pseudo-F ratios Adonis; $p = 0.001$). **(C,D)** PCoA based on unweighted UniFrac distances between gut microbiota structure of animals fed with L21 diet and exposed, respectively, to summer shift (LH transition) and autumn shift (HL transition). Samples are significantly separated in both conditions (permutation test with pseudo-F ratios Adonis; $p = 0.002$, $p = 0.002$). Black arrows are obtained by fitting the genus relative abundance values for each sample within the ordination space (function envfit of the vegan R package, with a p-value < 0.01). In each panel, boxplots show alpha diversity values, measured by Faith's Phylogenetic Diversity (PD_whole_tree), Chao1 index, and amplicon sequence variants (observed_ASVs). Only for the HL group **(B)**, all metrics showed a significant reduction (Kruskal–Wallis test $p < 0.01$) of alpha diversity in the final condition of group fed with L16 diet and subjected to a temperature switch toward autumn temperature (HL). L16, low-lipid 16% diet; L21, high-lipid 21% diet; HL, constant temperature exposure to high (H) temperature of 23°C before, and to low (L) temperature of 17°C after, temperature switch; LH, constant temperature exposure to low (L) temperature of 17°C before, and to high (H) temperature of 23°C after, temperature switch. Temperature switch occurred on day 58.

We next investigated whether the different diets were associated with specific gut microbiome compositional structure in fishes grown at high or low temperatures. To this end, the PCoA of the unweighted UniFrac distances of the gut microbiome composition of fishes consuming L16 or L21 diet is provided at both warm (**Figure 3A**) and cold (**Figure 3B**) growth temperatures. For each temperature, the internal gut microbiome diversity corresponding to both diets is also provided. According to our findings, only at a warm temperature did the different diets show a significantly different gut microbiome layout ("Adonis", p = 0.01), with L16 diet resulting in a higher load of *Lactobacillus*. On the contrary, no dietary impact on ecosystem diversity was observed, independently from the temperature.

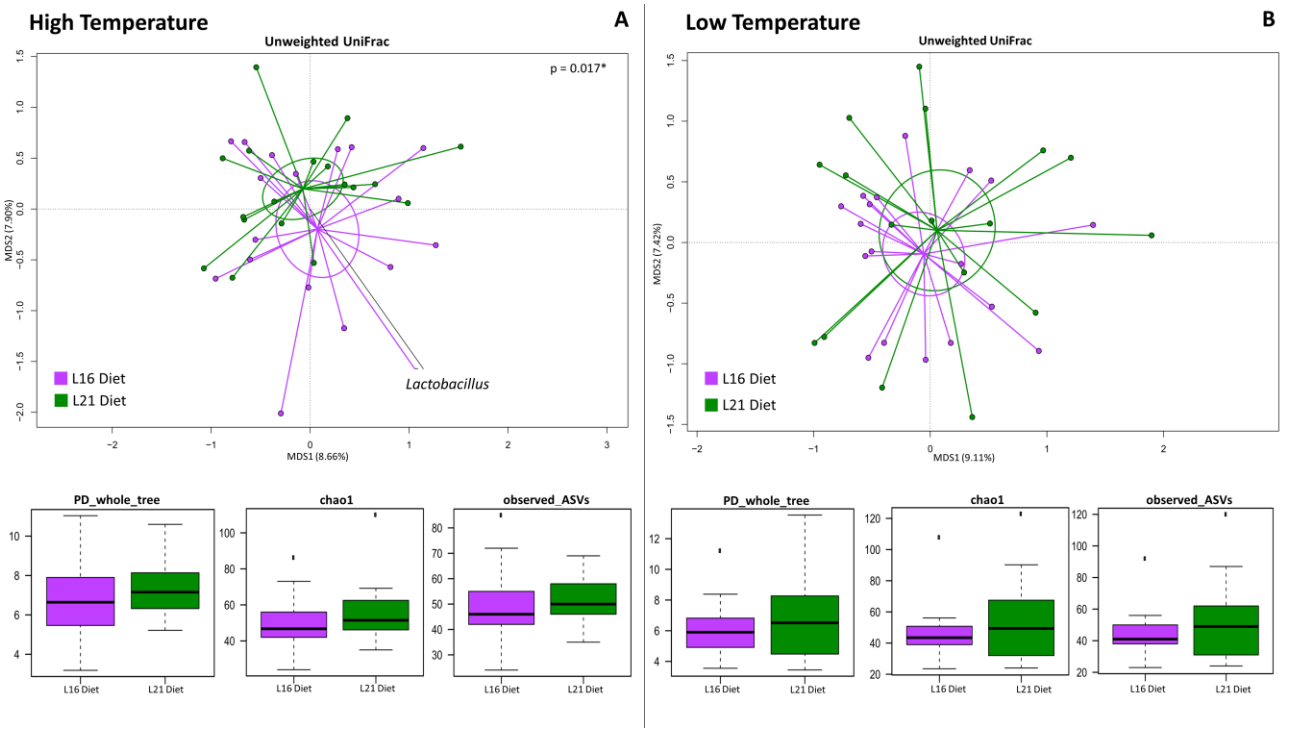

Figure 3. Beta diversity and alpha diversity of gut microbiota of gilthead sea bream fed with the experimental diets at both warm and cold temperatures. (A) PCoA based on unweighted UniFrac distances between gut microbiota structure of animals fed with L16 and L21 diets and grown at warm temperature. Samples are significantly separated (permutation test with pseudo-F ratios Adonis; $p = 0.017$). (B) PCoA based on unweighted UniFrac distances between gut microbiota structure of animals fed with L16 and L21 diets and grown at cold temperature. Samples are not significantly separated (permutation test with pseudo-F ratios Adonis; $p > 0.05$). Black arrows are obtained by fitting the genus relative abundance values for each sample within the ordination space (function envfit of the vegan R package, with a p-value < 0.01). For both temperature conditions, all metrics used to assess alpha diversity did not show a significant variation between the two experimental diets (as highlighted by the boxplots in both panels). L16, low-lipid 16% diet; L21, high-lipid 21% diet.

The overall composition of the sea bream gut microbiome at different phylogenetic levels is represented in **Fig. 4**: phylum in panel (A) and family in panel (B). For all experimental groups, the most abundant taxa were Firmicutes, Proteobacteria and Actinobacteria, which represented about 88% of the whole gilthead sea bream gut microbiota (**Fig. 4A, Supplementary Table 5**). At family level, the gilthead sea bream gut bacterial community was dominated almost entirely by *Lactobacillaceae*, which represented around 60% of the whole ecosystem in all groups (**Fig. 4B**). Interestingly, focusing on the genus level, specific compositional differences were detectable among the groups studied (Wilcoxon rank-sum test $p < 0.05$) (**Figure 5**). In particular, according to our data, for sea bream receiving L16 diet the HL transition (autumn shift) resulted in a significant decrease of *Bacillus* and *Planctomycetaceae*, while for fish fed with L21 diet the same shift resulted in the reduction of *Planctomyces* (Wilcoxon rank-sum test $p = 0.008$, $p = 0.016$, $p = 0.033$, respectively). On the other hand, for both diets, the LH transition (summer shift) gave a significant increase of *Methylobacterium* (Wilcoxon rank-sum test $p = 0.012$, $p = 0.033$). Finally, the L16 diet in the HL transition (autumn shift) favoured an increase of *Weissella* and *Bradyrhizobium* genera in the gut microbiome, resulting in a significantly higher relative abundance of these genera in the final condition compared to fish fed with L21 diet in the same condition (Wilcoxon $p = 0.014$, $p = 0.026$), while L21 diet in the final condition of LH (summer shift) transition favoured a significant increase of *Streptococcus* and *Bacillus* genera compared to L16 diet in the corresponding condition (Wilcoxon $p = 0.015$, $p = 0.011$).

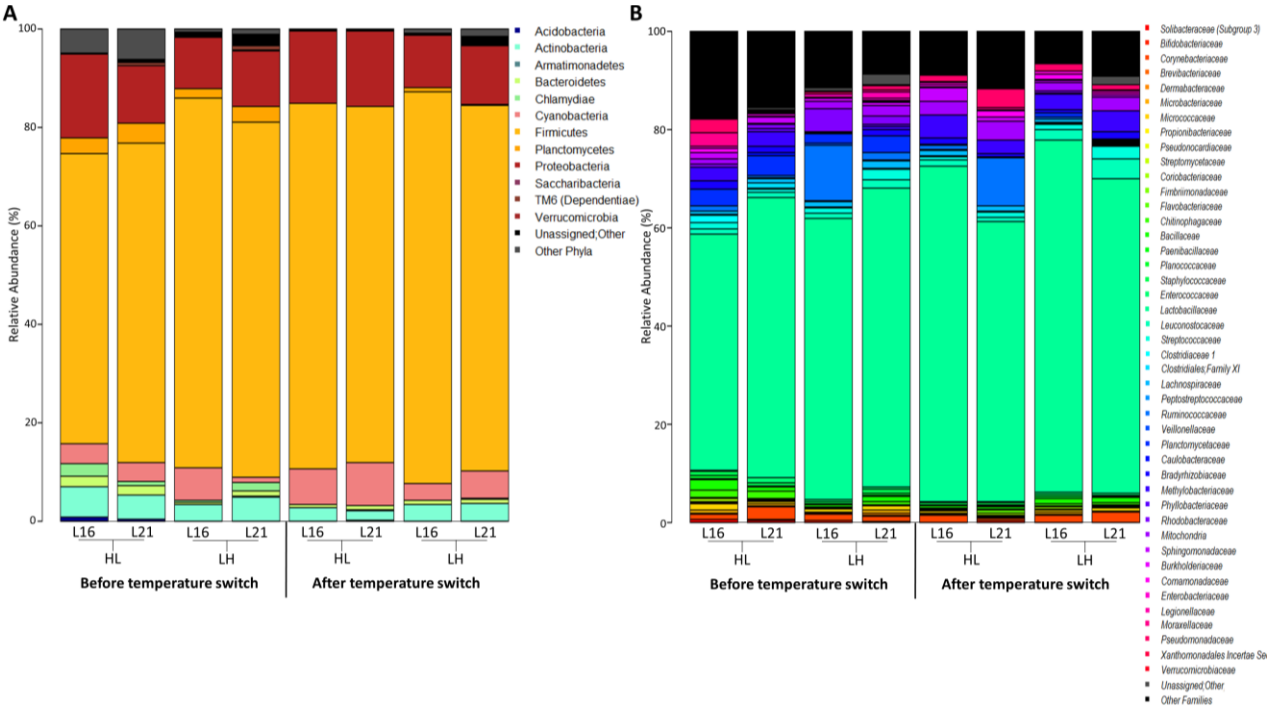

Figure 4. Microbiota composition of distal gut content of gilthead sea bream fed with the experimental diets and exposed to temperature switch over 121 days. Bar plot summarizing the microbiota composition at phylum **(A)** and family level **(B)** of fish intestinal content. Only phyla with a relative abundance ≥ 0.1% in at least 10 samples, and families with relative abundance ≥ 0.1% in at least 10 samples are represented. L16, low-lipid 16% diet; L21, high-lipid 21% diet; HL, constant temperature exposure to high (H) temperature of 23°C before, and to low (L) temperature of 17°C after, temperature switch; LH, constant temperature exposure to low (L) temperature of 17°C before, and to high (H) temperature of 23°C after, temperature switch. Temperature switch occurred on day 59.

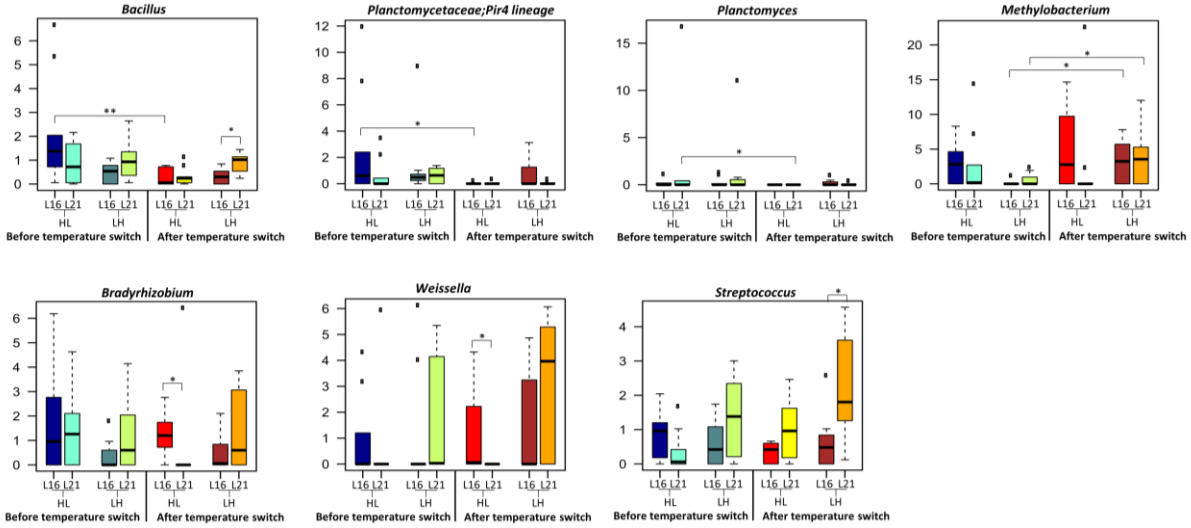

Figure 5. Taxonomic composition of bacterial communities of distal gut content of gilthead sea bream fed with the experimental diets and exposed to temperature switch over 121 days. Distributions of relative abundance of genera that showed a significant variation between groups fed with different diets or after the temperature switch (Wilcoxon rank-sum test, $^{**}p \leq 0.01$; $^{*}p \leq 0.05$), only genera with a mean relative abundance ≥ 1.0% in at least one group were represented. The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line. L16, low-lipid 16% diet; L21, high-lipid 21% diet; HL, constant temperature exposure to high (H) temperature of 23°C before, and to low (L) temperature of 17°C after, temperature switch; LH, constant temperature exposure to low (L) temperature of 17°C before, and to high (H) temperature of 23°C after, temperature switch. Temperature switch occurred on day 59.

Discussion

Though many studies have been conducted on the effect of water temperature on growth, physiological responses and health in gilthead sea bream, so far very few have investigated the possible interaction between

seasonal temperature fluctuations and dietary lipid level, and no work exists on its capability to affect gut microbiota. The growth parameters observed throughout the overall trial (fish encountering seasonal temperature change between 23 °C and 17 °C and vice versa), within 16 % or 21 % dietary lipid levels showed similar performance in terms of growth (FBW, WG and SGR). However, overall FCR was higher in animals entering autumn temperature (17°C, HL) in both diets. This significant difference was mainly due to the observed negative effect of temperature on FCR after seasonal changes (day 59-121), when fish moving from high to low temperature exhibited higher values and higher relative increments. Similarly, our study agrees with the "winter growth arrest" described by (Sánchez-Nuño, Eroldogan, et al., 2018), where gilthead sea bream brought from 22 °C down to 14 °C showed a doubling of FCR and a 4-fold drop of SGR. Before the seasonal change (day 0-58), temperature alone regulated fish growth rather than dietary lipid, and no differences in overall performance were detected within the temperature regimes tested. Our findings are in agreement with previous studies which found no differences in growth and feed utilization when feeding sea bream juveniles at increasing dietary lipid levels at constant high temperatures (Bonaldo et al., 2010; Mongile, Bonaldo, Fontanillas, Mariani, Badiani, Bonvini, Parma, et al., 2014; Velázquez et al., 2006), or as reported by Sánchez-Nuño et al., (2018a), which found no differences related to dietary lipid (18 *vs* 14%) level during water temperature fluctuation within 22 °C and 14 °C (22 °C for 30 days, then 14 °C for 50 days, finally 22 °C for 35 days). Interestingly, in our study, after seasonal temperature change (day 59-121) low dietary lipid gained more influence, bringing a compensatory growth effect. In fact, 62 days after seasonal temperature change, L16 diet seemed to compensate better for the differences in SGR occurring between days 0-59. Further, L16 was better accepted (higher FI values and higher relative increment) by animals entering the spring change, passing from 17 °C to 23 °C. In contrast, Sánchez-Nuño et al., (2018b) did not observe a compensatory growth and redox status at the end of the recovery period when fish were brought back from 14 °C to 22 °C after a cold period. However, the authors stated that the duration of their recovery period, which lasted 35 days, was not long enough to let the animals finish the rearrangement of FCR due to the delayed recovery bound to putative oxidative stress and fat deposition physiological reassessment. Concerning the nutritional efficiency of lipids, overall LER and GLE were higher in animals entering into spring (LH) than in those going towards the fall season (HL). At the same temperature regime, low dietary lipids guaranteed better lipid utilization rather than high ones. Both before and after seasonal change, again low lipid diet and warmer temperatures led to better lipid utilization by fish, confirming previous study statements (Bonaldo et al., 2010; Mongile, Bonaldo, Fontanillas, Mariani, Badiani, Bonvini, & Parma, 2014; Velázquez et al., 2006). PER and GPE were not statistically influenced by dietary lipid levels but were positively influenced by high rearing temperature both before and after seasonal change. Feeding 16% or 21% lipid diets did not make any difference to HSI, as observed in other previous studies (Bonaldo et al., 2010; Gómez-Milán & Lozano, 2007; Melis et al., 2017; Mongile, Bonaldo, Fontanillas, Mariani, Badiani, Bonvini, Parma, et al., 2014; Velázquez et al., 2006). However, HSI increased by 22-35% from H to L temperature and though no statistical difference occurred in fat liver content, liver lipid content tended to be higher at 23 °C. In contrast, most of the current literature focused on metabolic and physiological responses of this species to low temperatures observed an increase in

the hepatosomatic index (HSI), explained by a higher mobilization of lipids (due to fat mobilization and hepatic deposition caused by cold temperatures) (A Ibarz et al., 2007b; Antoni Ibarz et al., 2007; Ibarz et al., 2005; Mateus et al., 2017; Mininni et al., 2014). However, in other studies HSI variations related to water temperature were mostly attributed to changes in hepatic carbohydrate content (Melis et al., 2017; Richard et al., 2016; Silva et al., 2014). In our study, before seasonal temperature change MFI, VSI and their relative variations were not influenced by diet nor temperature. These findings are consistent with Mongile et al., (2014b), where dietary lipid from 20% up to 24% did not have any effect in gilthead sea bream maintained at 27 °C. However, after seasonal temperature change, 21% lipid diet caused slightly higher MFI and VSI levels, in contrast with the above-mentioned author's findings. To further explain the growth responses of gilthead sea bream after undertaking seasonal thermal changes, a spotlight on digestive enzymatic activity was performed. In our study, before the seasonal change pepsin activity was higher in fish fed high lipid (L21). However, after the seasonal change a significant interaction indicated that the combined effect of low lipid (L16) and low temperature guaranteed higher activity of this enzyme in animals subjected to a lower temperature of 17 °C (HL). Both before and after seasonal temperature change, our study reported no temperature-significant influence on pepsin activity. However, L16 seems to promote ($p=0.077$) the relative increase of pepsin activity after temperature changes (especially when going from H to L temperature) in comparison to L21. On the contrary, in on-growing cobia (*Rachycentron canadum*) reared at two different temperatures (30 °C and 34 °C) higher pepsin activity was attributed to animals reared at a high temperature of 34 °C (Nguyen et al., 2019; Yúfera et al., 2019). Yet those subjects had higher FCR rather than others reared at 30 °C and fed the same daily ration (Nguyen et al., 2019; Yúfera et al., 2019). The authors stated that in cobia reared at a higher temperature, increased pepsin activity could not improve growth owing to increased gastric transit rate (Yúfera et al., 2019). Unlike pepsin, trypsin appeared to be influenced by seasonal thermal changes. Before the seasonal change trypsin level was slightly higher at 17 °C in each diet, then after the thermal switch its activity displayed higher values at 23 °C. Thus, after the seasonal change, trypsin response to temperature showed the opposite trend, being higher at 23 °C, while it was slightly higher in animals fed higher lipid diet content (L21). After temperature changes, similar relative decreasing changes ranging from 40 to 59% were observed for all the treatment. Our trypsin levels found before thermal change are consistent with results found in European sea bass (*Dicentrarchus labrax*) reared at three different water temperatures (17 °C, 20 °C and 23 °C) where the trypsin activity peaked at the lowest temperature of 17 °C (Pereira et al., 2018). Also, Hani et al., (2018) on fed ad-libitum three-spine sticklebacks (*Gasterosteus aculeatus*) exposed to 16 °C, 18 °C and 21 °C, found that lower temperatures (16 °C and 18 °C) favoured higher growth and trypsin activity, while higher temperatures decreased them. Given these results, the authors suggested using trypsin as a marker of warm thermal stress in the three-spine stickleback. In accordance, our observed reduction of trypsin activity after thermal changes (especially from high to low temperature) supports trypsin as a valuable marker during thermal stress occurrences. Temperature and dietary lipid level also affected FI which could have resulted in variation of enzymatic activity. In addition, dietary lipid level may affect gastric transit rate as reported by Bonvini et al., (2018) in European sea bass and by García-Meilán et al., (2013) in gilthead sea bream, or

contribute to low transit rate at high dietary content as Fountoulaki et al., 2005 observed in sea bream, with possible consequences on enzyme activity. In the present study chymotrypsin activity was influenced only by diet, being more elevated in fish fed 21% dietary lipid level rather than 16% both before and after seasonal change. Similarly, while temperature did not affect chymotrypsin, dietary regime (probiotic supplementation versus blind control) was shown to improve consistently its activity in European sea bass reared at 17 °C (Pereira et al., 2018). As regards amylase activity, while before the seasonal change no dietary or thermal effect occurred, after the seasonal change it was significantly impeded, with lower activity values and higher relative reduction in fish brought to 17 °C (HL). This temperature influence reinforces the hypothesis that when fish are subjected to colder temperatures, feeding absorption drops, while in the liver a metabolic reassessment takes place for glycogen synthesis, accumulation and storage (Melis et al., 2017; Sánchez-Nuño, Sanahuja, et al., 2018; Silva et al., 2014). In the present study, the trend to higher amylolytic activity at higher temperatures after the seasonal change is consistent with the increased activity found during warm periods with a long photoperiod tested on three-spine sticklebacks (Hani et al., 2018). Similarly, lipase activity also was not influenced by diet or temperature before seasonal change. Afterwards, lipase showed a general reduction of activity with higher values in L21 and at H temperature. This is in contrast with Arantzamendi et al., (2019), where bile salt-activated lipase activity (BAL) of gilthead sea bream maintained at constant optimal water temperature (within 20 °C and 24.2 °C) tended to increase with age throughout the life cycle. Plasma cortisol and glucose levels are the first and main metabolites being released into the plasma as response markers to stress (Barton, 2002). Before the seasonal change, while cortisol level in same temperature regimes was found to be almost twice as high in fish fed low lipid, glucose was not influenced by either of the two factors considered. Afterwards, cortisol was not influenced by any factor, while glucose increased in animals brought from 17 °C to 23 °C (LH). Our findings on glucose levels are in contrast with cold induced-hyperglycaemia observed in studies on gilthead sea bream undertaken in both outdoor and indoor conditions (Faggio et al., 2014; Matias et al., 2018; Rotllant et al., 2001). On the other hand, in our study cortisol was seen to be unrelated to water temperature but linked mostly to feeding regime or diet (while its relative variation was not significantly related to diet nor temperature change), which is consistent with other works in sea bream (Matias et al., 2018; Montoya et al., 2010). Total protein is a liver impairment marker, and increase in concentration can be caused by structural liver alterations such as aminotransferase activity reduction, leading to a concurrent reduction of deamination capacity (Bernet et al., 2001). Among them, albumin was found to be the major plasma protein in 16 - 18 °C acclimated gilthead sea bream, representing 25% - 30% of the TP, regulating colloidosmotic pressure, participating in ionic balance, contributing to the elimination of toxicants and to the transport of organic molecules, and serving for protein synthesis after degradation (Gras, 1983; Sala-Rabanal et al., 2003). In our study, TP level was greater at lower temperatures, and after seasonal change both TP and ALB were found to be higher in animals fed lower lipid (L16). As stated by Mateus et al., (2017), plasma levels of total protein were also modified in fish exposed to cold: field-based (Guijarro et al., 2003; Vargas-Chacoff et al., 2009) and laboratory (Gallardo et al., 2003) studies have previously reported increased total plasma protein concentrations during winter or under lower temperatures, respectively, as a result of increased

globulins (Cataldi et al., 1998; Gallardo et al., 2003). Our findings are in accordance with the significantly higher plasma TP levels of gilthead sea bream exposed to 13°C compared with those with the same thermal history maintained at 23°C described by Mateus et al., (2017). On the other hand, Sala-Rabanal et al., (2003) reported a fall of plasma TP in gilthead sea bream exposed to 8 °C- and 12 °C in cage farms. AST, ALP and LDH are nonspecific plasma enzymes, indicators of tissue damage owing to pathological processes, toxic chemical exposure, or traumatic fish handling (Peres et al., 2013). Elevated plasma LHD may imply a liver parenchyma, renal failure, muscle tissue breakdown, or haemolysis (Peres et al., 2013). In the present study, AST, ALP and LDH were influenced only by temperature before the seasonal change. While LDH and AST levels were higher at 17 °C, ALP was very high in animals kept at 23 °C. Then, after the seasonal change ALP, AST and LDH were found to be influenced by dietary lipids, with greater levels in response to high dietary lipid diet (L21). In the present study, the elevated blood LDH activity found at low temperatures before seasonal changes could probably be caused by lactate accumulation in aerobic tissues such as red muscle and heart indicating an activation of the anaerobic component of metabolism during exposure to cold (Faggio et al., 2014). CHOL levels and its relative variation were not statistically different throughout the trial, and the same was observed for TRIG. Before the seasonal change occurred, TRIG and HDL were more elevated at 17 °C. After the seasonal change, they showed smaller differences among groups but with opposite trends to these two analyses. Our results are consistent with increased triglyceride levels found during the colder months, interpreted as a mobilization of the lipid deposits to use as fuels by Faggio et al., (2014).Though most previous studies revealed that cold water conditions for gilthead sea bream caused an imbalance in plasma ions levels (Gallardo et al., 2003; Mateus et al., 2017; Rotllant et al., 2001; Sala-Rabanal et al., 2003; Vargas-Chacoff et al., 2009), in the present study they mostly changed according to dietary lipid content rather than temperature changes. Indeed, before the seasonal change, calcium, phosphorus, and sodium were more elevated in animals fed high lipid diet (L21). Then, after temperature change, while potassium, sodium and chloride remained higher in accordance with a high lipid diet, calcium was found to be more elevated in fish fed L16 diet. In our study, iron was the only ion influenced by temperature with higher values in animals kept at 23 °C before the seasonal change. Concerning the relative variations in ions, only Ca^{2+} and Cur Ca^{2+} displayed significant dietary effect, with higher values in fish fed L16, while all the other elements considered were not statistically significant. The study of gut microbiota has received great attention in the aquaculture sector as an indicator of productivity and fish health and it is likely that its manipulation will be achieved in the near future in several fish species of commercial interest. While great efforts have been made to analyse the effects of different raw materials in feed ingredients on the structure of the gut microbiome in marine species, more studies to detect dynamical changes of microbial composition during the farming cycle are necessary (Infante-Villamil et al., 2020). In the present study, at high phylogenetic levels, the overall GM structure was similar among groups and the main represented taxa at phylum (Firmicutes, Proteobacteria and Actinobacteria) and family (*Lactobacillaceae*) level are consistent with previous trials on this species reared on similar aquafeed formulation and feeding protocols (Parma et al., 2016, 2020). According to our findings, the impact of the L16 and L21 diets on the overall gut microbiome was dependent on growth temperature. Indeed, only warm

temperature lead the two diets associated with different gut microbiome compositional layouts with L16 diet resulting in a higher load of *Lactobacillus*. The dominance of *Lactobacillaceae* mainly *Lactobacillus* has been considered a valid indicator of optimal gut health condition in sea bream (Parma et al., 2016, 2020). Interestingly, the two diets performed differently in term of microbiome response to the temperature transitions. In particular, while fish fed with L16 diet showed significant gut microbiome changes only at the autumn shift, parallel with a reduction of ecosystem diversity, for L21 diet both summer and autumn temperature shifts resulted in significant variations of the ecosystem. Temperature is known to modulate microbial diversity in animals especially in poikilothermic fish species (Sepulveda & Moeller, 2020); however data explaining the interaction between diet and temperature changes in fish are scarce (Busti et al., 2020; Pelusio et al., 2020; Soriano et al., 2018). Interestingly, among the few studies which underlined the combined effect of temperature and dietary lipid level, Soriano et al., (2018) in yellowtail kingfish detected a reduced bacterial abundance and richness associated to a suboptimal low temperature and low dietary lipid level, suggesting that GM composition could maintain high relative abundance after the decrease of temperature only in the presence of appropriate nutritional conditions, pointing out the importance of optimal lipid level at low temperatures. On the other hand, in the present study the temperature increase from 17 to 23 °C showed a significant impact on the diversity (β-diversity) only in a high lipid diet. As concerns the specific gut microbiome compositional changes, the decreasing of temperature from 23 to 17 °C leads to a significant reduction of *Planctomycetaceae* and *Bacillus*. *Bacillus* is one of the most important beneficial taxa in fish species which can make a positive contribution to nutrition, to the immune system and to disease resistance towards pathogens by producing bacteriocins. This decreasing effect may be in line with the sensibility of sea bream to thermal reduction; however, it should be mentioned that although there has been a significant decrease of this bacterial taxa only under L16, its value was higher in comparison to L21 at the same time point examined. After the temperature decrease from 23 °C to 17°C, fish fed L16 also showed a significantly higher abundance of *Weissella* in comparison to L21. This taxon belonging to LAB is of potential interest for its application as a probiotic in aquaculture (Mortezaei et al., 2020; Ringø et al., 2018, 2020) and has been shown to improve intestinal health and the hemato-parameters of hybrid surubim (*Pseudoplatystoma reticulatum* female × *P. corruscans*) male (Jesus et al., 2017). In L16, the change towards high temperature was characterized by a significant increase in the relative abundance of *Methylobacterium*. Although with contradictory results, the abundance of *Methylobacteriaceae* in fish gut has been previously associated with environmental temperature change in tench, *Tinca tinca*, and the sparids pinfish, *Lagodon rhomboids* (Dulski et al., 2020; Givens, 2012). *Methylobacterium* have also been associated as beneficial microbial taxa in Nile Tilapia fed functional ingredients (Zheng et al., 2018). Focusing on the dietary effect after the increase of temperature, L21 showed a significantly higher abundance of *Bacillus* and *Streptococcus* compared to L16. Dietary lipid content and composition is known to potentially affect gut microbiota composition of animals, although very few studies in aquatic species are available. In mice, high-calorie diets can affect gut microbiota, reducing bacterial diversity and altering the ecosystem in favour of opportunistic taxa (Bruce-Keller et al., 2020). Also in zebrafish the increase of dietary fat from 5 to 15% led to reduced GM diversity (Falcinelli et

al., 2015) and a high-fat diet (24% vs 8%) fed to overfeeding affected the GM composition (Navarro-Barró et al., 2019). In this last-mentioned study, the authors revealed an increase in the abundance of Proteobateria which have been proposed as a possible sign of GM imbalance in fish species. This is also in agreement with the observed increased taxa (*Enterobacteriaceae*) belonging to this phylum in sea bass GM experienced inflammatory gut mucosal after the exposure to high temperature and low oxygen condition (Busti, Rossi, et al., 2020). In the present study we did not observe a GM imbalance related to the lipid level tested, which remains within a general optimal requirement for this species. However, the significant increase in *Streptoccoccus* under L21 compared to L16 at the end of the trial may deserve specific attention. *Streptoccoccus* is considered one of the most common pathogens in aquaculture (Ringø et al., 2018). These taxa were indicative of dysbiosis in olive flounder, *Paralichthys olivaceus,* after antibiotic treatment (Kim et al., 2019) and its significant increase was associated with low fish diet and high rearing density conditions.

Conclusion

In conclusion, high dietary lipid levels 21% did not improve growth and feed efficiency during seasonal temperature changes in comparison to low dietary lipid (16 %). On the other hand, low dietary lipid improved feed intake, growth and nutrient utilization after seasonal temperature changes, especially in fish entering optimal temperature (23 °C) which simulated the spring to summer water temperature switch. In addition, after seasonal temperature changes, L16 reduced perivisceral fat. Low temperature (17 °C) strongly affected overall growth performance and nutrient efficiency parameters in comparison to 23 °C with major negative effects in fish experiencing summer to autumn temperature changes. After temperature changes, the combined effects of low lipid diet and low temperature conditions resulted in higher pepsin activity while trypsin, chymotrypsin and lipase were generally higher at high lipid content. The absence of a significant interaction in most of the plasma parameters examined supports the hypothesis that the combined effect of diet and temperature did not alter the metabolic plasma profile. However, the higher AST and ALP observed at the end of the trial in L21 may deserve further attention of possible negative effect on liver status when combining high dietary lipid and temperature changes. GM composition were similar among all groups with the dominance of beneficial taxa (such as *Lactobacillus*) representative of a healthy ecosystem in this species. However, after the temperature reduction L16 was characterized by a higher abundance of the potential beneficial taxa *Weisella* spp while the increase of temperature L21 supports the growth of the potential pathogens *Streptococcus* spp. According to the results, the utilization of low dietary lipid levels in gilthead sea bream should be preferred during seasonal temperature changes in order to optimize feed utilization and fish welfare.

References

Albro, P. W., Hall, R. D., Corbett, J. T., & Schroeder, J. (1985). Activation of nonspecific lipase (EC 3.1.1.-) by bile salts. *Biochimica et Biophysica Acta (BBA)/Lipids and Lipid Metabolism*, *835*(3), 477–490. https://doi.org/10.1016/0005-2760(85)90117-1

Anson, M. L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with hemoglobin. *Journal of General Physiology*, *22*, 79–89.

Arantzamendi, L., Roo, F., Hernández-Cruz, C. M., Fernández-Palacios, H., & Izquierdo, M. (2019). Lipid digestion capacity

in gilthead seabream (Sparus aurata) from first feeding to commercial size. *Fish Physiology and Biochemistry*, *45*(1), 469–484. https://doi.org/10.1007/s10695-018-0577-y

Barton, B. A. (2002). Stress in Fishes: A Diversity of Responses with Particular Reference to Changes in Circulating Corticosteroids. *Integrative and Comparative Biology*, *42*(3), 517–525. https://doi.org/10.1093/icb/42.3.517

Bell, J. G., & Koppe, W. (2010). Lipids in aquafeeds. In D. R. Turchini, Giovanni M., Ng, Wing-Keong , Tocher (Ed.), *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*. CRC Press, 2010.

Bernet, D., Schmidt, H., Wahli, T., & Burkhardt-Holm, P. (2001). Effluent from a sewage treatment works causes changes in serum chemistry of brown trout (Salmo trutta L.). *Ecotoxicology and Environmental Safety*, *48*(2), 140–147. https://doi.org/10.1006/eesa.2000.2012

Bernfeld, P. (1955). Amylases, alpha and beta. *Methods in Enzymology*, *1*, 149–154.

Biagi, E., D'Amico, F., Soverini, M., Angelini, V., Barone, M., Turroni, S., Rampelli, S., Pari, S., Brigidi, P., & Candela, M. (2019). Faecal bacterial communities from Mediterranean loggerhead sea turtles (Caretta caretta). *Environmental Microbiology Reports*, *11*(3), 361–371. https://doi.org/10.1111/1758-2229.12683

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–917. https://doi.org/10.1139/o59-099

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., … Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. In *Nature Biotechnology* (Vol. 37, Issue 8, pp. 852–857). Nature Publishing Group. https://doi.org/10.1038/s41587-019-0209-9

Bonaldo, A., Isani, G., Fontanillas, R., Parma, L., Grilli, E., & Gatta, P. P. (2010). Growth and feed utilization of gilthead sea bream (Sparus aurata, L.) fed to satiation and restrictively at increasing dietary energy levels. *Aquaculture International*, *18*(5), 909–919. https://doi.org/10.1007/s10499-009-9312-0

Bonvini, E., Bonaldo, A., Mandrioli, L., Sirri, R., & Dondi, F. (2018). Effects of feeding low fishmeal diets with increasing soybean meal levels on growth, gut histology and plasma biochemistry of sea bass. *Animal*, *12*(5), 923–930.

Bruce-Keller, A. J., Richard, A. J., Fernandez-Kim, S.-O., Ribnicky, D. M., Salbaum, J. M., Newman, S., Carmouche, R., & Stephens, J. M. (2020). Fenugreek counters the effects of high fat diet on gut microbiota in mice: Links to metabolic benefit. *Scientific Reports*, *10*(1).

Busti, S., Bonaldo, A., Dondi, F., Cavallini, D., Yúfera, M., Gilannejad, N., Moyano, F., Gatta, P., & Parma, L. (2020). Effects of different feeding frequencies on growth, feed utilisation, digestive enzyme activities and plasma biochemistry of gilthead sea bream (Sparus aurata). *Aquaculture*, *529*.

Busti, S., Rossi, B., Volpe, E., Ciulli, S., Piva, A., D'Amico, F., Soverini, M., Candela, M., Gatta, P. P., Bonaldo, A., Grilli, E., & Parma, L. (2020). Effects of dietary organic acids and nature identical compounds on growth, immune parameters and gut microbiota of European sea bass. *Scientific Reports*, *10*(1).

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Cataldi, E., Marco, P. Di, Mandich, A., & Cataudella, S. (1998). Serum parameters of Adriatic sturgeon Acipenser naccarii (Pisces: Acipenseriformes): effects of temperature and stress. In *Comparative Biochemistry and Physiology Part A* (Vol. 121).

Couto, A., Enes, P., Peres, H., & Oliva-Teles, A. (2012). Temperature and dietary starch level affected protein but not starch digestibility in gilthead sea bream juveniles. *Springer*. https://doi.org/10.1007/s10695-011-9537-5

Culhane, A., Thioulouse, J., & … G. P. (2005). MADE4: an R package for multivariate analysis of gene expression data. *Bioinformatics*, *21*(11), 2789–2790.

Davis, P. S. (1988). Two occurrences of the gilthead, Sparus aurata Linnaeus 1758, on the coast of Northumberland, England. *Journal of Fish Biology*, *33*(6), 951–951. https://doi.org/10.1111/j.1095-8649.1988.tb05545.x

Dulski, T., Kozłowski, K., & Ciesielski, S. (2020). Habitat and seasonality shape the structure of tench (Tinca tinca L.) gut microbiome. *Scientific Reports*, *10*(4460). https://doi.org/10.1038/s41598-020-61351-1

Erlanger, B. F., Cooper, A. G., & Cohent, W. (1966). The Inactivation of Chymotrypsin by Diphenylcarbamyl Chloride and Its Reactivation by Nucleophilic Agents. *Biochemistry*, *5*(1), 190–196. https://doi.org/10.1021/bi00865a025

Erlanger, B., Kokowsky, N., & Cohen, N. (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Archives of Biochemistry and Biophysics*, *95*(2), 271–278.

Faggio, C., Piccione, G., Marafioti, S., Arfuso, F., Fortino, G., & Fazio, F. (2014). Metabolic response to monthly variations of Sparus aurata reared in mediterranean on-shore tanks. *Turkish Journal of Fisheries and Aquatic Sciences*, *14*(2), 567–574. https://doi.org/10.4194/1303-2712-v14_2_28

Falcinelli, S., Picchietti, S., Rodiles, A., Cossignani, L., Merrifield, D., & Taddei, A. (2015). Lactobacillus rhamnosus lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. *Scientific Reports*, *5*(9336).

Fountoulaki, E., Alexis, M. N., Nengas, I., & Venou, B. (2005). Effect of diet composition on nutrient digestibility and digestive enzyme levels of gilthead sea bream (Sparus aurata L.). *Aquaculture Research*, *36*(13), 1243–1251. https://doi.org/10.1111/j.1365-2109.2005.01232.x

Gallardo, M. A., Sala-Rabanal, M., Ibarz, A., Padrós, F., Blasco, J., Fernández-Borras, J., & Sánchez, J. (2003). Functional alterations associated with "winter syndrome" in gilthead sea bream (Sparus aurata). *Aquaculture*, *223*(1–4), 15–27.

García-Meilán, I., Valentín, J. M., Fontanillas, R., & Gallardo, M. A. (2013). Different protein to energy ratio diets for gilthead sea bream (Sparus aurata): Effects on digestive and absorptive processes. *Aquaculture*, *412–413*, 1–7. https://doi.org/10.1016/j.aquaculture.2013.06.031

Givens, C. E. (2012). *A fish tale: comparison of the gut microbiome of 15 fish species and the influence of diet and temperature on its composition*. University of South Carolina.

Gómez-Milán, E., & Lozano, M. J. S. M. (2007). Daily and annual variations of the hepatic glucose 6-phosphate dehydrogenase activity and seasonal changes in the body fats of the gilthead seabream Sparus aurata. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *307*(9), 516–526. https://doi.org/10.1002/jez.406

Gras, J. (1983). *Proteínas plasmáticas: fisicoquímica, metabolismo, fisiopatología y clínica de las proteínas extracelulares* (4th ed.). Editorial JIMS, Barcelona.

Guerreiro, I., Enes, P., Rodiles, A., Merrifield, D., & Oliva-Teles, A. (2016). Effects of rearing temperature and dietary short-chain fructooligosaccharides supplementation on allochthonous gut microbiota, digestive enzymes activities and intestine health of turbot (Scophthalmus maximus L.) juveniles. *Aquaculture Nutrition*, *22*(3), 631–642. https://doi.org/10.1111/anu.12277

Guijarro, A. I., Lopez-Patiño, M. A., Pinillos, M. L., Isorna, E., De Pedro, N., Alonso-Gómez, A. L., Alonso-Bedate, M., & Delgado, M. J. (2003). Seasonal changes in haematology and metabolic resources in the tench. *Journal of Fish Biology*, *62*(4), 803–815. https://doi.org/10.1046/j.1095-8649.2003.00066.x

Guillen, J. (2019). European and Mediterranean Aquaculture data collection and reporting under the STECF. In *medaid-h2020.eu*.

Hani, Y. M. I., Marchand, A., Turies, C., Kerambrun, E., Palluel, O., Bado-Nilles, A., Beaudouin, R., Porcher, J. M., Geffard, A., & Dedourge-Geffard, O. (2018). Digestive enzymes and gut morphometric parameters of threespine stickleback (Gasterosteus aculeatus): Influence of body size and temperature. *PLoS ONE*, *13*(4). https://doi.org/10.1371/journal.pone.0194932

Hasan, M. R. (2007). *Global synthesis of feeds and nutrients for sustainable aquaculture development Food and Agriculture*

Organization of the United Nations.

Ibarz, A., Beltrán, M., Fernández-Borràs, J., & Gallardo, M. (2007a). Alterations in lipid metabolism and use of energy depots of gilthead sea bream (Sparus aurata) at low temperatures. *Aquaculture*, *262*(2–4), 470–480. https://doi.org/10.1016/j.aquaculture.2006.11.008

Ibarz, A., Beltrán, M., Fernández-Borràs, J., & Gallardo, M. (2007b). Alterations in lipid metabolism and use of energy depots of gilthead sea bream (Sparus aurata) at low temperatures. *Aquaculture*.

Ibarz, A., Blasco, J., Beltrán, M., Gallardo, M., & Sánchez, J. (2005). Cold-induced alterations on proximate composition and fatty acid profiles of several tissues in gilthead sea bream (Sparus aurata). *Aquaculture*.

Ibarz, A., Blasco, J., Sala-Rabanal, M., Gallardo, Á., Redondo, A., & Fernández-Borràs, J. (2007). Metabolic rate and tissue reserves in gilthead sea bream (Sparus aurata) under thermal fluctuations and fasting and their capacity for recovery. *Canadian Journal of Fisheries and Aquatic Sciences*, *64*(7), 1034–1042. https://doi.org/10.1139/F07-079

Ibarz, A., Padrós, F., Ngeles Gallardo, M. A. ´, Fernández-Borrà, J., Blasco, J., & Tort, L. (2010). Low-temperature challenges to gilthead sea bream culture: review of cold-induced alterations and "Winter Syndrome." *Springer*. https://doi.org/10.1007/s11160-010-9159-5

Infante-Villamil, S., Huerlimann, R., & Jerry, D. R. (2020). Microbiome diversity and dysbiosis in aquaculture. In *Reviews in Aquaculture*. Wiley-Blackwell. https://doi.org/10.1111/raq.12513

Jesus, G. F. A., Vieira, F. D. N., Silva, B. C., Junior, M. M. D. S., Ushizima, T. T., Schmidt, E. C., Bouzon, Z. L., Pereira, S. A., Pereira, G. V., Martins, M. L., & Mouriño, J. L. P. (2017). Probiotic bacteria may prevent haemorrhagic septicaemia by maturing intestinal host defences in Brazilian native surubins. *Aquaculture Nutrition*, *23*(3), 484–491. https://doi.org/10.1111/anu.12416

Jobling, M., & Peruzzi, S. (2010). Seabreams and Porgies (Family: Sparidae). In M. Jobling & C. Carter (Eds.), *Finfish Aquaculture Diversification* (p. 681). CABI.

Kim, A., Kim, N., Roh, H. J., Chun, W.-K., Ho, D. T., Lee, Y., & Kim, D.-H. (2019). Administration of antibiotics can cause dysbiosis in fish gut. *Aquaculture*, *512*.

Klindworth, A., Pruesse, E., Schweer, T., Rg Peplies, J., Quast, C., Horn, M., & Glö Ckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), e1. https://doi.org/10.1093/nar/gks808

Koven, W. (2002). Gilthead sea bream, Sparus aurata. In C. Webster, Carl D., Lim (Ed.), *Nutrient Requirements and Feeding of Finfish for Aquaculture* (p. 448). CABI.

Leaver, M. J., Bautista, J. M., Björnsson, T., Jönsson, E., Krey, G., Tocher, D. R., Torstensen, B. E., Jos´, J., Bj¨ Bj¨orn Thrandur Bj¨ornssonbj¨ Bj¨ornsson, B., & Onsson, E. J. ¨. (2008). *Reviews in Fisheries Science Towards Fish Lipid Nutrigenomics: Current State and Prospects for Fin-Fish Aquaculture Towards Fish Lipid Nutrigenomics: Current State and Prospects for Fin-Fish Aquaculture*. https://doi.org/10.1080/10641260802325278

Luna, M., Llorente, I., Cobo, · Angel, & Es, M. L. (2019). Determination of feeding strategies in aquaculture farms using a multiple-criteria approach and genetic algorithms. *Annals of Operations Research*. https://doi.org/10.1007/s10479-019-03227-w

Mateus, A. P., Costa, R., Gisbert, E., Pinto, P. I. S., Andree, K. B., Estévez, A., & Power, D. M. (2017). Thermal imprinting modifies bone homeostasis in cold-challenged sea bream (Sparus aurata). *Journal of Experimental Biology*, *220*(19), 3442–3454. https://doi.org/10.1242/jeb.156174

Matias, A. C., Ribeiro, L., Araujo, R. L., & Pousão-Ferreira, P. (2018). Preliminary studies on haematological and plasmatic parameters in gilthead sea bream (Sparus aurata) held under day/night temperature variations. *Fish Physiology and Biochemistry*, *44*(1), 273–282. https://doi.org/10.1007/s10695-017-0432-6

Melis, R., Sanna, R., Braca, A., Bonaglini, E., Cappuccinelli, R., Slawski, H., Roggio, T., Uzzau, S., & Anedda, R. (2017). Molecular details on gilthead sea bream (Sparus aurata) sensitivity to low water temperatures from 1H NMR metabolomics. *Comparative Biochemistry and Physiology - Part A : Molecular and Integrative Physiology*, *204*, 129–136. https://doi.org/10.1016/j.cbpa.2016.11.010

Mininni, A. N., Milan, M., Ferraresso, S., Petochi, T., Di Marco, P., Marino, G., Livi, S., Romualdi, C., Bargelloni, L., & Patarnello, T. (2014). Liver transcriptome analysis in gilthead sea bream upon exposure to low temperature. *BMC Genomics*, *15*(1). https://doi.org/10.1186/1471-2164-15-765

Mongile, F., Bonaldo, A., Fontanillas, R., Mariani, L., Badiani, A., Bonvini, E., & Parma, L. (2014). Effects of Dietary Lipid Level on Growth and Feed Utilisation of Gilthead Seabream (*Sparus Aurata* L.) Reared at Mediterranean Summer Temperature. *Italian Journal of Animal Science*, *13*(1), 2999. https://doi.org/10.4081/ijas.2014.2999

Mongile, F., Bonaldo, A., Fontanillas, R., Mariani, L., Badiani, A., Bonvini, E., Parma, L., Mediche, S., Bologna, U., & Bo, E. (2014). *utilisation of gilthead reared at Mediterranean on co m er ci al us e on l Lipid level seabream at summer temperature on co m m er al*. *13*. https://doi.org/10.4081/ijas.2014.2999

Montoya, A., López-Olmeda, J. F., Garayzar, A. B. S., & Sánchez-Vázquez, F. J. (2010). Synchronization of daily rhythms of locomotor activity and plasma glucose, cortisol and thyroid hormones to feeding in Gilthead seabream (Sparus aurata). *Physiol Behav*, *101*, 101–107.

Mortezaei, F., Royan, M., Noveirian, H. A., Babakhani, A., Kordghashlaghi, H. A., & Balc Azar, J. L. (2020). In vitro assessment of potential probiotic characteristics of indigenous Lactococcus lactis and Weissella oryzae isolates from rainbow trout (Oncorhynchus mykiss Walbaum). *Journal of Applied Microbiology*, *129*(4), 1004–1019. https://doi.org/10.1111/jam.14652

Musella, M., Wathsala, R., Tavella, T., Rampelli, S., Barone, M., Palladino, G., Biagi, E., Brigidi, P., Turroni, S., Franzellitti, S., & Candela, M. (2020). Tissue-scale microbiota of the Mediterranean mussel (Mytilus galloprovincialis) and its relationship with the environment. *Science of the Total Environment*, *717*.

Navarro-Barró, E., Herná Ndez, C., Llera-Herrera, R. L., García-Gasca, A., & Gó Mez-Gil, B. (2019). Overfeeding a High-Fat Diet Promotes Sex-Specific Alterations on the Gut Microbiota of the Zebrafish (Danio rerio). *Zebrafish*, *16*(3), 268–279. https://doi.org/10.1089/zeb.2018.1648

Nguyen, M. Van, Espe, M., Conceição, L. E. C., Le, H. M., Yúfera, M., Engrola, S. A. D., Jordal, A. E. O., & Rønnestad, I. (2019). The role of dietary methionine concentrations on growth, metabolism and N-retention in cobia (Rachycentron canadum) at elevated water temperatures. *Aquaculture Nutrition*, *25*(2), 495–507. https://doi.org/10.1111/anu.12875

Parma, L., Candela, M., Soverini, M., Turroni, S., Consolandi, C., Brigidi, P., Mandrioli, L., Sirri, R., Fontanillas, R., Gatta, P. P., & Bonaldo, A. (2016). Next-generation sequencing characterization of the gut bacterial community of gilthead sea bream (Sparus aurata, L.) fed low fishmeal based diets with increasing soybean meal levels. *Animal Feed Science and Technology*, *222*, 204–216. https://doi.org/10.1016/j.anifeedsci.2016.10.022

Parma, L., Pelusio, N. F., Gisbert, E., Esteban, M. A., D'Amico, F., Soverini, M., Candela, M., Dondi, F., Gatta, P. P., & Bonaldo, A. (2020). Effects of rearing density on growth, digestive conditions, welfare indicators and gut bacterial community of gilthead sea bream (Sparus aurata, L. 1758) fed different fishmeal and fish oil dietary levels. *Aquaculture*, *518*, 734854. https://doi.org/10.1016/j.aquaculture.2019.734854

Pelusio, N. F., Rossi, B., Parma, L., Volpe, E., Ciulli, S., Piva, A., D'Amico, F., Scicchitano, D., Candela, M., Gatta, P. P., Bonaldo, A., & Grilli, E. (2020). Effects of increasing dietary level of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (Oncorhynchus mykiss) reared at normal and high temperature. *Fish & Shellfish Immunology*, *107*, 324–335. https://doi.org/10.1016/J.FSI.2020.10.021

Pereira, L. F., Peixoto, M. J., Carvalho, P., Sansuwan, K., Santos, G. A., Gonçalves, J. F. M., & Ozório, R. O. A. (2018). Cross-effects of dietary probiotic supplementation and rearing

temperature on growth performance, digestive enzyme activities, cumulative mortality and innate immune response in seabass (Dicentrarchus labrax). *Aquaculture Nutrition*, *24*(1), 453–460. https://doi.org/10.1111/anu.12578

Peres, H., Santos, S., & Oliva-Teles, A. (2013). Selected plasma biochemistry parameters in gilthead seabream (Sparus aurata) juveniles. *Journal of Applied Ichthyology*, *29*(3), 630–636. https://doi.org/10.1111/j.1439-0426.2012.02049.x

QIIME 2. (n.d.).

Quast, C., Klindworth, A., Pruesse, E., Schweer, T., Horn, M., & Glo, F. O. (2013). *Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies*. *41*(1), 1–11. https://doi.org/10.1093/nar/gks808

Richard, N., Silva, T., Wulff, T., Schrama, D., Dias, J., P, R., & L, C. (2016). Nutritional mitigation of winter thermal stress in gilthead seabream: associated metabolic pathways and potential indicators of nutritional state. *Journal of Proteomics*, *142*, 1–14. https://doi.org/10.1016/j.jprot.2016.04.037

Ringø, E., Hoseinifar, S. H., Ghosh, K., Doan, H. Van, Beck, B. R., & Song, S. K. (2018). Lactic acid bacteria in finfish-An update. In *Frontiers in Microbiology* (Vol. 9, Issue AUG, p. 1818). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2018.01818

Ringø, E., Van Doan, H., Lee, S. H., Soltani, M., Hoseinifar, S. H., Harikrishnan, R., & Song, S. K. (2020). Probiotics, lactic acid bacteria and bacilli: interesting supplementation for aquaculture. *Journal of Applied Microbiology*, *129*(1), 116–136. https://doi.org/10.1111/jam.14628

Rognes, T., Flouri, T., Nichols, B., Quince, C., & Mahé, F. (2016). VSEARCH: A versatile open source tool for metagenomics. *PeerJ*, *2016*(10), e2584. https://doi.org/10.7717/peerj.2584

Rotllant, J., Balm, P. H. M., & Pe, J. (2001). *Pituitary and Interrenal Function in Gilthead Sea Bream (Sparus aurata L ., Teleostei) after Handling and Confinement Stress*. *342*, 333–342. https://doi.org/10.1006/gcen.2001.7604

Sala-Rabanal, M., Sánchez, J., Ibarz, A., Fernández-Borràs, J., Blasco, J., & Gallardo, M. A. (2003). Effects of low temperatures and fasting on hematology and plasma composition of gilthead sea bream (Sparus aurata). In *Fish Physiology and Biochemistry* (Vol. 29).

Sánchez-Nuño, S., Eroldogan, O. T., Sanahuja, I., özşahinoğlu, I., Blasco, J., Fernández-Borràs, J., Fontanillas, R., Guerreiro, P. M., & Ibarz, A. (2018). Cold-induced growth arrest in gilthead sea bream Sparus aurata: Metabolic reorganisation and recovery. *Aquaculture Environment Interactions*, *10*, 511–528. https://doi.org/10.3354/AEI00286

Sánchez-Nuño, S., Sanahuja, I., Fernández-Alacid, L., Ordóñez-Grande, B., Fontanillas, R., Fernández-Borràs, J., Blasco, J., Carbonell, T., & Ibarz, A. (2018). Redox challenge in a cultured temperate marine species during low temperature and temperature recovery. *Frontiers in Physiology*, *9*(JUL). https://doi.org/10.3389/fphys.2018.00923

Sepulveda, J., & Moeller, A. H. (2020). The Effects of Temperature on Animal Gut Microbiomes. In *Frontiers in Microbiology* (Vol. 11). Frontiers Media S.A. https://doi.org/10.3389/fmicb.2020.00384

Silva, T., da Costa, A., Conceição, L., Dias, J., Rodrigues, P., & Richard, N. (2014). Metabolic fingerprinting of gilthead seabream (Sparus aurata) liver to track interactions between dietary factors and seasonal temperature variations. *PeerJ*, *2*, e527. https://doi.org/10.7717/peerj.527

Soriano, E. L., Ramírez, D. T., Araujo, D. R., Gómez-Gil, B., Castro, L. I., & Sánchez, C. G. (2018). Effect of temperature and dietary lipid proportion on gut microbiota in yellowtail kingfish Seriola lalandi juveniles. *Aquaculture*, *497*, 269–277. https://doi.org/10.1016/j.aquaculture.2018.07.065

Vargas-Chacoff, L., Arjona, F. J., Polakof, S., del Río, M. P. M., Soengas, J. L., & Mancera, J. M. (2009). Interactive effects of environmental salinity and temperature on metabolic responses of gilthead sea bream Sparus aurata. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *154*(3), 417–424.

Vasconi, M., Caprino, F., Bellagamba, F., & Moretti, V. M. (2017). Fatty acid composition of gilthead sea bream (Sparus aurata) fillets as affected by current changes in aquafeed formulation. *Turkish Journal of Fisheries and Aquatic Sciences*, *17*(3), 51–459. https://doi.org/10.4194/1303-2712-v17_3_01

Velázquez, M., Zamora, S., & Martínez, F. J. (2006). Effect of dietary energy content on gilthead sea bream (Sparus aurata) feeding behaviour and nutritional use of the diet. *Aquaculture Nutrition*, *12*(2), 127–133. https://doi.org/10.1111/j.1365-2095.2006.00391.x

Yúfera, M., Nguyen, M. V., Navarro-Guillén, C., Moyano, F. J., Jordal, A. E. O., Espe, M., Conceição, L. E. C., Engrola, S., Le, M. H., & Rønnestad, I. (2019). Effect of increased rearing temperature on digestive function in cobia early juvenile. *Comparative Biochemistry and Physiology -Part A : Molecular and Integrative Physiology*, *230*, 71–80. https://doi.org/10.1016/j.cbpa.2019.01.007

Zarkasi, K. Z., Taylor, R. S., Abell, G. C. J., Tamplin, M. L., Glencross, B. D., & Bowman, J. P. (2016). Atlantic Salmon (Salmo salar L.) Gastrointestinal Microbial Community Dynamics in Relation to Digesta Properties and Diet. *Microbial Ecology*, *71*(3), 589–603. https://doi.org/10.1007/s00248-015-0728-y

Zheng, Y., Wu, W., Hu, G., Qiu, L., Meng, S., Song, C., Fan, L., Zhao, Z., Bing, X., & Chen, J. (2018). Gut microbiota analysis of juvenile genetically improved farmed tilapia (Oreochromis niloticus) by dietary supplementation of different resveratrol. *Fish and Shellfish Immunology*, *77*, 200–207.

2.5 Study III - Dietary supplementation with a blend of chestnut and quebracho extracts improves intestinal morphology, microbiota, inflammatory status, and innate immune response in zebrafish (*Danio rerio*)

Imperatore, R., Fronte, B., Scicchitano, D., Orso, G., Marchese, M., Mero, S., ... & Paolucci, M. (2022). Dietary Supplementation with a Blend of Hydrolyzable and Condensed Tannins Ameliorates Diet-Induced Intestinal Inflammation in Zebrafish (Danio rerio). Animals, 13(1), 167.

Introduction

The increasing demand for fish products (FAO report, 2020) has prompted aquaculture towards solutions, such as intensive and super intensive systems, which are often adverse both for the environment and fish health and welfare (Naylor et al., 2021). The use of plant-based aquafeeds, as part of a strategic approach to reduce the use of fishmeal (FM) and ameliorating aquaculture carbon footprint, holds drawbacks on carnivorous fish. Indeed, plant sources in aquaculture diets can induce an unbalance in amino acid, fatty acid and mineral profiles and anti-nutritional factors leading to intestinal inflammation. This is a harmful condition that negatively affects feed digestion and nutrient absorption, resulting in an impaired fish growth and health (Bravo-Tello et al., 2017), and economic losses. In fish, the intestinal barrier consists of a single layer of epithelial cells selectively permeable to nutrients and secreting chemokines, cytokines, and antimicrobial proteins, essential for the intestinal mucosal immunity, while goblet cells protect the intestinal barriers producing mucus (Knoop and Newberry, 2018). At first, the intestinal inflammation can be mild and controllable, but if external stressors, such as feed, persist, the inflammation may turn into more serious epithelial tissue disruption and intestinal dysfunction. Moreover, inflamed epithelial cells produce chemokines to recruit immune cells that produce pro-inflammatory cytokines, including interleukin (IL)-1 and tumor necrosis factor-alpha (TNFα). The high concentrations of inflammatory cytokines impair epithelial cells inducing the destruction of the intestinal barrier (Shimizu, 2017). Together with microbial diversity, cytokines, intestinal barrier, and gut morphology, represent useful markers to study the effect of nutrients and nutraceuticals on fish health status (Randazzo et al., 2021). Recently, the scientific interest in "functional" or "nutraceutical" ingredients as modifiers of the organism biological response has increased (Brogi et al., 2021). It is well-known that proper nutrition can exert anti-inflammatory effects and lead to epigenetic imprinting through changes on gene expression patterns and nutrient-sensitive signaling pathways, this resulting in a lifelong contribution to health status (Rocha et al., 2014). Nowadays several studies are investigating the effects of the terrestrial plant-based fish diets on the intestinal status of farmed fish due to the reported pro-inflammatory effects. Plant grain meal and oil have been largely used to cope with fishmeal and fish oil market shortage and their environmentally unsustainable production (Gatlin et al., 2007; Naylor et al., 2009). It has been reported that such diets frequently have detrimental effects on the intestine functionality, reducing overall gut and fish health (Estruch et al., 2018). In these cases, the diet inclusion of natural anti-inflammatory molecules may counteract the described negative effects of terrestrial plant-based diets in fish (Ahmadifar et al., 2020; 2021).

In this context, tannins are important vegetable bioactive compounds worthy to be investigated for both human and animal nutrition purposes (Das et al., 2020). Tannins are phenolic compounds, secondary chemicals ubiquitous in woody plants. They are classified on the basis of structural characteristics into two main groups: hydrolysable tannins (HT) and condensed tannins (CT), while the third group of tannins, called phlorotannins, is present in brown algae and has less structural complexity than HT and CT (Khanbabaee and van Ree, 2001; Bule et al., 2020; Li et al., 2011). In HT phenolic groups such as gallic acid or ellagic acid are linked to a partially or totally esterified carbohydrate, usually represented by D-glucose, giving rise to the esters of the gallic or ellagic acid of glucose. The CT consist of flavan-3-ol units, oligomeric flavonoids, essentially catechin, epicatechin, gallocatechin, and epigallocatechin bonded via a carbon-carbon bond (Khanbabaee and van Ree, 2001). Recently, tannins have received more attention due to numerous beneficial actions such

as antioxidant (Okuda, 2005), anticancer (Cai et al., 2016), antimicrobial and antiviral (Buzzini et al., 2008) activity. However, the potential effects of tannins on human and animal health remain largely unexplored. Research has shown the presence of positive effects linked to the administration of tannins in farmed animals (Mueller-Harvey, 2006; Huang et al., 2018). The most successful supplementation of tannins is attributed to the mitigation of the frothy bloat in ruminants (Wang et al., 2012), and intestinal inflammation in terrestrial farm animals (Varricchio et al., 2019). The literature on the effect of tannins on farmed fish production is limited. Previous studies indicate that dietary tannins have a general health-promoting effect in fishes, although it may vary with the concentration employed. Indeed, dietary chestnut tannin supplementation had the highest efficacy on growth performance, innate immunity parameters, and antioxidant defenses in juvenile beluga sturgeon, *Huso huso* (Safari et al., 2020), Nile tilapia, *Oreochromis niloticus* (Van Doan et al., 2020), common carp, *Cyprinus carpio* (Jahazi et al., 2020), and convict cichlid, *Amatitlania nigrofasciata* (Hoseinifar et al., 2020) at a concentration of 2 g kg−1; while diets supplemented with 10, 20, or 30 g kg−1 of tannic acid resulted in a decrease in growth parameters in juvenile European seabass, *Dicentrarchus labrax L.* (Omnes et al., 2017).

The exploitation of inedible parts of plants, rich in tannins, such as wood, is a successful strategy to obtain low-cost and sustainable highly valuable polyphenols and to this purpose, zebrafish (*Danio rerio*) is a well-established multipurpose biomedicine and aquaculture research model organism (Goldsmith and Jobin, 2012; Aleström and Winther-Larsen, 2016; Ogi et al., 2021; Licitra et al., 2021a). Zebrafish (*D. rerio*) is nowadays successfully employed for aquaculture studies aiming at assessing the relationship between nutrition and health (Ulloa et al., 2014; Watts et al., 2017; Fronte et al., 2021). Moreover, the zebrafish (*D. rerio*) model enables lower research cost (Ulloa et al., 2014; Fronte et al., 2021) and possess a well-known digestive system from the morpho-functional and microbiome point of view (Roeselers et al., 2011; Brugman, 2016). On these bases, the aim of the present study was to investigate the effect of a commercially available blend of sensory flavouring additives (Silvafeed® TSP), rich in tannins, extracted from chestnut and quebracho wood, on fish intestinal health status. This study has been the first to investigate the effects of increasing TSP contents in diets rich in plant products, on gut morphology and inflammation, cytokines gene expression and microbiota composition in zebrafish (*D. rerio*).

Materials and Methods

Ethics Statement

The study was performed in accordance with the European Union (EU) Directive 2010/63/EU for animal experiments and upon approval of Italian Authority for Animal Care and Use Committee (B290E.N.F7X).

Fish husbandry

The present study was carried-out at the "zebrafish facility" of the Department of Veterinary Science of the University of Pisa, Pisa (Italy). Height-month-old wild type AB strain zebrafish (*D. rerio*) were used. Fish body weight was measured at the beginning (310,0±118,28 mg; mean±s.d.) and at the end of the experimental period. Animals were maintained at 28 °C in a water recirculating system, as described by Fronte et al., 2021, according to the indications of Westerfield (2007). During the experimental period (12 days), water temperature, pH, electrical conductivity, and dissolved oxygen were daily monitored. No deaths occurred in the facilities before the euthanasia of animals used for the experiments.

Experimental design and feeding protocol

Four dietary treatments characterized by an increasing level of polyphenols were used. TSP (SilvateamS.p.A., San Michele Mondovì, Italy), a blend of HT and CT obtained from chestnut and quebracho wood, was used as polyphenol source and included into 4 terrestrial plant-based diets. The dietary treatments are shown in Table 1. The ingredients and chemical compositions of the diets were analyzed using an AOAC (2000) protocol.

Table 1: Formulation and proximate composition of the experimental diets

		control	TSP I	TSP II	TSP III
Ingredient		**%**	**%**	**%**	**%**
Soybean meal 48		43,00	43,00	43,00	43,00
Corn meal		22,00	22,00	22,00	22,00
Corn gluten		15,00	15,00	15,00	15,00
Wheat Gluten		5,20	5,11	5,03	4,86
Rapeseed meal		5,00	5,00	5,00	5,00
Dicalcium phosphate		3,40	3,40	3,40	3,40
Binder (guar gum)		2,20	2,20	2,20	2,20
Soybean oil		2,00	2,00	2,00	2,00
Choline chloride		1,30	1,30	1,30	1,30
Vit & Min Premix[a]		0,50	0,50	0,50	0,50
Sodium propionate		0,10	0,10	0,10	0,10
L-Lysine		0,10	0,10	0,10	0,10
L-Threonine		0,10	0,10	0,10	0,10
DL-Methionine		0,10	0,10	0,10	0,10
TSP		0,00	0,09	0,17	0,34
Total		**100,0**	**100,0**	**100,0**	**100,0**
Proximate composition		**As fed**			
Dry Matter	%	96,11	96,1	96,08	96,06
Crude protein	%	36,95	36,88	36,81	36,67
Crude fat	%	4,53	4,53	4,53	4,53
Fiber	%	3,59	3,59	3,59	3,59
Starch	%	18,65	18,64	18,64	18,62
Ash	%	4,99	4,98	4,98	4,98
Gross Energy	MJ/kg	17,05	17,03	17,01	16,97

[a] Vitamin and mineral premix (kg of product): vitamin A = 1,200,000 IU; vitamin D3 = 200,000 IU; vitamin E = 12,000 mg; vitamin K3 = 2,400 mg; vitamin B1 = 4,800 mg; vitamin B2 = 4,800 mg; vitamin B6 = 4,000 mg; vitamin B12 = 4,800 mg; folic acid =1,200 mg; calcium pantothenate =12,000 mg; biotin =48 mg; nicotinic acid =24,000 mg; Mn =4.000 mg; Zn =6.000 mg; I = 20 mg; Co =2 mg; Cu =4 mg; and Se =20 mg.

The different feeds were prepared as described by Royes and Chapman (2003) and Fronte et al. (2021). Briefly, raw ingredients and TSP were ground, homogenized in a mixer, moisturized, pelletized, and dried in a forced air oven (40 °C; 24 h). After the drying process, the diets were standardized into convenient pellet size (400–600 μm) and stored at 4 °C for further use. The diets were then supplied to the four dietary groups (n= 21 fish per group) for a total of 12 days. The TSP feed supplementation was calculated for ensuring the administration of 12, 24 and 48 μg/fish/day for TSP group I, II and III, respectively. To this purpose, the voluntary fish feed intake was measured during a two-week adaptation period, when control diet only was supplied to all the fishes (4,5% of the BW on average). Experimental feeds were distributed four times per day (8:00 a.m., 11:00 a.m., 2:00 p.m. and 5:00 p.m.) ad libitum, according to the "five minutes" rule, as described by Lawrence (2007). After the trial, zebrafish (*D. rerio*) were fasted overnight and sacrificed by overdose of anesthesia (0.25 mg/mL, MS-222, Sigma©, St. Louis, MO, USA). For each treatment, fish intestines were collected: six for histological and immunohistochemical analysis, five for inflammatory factors analysis and ten for microbiome analysis.

Histological analysis

The intestine samples were processed as reported in Orso et al. (2021). Specifically, tissues were fixed for 24hrs at 4°C in 4% formalin in 0.01 M phosphate-buffered saline (PBS) pH 7.4., dehydrated in a graded series of ethanol, cleared with xylene and embedded in paraffin. Embedded samples were cut with a microtome (Leica Microsystems, Wetzlar, Germany) into 5µm sections and anatomically comparable sections of intestine were deparaffinized with xylene and stained with haematoxylin-eosin (H&E) for the morphological analysis or Alcian blue for the quantitative estimation of the goblet cell number as reported by Orso et al. 2021. The stained intestinal sections were examined under a Leica DMI6000 light microscope equipped with Leica DFC340 digital camera (Leica Microsystems, Wetzlar, Germany) at 10X or 20X magnification. Score and goblet cells quantification were performed as previously described in Orso et al. (2021).

Immunohistochemical analysis

The avidin-biotin immunohistochemical technique was performed as reported in Imperatore et al. (2020). Briefly, anatomically comparable sections of MI were deparaffinized and prepared to be stained with a monoclonal antibody raised in mouse against tumor-necrosis factor-α (TNFα) (code ab1793, Abcam, Cambridge, UK) or a polyclonal antibody raised in rabbit against cyclooxygenase 2 (COX2) (code 69720, NovaTeinBio, Woburn, MA). Briefly, sections were incubated with 0.1% H_2O_2 for 5 min to inactivate the endogenous peroxidase activity, followed by incubation with 10% normal goat serum (NGS) (Vector Laboratories, UK) in 0.1 M Tris-buffered saline, pH 7.6, containing 0.3% Triton X-100 for 30 min. Subsequently, sections were incubated with primary antibodies (1:200 in the NGS) overnight at 4°C. The day after, the sections were rinsed several times and then incubated for 2h, at room temperature, in biotinylated goat anti-mouse or goat anti-rabbit immunoglobulin with appropriate dilution (Vector Laboratories). The slides were incubated for 1h with the avidin-biotin complex diluted in Tris-buffered saline (ABC Kit; Vectastain, Vector) and then with 0.05% of 3'-diaminobenzidine (DAB) for 10 min (DAB Sigma Fast, Sigma-Aldrich). The antibodies specificity was validated with controls as reported in Imperatore et al. (2018; 2020). A Leica DMI6000 light microscope (Leica Microsystems, Germany) equipped with a digital camera working on gray levels (JCV FC 340FX, Leica) was used to acquire digital images under constant light illumination at 20x and 40x magnification.

RNA isolation, cDNA synthesis and Real-Time PCR

Total RNA was extracted from zebrafish (*D. rerio*) intestines using the Quick RNA miniprep (ZymoResearch, Irvine, USA) according to the manufacturer's instruction. cDNA was synthesized by reverse transcription of about 500 ng of total RNA using PrimeScript™ RT Reagent kit (Takara Bio Inc., Shiga, Japan), and quantitative real-time polymerase chain reaction (qRT-PCR) was performed in qPCRBIO SyGreen Mix Hi-ROX (PCR Biosystem, Wayne, NJ, USA), according to Licitra et al. (2021b). The sequences of the primers used are listed in **Supplementary Table S1**. Relative expression levels of the gene were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The results obtained were normalized to the expression of the housekeeping gene, β-actin (ENSDARG00000037746). The analysis of gene expression was calculated with fold change method. Each assay was done in triplicate and 5 samples per group were analyzed.

Table 2: Primer sequences for qRT-PCR

Gene	Sequence 5'-3'	Reference
beta actin	F: GCAGAAGGAGATCACATCCCTGGC	https://doi.org/10.3390/nu13030998
	R: CATTGCCGTCACCTTCACCGTTC	
cox2	F: CCCTGTCAGAATCGAGGTGT	https://doi.org/10.1038/nchembio.147
	R: TTGGGAGAAGGCTTCAGAGA	
il1b	F: GGACTTCGCAGCACAAAATGAA	https://doi.org/10.1101/2020.04.09.033837
	R: TTCACTTCACGCTCTTGGATGA	
cxcl8-l1	F: GTCGCTGCATTGAAACAGAA	https://doi.org/10.4049/jimmunol.1203266
	R: CTTAACCCATGGAGCAGAGG	
tnfa	F: GGGCAATCAACAAGATGGAAG	https://doi.org/10.1038/s41598-018-28511-w
	R: GCAGCTGATGTGCAAAGACAC	

Microbiome analysis

Total DNA was extracted and analyzed from individual whole intestine sampled from 6 fish for each treatment, as reported in Pelusio et al. (2021). Extracted DNA was quantified with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at –20°C until further processing. The amplification, purification and sequencing of the V3-V4 hypervariable regions of the 16S rRNA gene was performed as described in paragraph 2.2. Sequencing was then performed on Illumina MiSeq platform using a 2 × 250 bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA). At the end of the sequencing process, raw sequences were processed as described in paragraph 2.2. A p-value ≤ 0.05 was considered statistically significant, while p-values between 0.05 and 0.1 were considered as a trend.

Statistical analysis

Data related to the fish growth performance and score number were analyzed using One-way ANOVA and differences between groups tested by mean HSD Tukey-Kramer test (α=0.05). The data related to qRT-PCR were first analyzed with the Shapiro-Wilks test to evaluate the normality distribution. Post hoc comparisons were performed using the one-way ANOVA. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., CA, USA) and differences between treatments were considered significant for p≤0.05 (*), p≤0.01 (**), p≤0.001 (***) and p≤0.00001 (****).

Results

Growth performance

During the experimental period, all fish grew normally, and no statistically significant differences were observed between treatments, neither for the initial nor for the final BW (table 2).

Table 3: Initial and final fish BW (mg), according to the considered treatments

Parameters	**Initial BW (mg)**		**Final BW (mg)**		**BW increment**
Treatments	mean	SD	mean	SD	
Control	291,0	116,93	350,1	117,63	20,3%
TSP 12	316,9	108,85	375,1	140,84	18,4%
TSP24	307,2	126,57	372,3	120,54	21,2%
TSP 48	318,6	121,84	382,9	157,84	20,2%
SEM	14,617		16,427		
P	0,9021		0,9031		

Intestinal histology

Intestinal inflammation was morphologically detectable in zebrafish (*D. rerio*) fed with control diet showing damage of the intestinal villi and disruption of the intestinal tissue integrity (**Fig. 1**). Specifically, the intestine showed clear signs of inflammation, such as expansion of gut lumen, irregular intestinal folds (villi) with loss of margins, abundant mucus presence, high goblet cells number and leukocyte infiltrates, loss of lamina propria integrity and accumulation of fat in the submucosa layer. Diets enriched with TSP partially prevented the morphological alterations in a dose-dependent manner. Indeed, intestine sections of zebrafish (*D. rerio*) fed a diet containing 12 µg/day (TSP I) and 24 µg/day (TSP II) of TSP kept showing an altered morphology of the villi, abundance of goblet cells, loss of integrity of lamina propria and fat accumulation in the submucosa layer. In zebrafish (*D. rerio*) fed TSP at 48 µg/day (TSP III), the integrity of the villi and lamina propria were preserved and a reduction of goblet cells and fat accumulation was observed (**Fig. 1 and 2**). Specifically, significant reduction of score number was found only in the TSP II ($p<0.001$) and TSP III ($p<0.0001$) groups compared with the control group; while the number of goblet cells number decreased significantly only in TSP III ($p<0.05$) compared with the zebrafish (*D. rerio*) group fed with control diet.

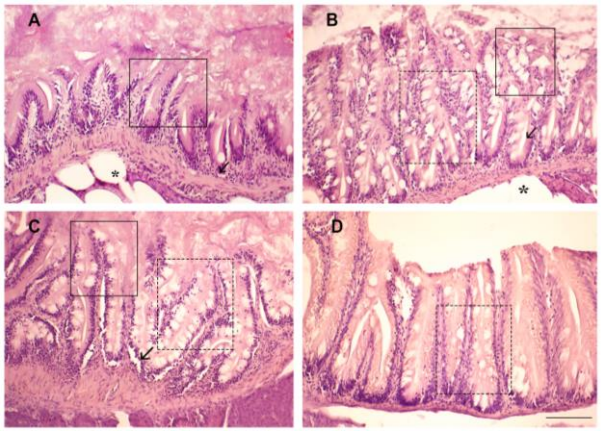

Figure 1: Haematoxylin-eosin (H&E) staining of mid intestine (MI) of (A) zebrafish fed control diet, (B) TSP I, zebrafish fed control diet supplemented with 12 µg/d di TSP, (C) TSP II, zebrafish fed control diet supplemented with 24 µg/d di TSP, (D) TSP III, zebrafish fed control diet supplemented with 48 µg/d di TSP. Scale bar: 100 µm. Arrows indicate loss of lamina propria integrity, linear boxes indicate loss of villi integrity, dashed boxes depict goblet cells, asterisks indicate submucosal fat accumulation.

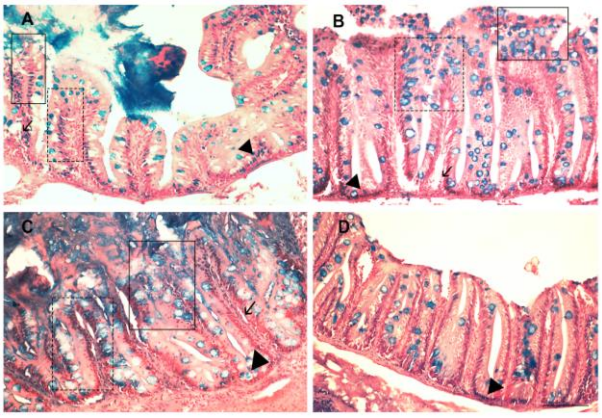

Figure 2: Alcian Blu staining of mid intestine (MI) of (A) zebrafish fed control diet, (B) TSP I, zebrafish fed control diet supplemented with 12 µg/d di TSP, (C) TSP II, zebrafish fed control diet supplemented with 24 µg/d di TSP, (D) TSP III, zebrafish fed control diet supplemented with 48 µg/d di TSP. Scale bar: 100 µm. Arrows indicate loss of lamina propria integrity, linear boxes indicate loss of villi integrity, dashed boxes depict goblet cells, arrowheads indicate leukocyte infiltrates.

Morphological alterations visible in the zebrafish (*D. rerio*) fed with control diet matched with the increased expression of the pro-inflammatory marker TNFα. TNFα immunoreactivity increased in the enteroendocrine and goblet cells and many infiltrated cells, such as eosinophils and fibroblasts, showed positivity for TNFα, highlighting the onset of an inflammatory state. TSP treatment partially counteracted such increase when added to the diet at high concentrations (**Fig. 3**). Specifically, intestine of zebrafish (*D. rerio*) fed diet supplemented with 12 µg/d and 24 µg/d of TSP showed intense TNFα immunoexpression along the villi and in both infiltrates and epithelial cells, demonstrating an intense overt inflammatory state. On the contrary, the intestine of zebrafish (*D. rerio*) fed with diet containing 48µg/d of TSP showed a significant reduction in the immune expression of TNFα which was found in a few infiltrated cells, indicating an active immune response but the absence of a real inflammatory state.

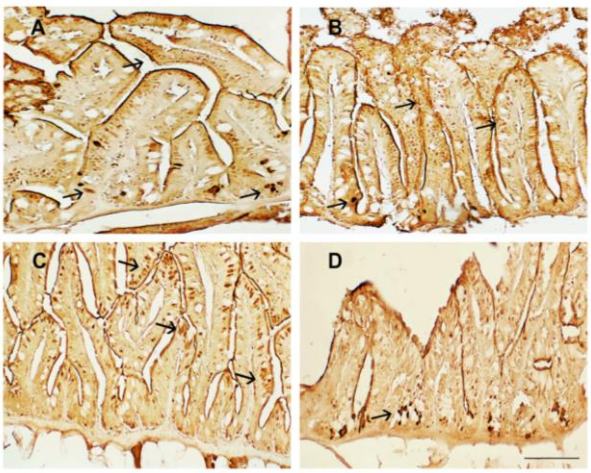

Figure 3: TNFα immunostaining in the mid intestine (MI) of (A) zebrafish fed control diet, (B) TSP I, zebrafish fed control diet supplemented with 12 µg/d di TSP, (C) TSP II, zebrafish fed control diet supplemented with 24 µg/d di TSP, (D) TSP III, zebrafish fed control diet supplemented with 48 µg/d di TSP. Scale bar: 100 µm. Arrows indicate epithelial cells or infiltrated eosinophils and fibroblasts expressing TNFα.

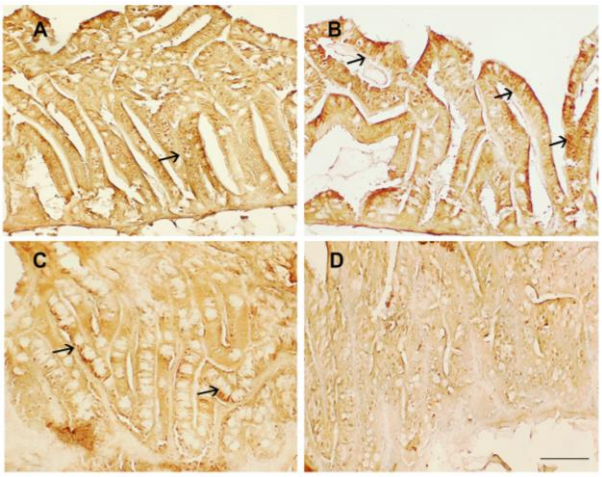

Figure 4: COX2 immunostaining in the mid intestine (MI) of (A) zebrafish fed control diet, (B) TSP I, zebrafish fed control diet supplemented with 12 µg/d di TSP, (C) TSP II, zebrafish fed control diet supplemented with 24 µg/d di TSP, (D) TSP III, zebrafish fed diet enriched with 48 µg/d di TSP. Scale bar: 100 µm. Arrows indicate COX2 expression at apical side of epithelial cells.

COX2 immunoexpression was found largely confined to the villus epithelium (**Fig. 4**). In particular, the epithelial cells of zebrafish (*D. rerio*) fed commercial diet showed COX2 immunoreactivity at the apical side of epithelial cells, showing a slight presence of oxidative stress. In zebrafish (*D. rerio*) fed a diet containing 12µg/d of TSP, an intense immunoexpression of COX2 along the villi on the apical layer of the epithelial cells was observed, highlighting an intense oxidative stress. However, the increase of TSP concentration in the diet was accompanied with a decrease of oxidative stress. In fact, COX2 expression was markedly reduced in zebrafish (*D. rerio*) fed with diet containing 24 µg/d of TSP, and almost disappeared in zebrafish (*D. rerio*) fed with diet containing 48 µg/d of TSP, indicating the absence of oxidative stress.

Inflammatory factors analysis

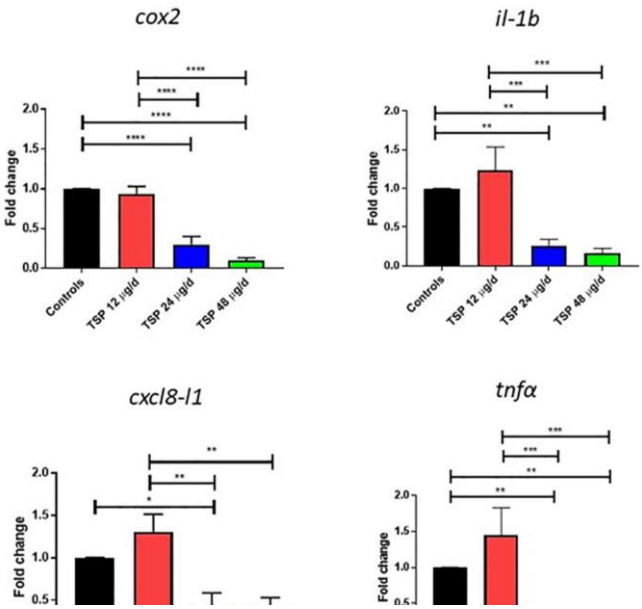

Figure 5: Relative mRNA expression of inflammatory mediators in intestine of zebrafish fed with control diet (black column) and control diet supplemented with 12 µg/d (TSP I), 24 µg/d (TSP II) and 48 µg/d (TSP III) of TSP. In the graph are reported only the significant differences. Error Bars represent Standard Error on the Mean (SEM). For statistical tests One-Way ANOVA was used.

The inflammatory factors analysis on zebrafish (*D. rerio*) intestines fed with increase concentration of TSP showed a dose dependent-manner response on oxidative stress. The inflammatory factors analyzed were *cox2*, *il-1b*, *cxcl8-l1*and *tnfα*. In particular, zebrafish (*D. rerio*) fed with the diet containing 12 µg/fish/day of TSP (TSP I) did not show an altered pattern of inflammatory mediators compared to control. Whereas a double and triple dosage of TSP (24 and 48 µg/fish/day for TSP II and III, respectively) reduced the expression of *cox2*, *il-1b*, *cxcl8-l1* and *tnfα* up to half compared to control group. This trend depicted a decreased oxidative stress related to TSP administration at doses higher than 24 µg/fish/day (**Fig. 5**).

Fecal Bacterial Community Profile

The 16S rRNA gene sequencing was performed on a total of 24 whole intestine samples, yielding 114,329 high-quality reads (mean ± SD, 4764 ± 2719) and clustered into a total of 334 ASVs. To assess whether the increasing treatment with TSP feed integrator could exert a beneficial effect on gut bacteria community during inflammatory events, the gut microbiome (GM) was analyzed for each dietary group. The variations in the GM profiles (beta-diversity) were assessed by the Principal Coordinate Analysis (PCoA) of the unweighted UniFrac distances calculated between samples. In addition, for each dietary group changes in the gut microbial community internal diversity were represented with three different metrics: PD_whole_tree, Chao1 index and observed_ASVs. According to our findings (**Figure 6**), none of the TSP® groups, showed a significant variation in the overall GM composition compared to control group, in terms of overall composition structure ("Adonis", $p > 0.05$) (**Figure 6A, B, C**). However, the TSP III group showed a higher p-value (Adonis) compared to the other TSP dietary groups, highlighting that bacterial community in TSP III group was more similar to the control group. Conversely, focusing on the microbial internal ecosystem diversity, the diet containing 24 ug/d of TSP determined a significant positive effect (Wilcoxon rank-sum test, $p < 0.05$). Indeed, we observed a higher value of internal ecosystem diversity in TSP II group, for all metrics (PD_whole_tree, Chao1, observed_ASVs), as compared with the control (**Figure 6D**).

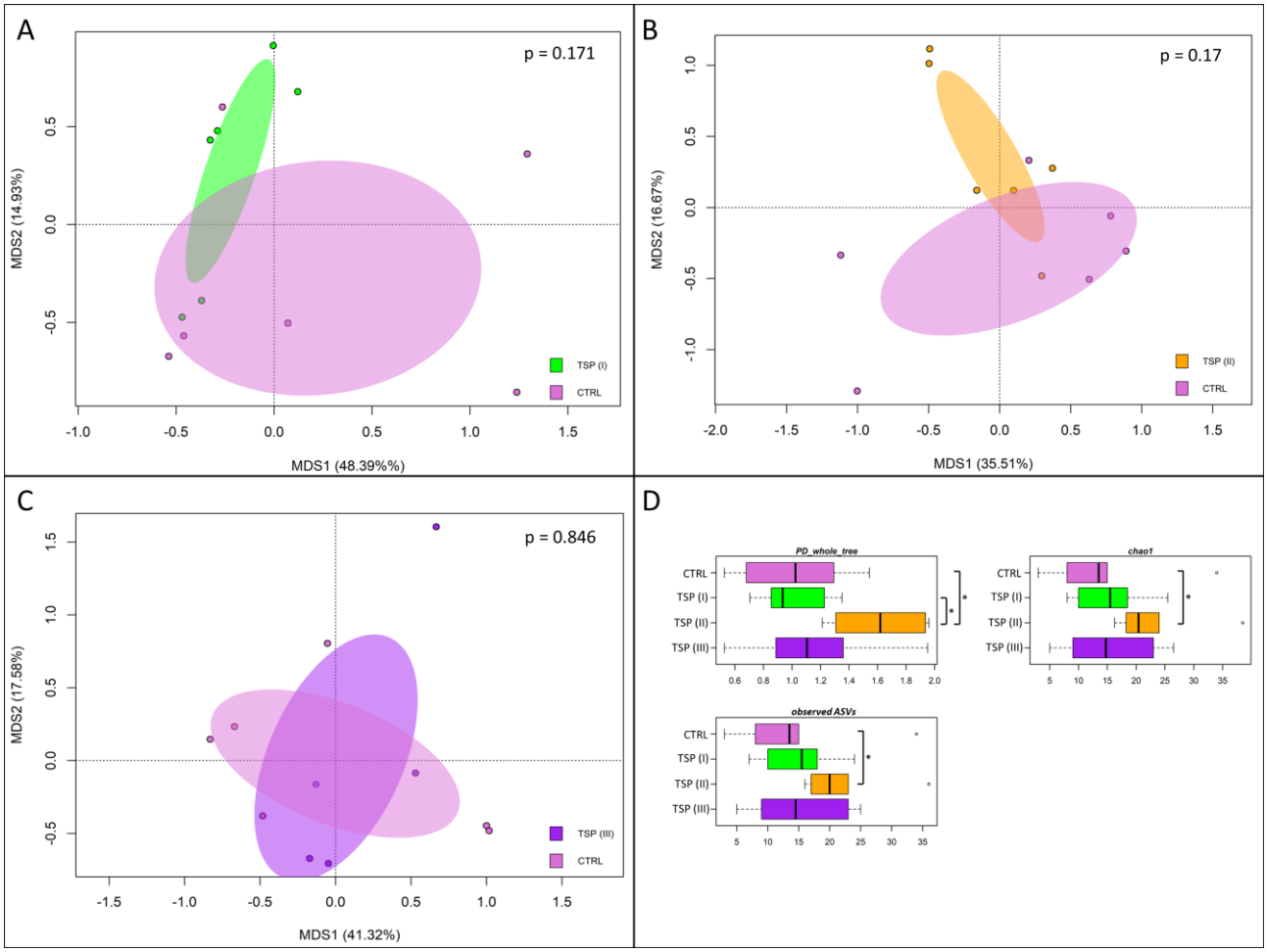

Figure 6: Beta diversity and alpha diversity of gut microbiota of zebrafish fed with the experimental diets over 12 days. PCoA based on unweighted UniFrac distances between gut microbiota structure of animals fed with control diet (CTRL) and zebrafish fed with control diet supplemented with 12 µg/d of TSP (TSP I) (A), with 24 µg/d of TSP (TSP II) (B), and with 48 µg/d of TSP (TSP III) (C). In all PCoA plots samples are not significantly separated (permutation test with pseudo-F ratio, $p>0.05$). (D) Boxplots of alpha diversity, measured with Faith's Phylogenetic Diversity (PD_whole_tree), Chao1 index, and observed_ASVs. Only for TSP II group a higher value of alpha diversity in all metrics is observed, compared to control group (Wilcoxon rank-sum test, $*p \leq 0.05$).

In order to further assess the GM composition of zebrafish (*D. rerio*) fed at different concentrations of TSP feed integrator (i.e., CTRL, TSP(I), TSP(II), TSP (III)), phylogenetic composition was assessed at phylum and genus levels, as highlighted in **Fig. 7A and 7B** respectively. Overall, the GM of each group showed a similar profile in terms of the most abundant bacterial taxa. More specifically, the most abundant phyla were Firmicutes, Fusobacteria and Proteobacteria, which represented about 94% of the whole intestinal bacterial ecosystem (**Fig. 7A**). On the other hand, the most represented genera were *Cetobacterium*, belonging to Fusobacteria phylum, *Plesiomonas* and *Sphingomonas*, belonging to Proteobacteria phylum and *Lactobacillus*, belonging to Firmicutes phylum (**Fig. 7B**).

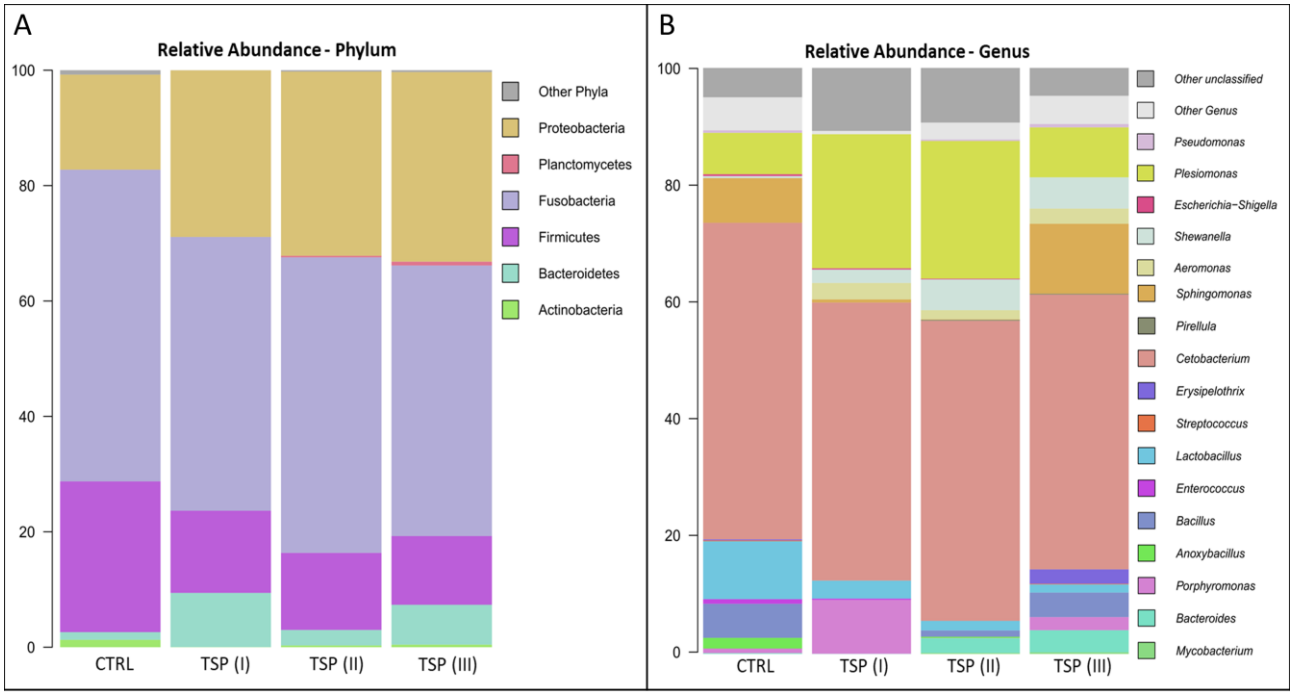

Figure 7: Microbiome composition of the whole intestine of zebrafish fed with the experimental diets over 12 days. Bar plot summarizing the microbiota composition at phylum (A) and genus level (B) of fish intestinal content. Only phyla with a relative abundance ≥ 0.5% in at least 2 samples and genera with relative abundance ≥ 0.5% in at least 2 samples are represented. CTRL, zebrafish fed with control diet; TSP I, zebrafish fed with control diet supplemented with 12 µg/d of TSP; TSP II, zebrafish fed with control diet supplemented with 24 µg/d of TSP; TSP III, zebrafish fed with control diet supplemented with 48 µg/d of TSP.

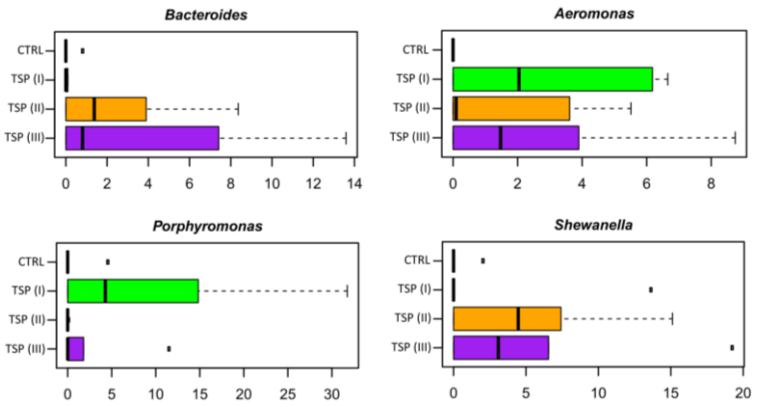

Figure 8 Distributions of relative abundance of genera that showed a tendency of variation between groups fed with different experimental diets (Wilcoxon rank-sum test, $p < 0.1$). Only genera with a mean relative abundance ≥ 0.5% in at least one sample were taken into account. The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line. CTRL, zebrafish fed with control diet; TSP I, zebrafish fed with control diet supplemented with 12 µg/d of TSP; TSP II, zebrafish fed with control diet supplemented with 24 µg/d of TSP; TSP III, zebrafish fed with control diet supplemented with 48 µg/d of TSP.

No statistically significant differences (Wilcoxon rank-sum test; $p > 0.05$) were detected between the dietary groups for the GM composition at genus level. However, the paired statistical analysis performed between each dietary group showed several tendencies of variations at the genus taxonomic level. More specifically, *Bacteroides* genus appeared to be more abundant in both TSP (II) and TSP (III) groups compared to control group (Wilcoxon rank-sum test; $p < 0.1$). Similarly, the abundance of *Aeromonas* genus was higher in all TSP groups compared to fish fed with control diet (Wilcoxon rank-sum test; $p < 0.1$). In addition, *Porphyromonas* and

Shewanella genera appeared to be more abundant in TSP groups, with the former more abundant in TSP (I) group compared to control group and the latter more abundant in TSP (II) group compared to fish fed with control diet (**Fig. 8**).

Discussion

In this study, zebrafish (*D. rerio*) was used as a model to investigate the effects of a tannin rich feed additive (Silvafeed® TSP) on fish intestinal health status, notably gut morphology and inflammation, cytokines gene expression and microbiota composition.

As previously reported in zebrafish (*D. rerio*) (Arias Jayo et al., 2018) and other fish species such as common carp (*Cyprinus carpio*) (Urán et al., 2008), and Atlantic salmon (*Salmo salar*) (Kroghdal et al., 2003), intestinal morphological alterations may occur due to the diet composition, such as gut lumen expansion, irregular intestinal villi with loss of margins, abundant mucus presence, negative variation in goblet cell number and leukocyte infiltrates, loss of lamina propria integrity and accumulation of fat in the submucosa layer (Torrecillas et al., 2007; Torrecillas et al., 2014; Fronte et al 2019).

It is very well-known that polyphenolic compounds have anti-inflammatory and antioxidant properties *in vitro* (Sorice et al., 2016), *ex-vivo* (Coccia et al., 2019), and *in vivo* (Orso et al., 2021) as well. It has been demonstrated that the addition of chestnut tannins (Sieniawska and Baj, 2017) is able to improve the general morphology of the intestine, restoring the intestinal structure and the organization of the villi, almost to normal feature. Recently, Orso et al. (2021), reported the morphological and functional recovery of k-carrageenan-induced intestinal inflammation in zebrafish (*D. rerio*) after treatment with 4 µg of chestnut tannins/fish/day. In the present study, the ameliorative effect of tannins was observed at higher concentrations (between 24 and 48 µg/fish/day), which could be due to various reasons. First, the severity of the basal inflammation must be considered. Based on a classification of inflammation symptoms in zebrafish (*D. rerio*) reported by Orso et al. (2021), the intensity of inflammation was more severe in this study, where the loss of integrity of the villi was particularly evident in the control group fed the feed rich in plants ingredients. Furthermore, the duration of the pro-inflammatory stimulus was longer in the present study, in which the specimens were fed the pro-inflammatory control feed for twelve days, while in the Orso et al. (2021), the inflammatory stimulus lasted three days only.

Polyphenols are powerful antioxidants that neutralize free radicals by donating an electron or hydrogen atom, contrasting the oxidative stress occurring in the cells when there is an excess of free radicals (Vladimir-Knežević et al., 2012; Hussain et al., 2016). Although free radicals are produced during normal metabolic processes, an excess of oxidative stress can activate a variety of transcription factors, which lead to the differential expression of genes involved in inflammation (Chen et al., 2006; Yu et al., 2016). Scientific research suggests that polyphenols exert their protective and therapeutic effects in the management of intestinal inflammation via down-regulation of pro-inflammatory and upregulation of anti-inflammatory cytokines and thus suppressing inflammatory pathways and their cellular signaling mechanisms (Santangelo et al., 2007; Fiesel et al., 2014).

The gastrointestinal tract of vertebrates performs important functions beyond those associated with the absorption of nutrients. These include defense, which is perhaps one of the most important functions. In fish the intestine acts both as a physical barrier to the entry of pathogens and as an immune barrier, thanks to the presence of the so-called Gut-associated lymphoid tissue (GALT), consisting of leukocyte populations located both intraepithelial and, in the lamina propria without a clear structural organization (Salinas, 2015). The present study indicated that, intestinal inflammation, is accompanied by the innate immune response which is involved and responsible for the increase in the number of leukocytes, as seen in this study. Leukocytes contribute to the recruitment of other immune cells and facilitate mucosal

healing by releasing molecules such as cytokines crucial for the orchestration of the defense response (Wang and Secombes, 2013).

In the present study, the inflammation status induced by the plant-based diet, characterized by intestinal morphological alterations and the increase of pro-inflammatory markers TNF-α and COX2, were ameliorated only in zebrafish (*D. rerio*) fed with TSP at 48 μg/fish/day. The study of gene expression of the inflammatory factors (*cox2, il-1b, cxcl8-l1* and *tnfa*) confirmed the beneficial effects of TSP on inflamed intestine. Indeed, the inflammation was reduced when TSP was administered at doses equal to 24 and 48 μg/fish/day. Worth of particular attention is the expression of TNF-α in the TSP II group (24 μg/fish/day), where the mRNA is reduced while the protein is highly immunoexpressed. The higher sensitivity of qRT-PCR compared to immunohistochemical analysis (Peinnequin, 2004) could justify this contradictory outcome. Moreover, there is a correlation between the expression of these inflammatory factors, because at sites of inflammation, in response to inflammatory stimuli, the inflammatory cells produce proinflammatory cytokines such as interleukin-1α/β, interferon-γ and tnf-α that stimulate the production of *cox2* (Wang, 2010).

In fact, besides the innate and adaptive immune components, also cytokines play vital roles in the intestinal immune function of fish. Research indicated that up-regulating the mRNA levels of pro-inflammatory cytokines (as *IL-1β* and *TNF-α*) could worsen the inflammatory responses of fish (Wang and Secombes, 2013). In the intestine of growing grass carp (*Ctenopharyngodon idella*), the majority of studied pro-inflammatory cytokines (*IFN-γ2, IL-1β, -6, -12p35, -12p40, -15 and -17D*) were significantly up-regulated when condensed tannins were added to the diet at levels higher than 30 g/kg, while the same condensed tannin levels had no significant impact on the relative mRNA level of *IL-8* and *TNF-α* (Li et al., 2020). Conversely, dietary inclusion equal to 0.1% of condensed tannins, mitigate the oxidative stress and maintain intestinal health in the spotted sea bass (*Lateolabrax maculatus*) (Peng et al., 2022). Recently, the quantitative analysis of the relative gene expression of *TNFα, COX-2, IL-1β* and *IL-8* was analyzed in the intestines of zebrafish (*D. rerio*) fed with chestnut shell tannins at doses equal to 4 μg/fish/day, suggesting that these tannins are able to reduce the analyzed pro-inflammatory factors, ameliorating the fish intestinal inflammation status (Orso et al., 2021), in line with our results. Similarly, another study on the effects of polyphenols on a model of zebrafish (*D. rerio*) intestinal inflammation, showed that, these compounds can counteract a large number of cytokines (*IL-4, IL-10,* and *TNFα*) induced by inflammation (Gong et al., 2020). Therefore, these results support the hypothesis that the supplementation of TSP obtained from chestnut wood, ameliorated the inflammatory responses in the intestinal tissue of zebrafish (*D. rerio*).

Evidences have been accumulated on the existence of an interplay between polyphenols and intestinal microbiota. The intestinal microbiota plays an important role in the metabolism of polyphenols (Kawabata et al., 2019), contributing to the degradation of high molecular weight tannins into various phenolic products of low molecular weight that can be easily absorbed (Goel et al., 2005)

In this study, the metagenomics analysis was in agreement with the report that the phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Fusobacteria are dominant in fish gut microbiota (Larsen et al., 2014; Eichmiller et al., 2016). Literature studies report that zebrafish (*D. rerio*) intestinal microbiota is dominated by members of the phyla Fusobacteria, Firmicutes and Proteobacteria (Roeselers et al., 2011; Da Silva et al., 2020), which represent most phyla found in the zebrafish (*D. rerio*) of this study. Fusobacteria are identified as the most abundant in the gut of adult zebrafish (*D. rerio*) and major contributors to the gut microbiome of commercial fish feed (Stephens et al., 2016; Walburn et al., 2019). In agreement with these data, the metagenomic analysis of this study showed that our control group fed a diet rich in plant ingredients, had Fusobacteria as the predominant phylum, followed by Firmicutes and Proteobacteria. Interestingly, Orso et al. (2021) report Fusobacteria <5% in zebrafish (*D. rerio*) fed on *Artemia salina*.

The Fusobacteria are anaerobic, Gram-negative bacilli that produce the short-chain fatty acid butyrate (Bennett and Eley 1993). In mammals, butyrate provides many benefits to the host, enhancing mucus production, thus acting as an anti-inflammatory (Andoh et al. 1999; Hamer et al. 2008). This outcome can explain the abundance of mucus lining the intestinal mucosa in all zebrafish (*D. rerio*) groups of this study. However, Fusobacteria have also been reported as the most abundant phylum in adult zebrafish (*D. rerio*) with intestinal inflammation (Orso et al., 2021). Similarly, in this study the control group shows morphological signs of intestinal inflammation, partially reversed by the administration of tannins which caused a slight decrease in Fusobacteria, a result similar to that reported by Orso et al. (2021). Moreover, most of the Fusobacteria in freshwater herbivorous fish species is mainly represented by the genera of Cetobacterium (Larsen et al., 2014), an outcome in agreement with our data. Interestingly, the abundance of genus *Shewanella* was slightly increased in the group fed with high concentration of TSP (48 μg/fish/day) compared to control group. Some *Shewanella* species can act as fish health modulator thanks to their potential probiotic activity (Cámara-Ruiz et al., 2020), so a higher presence of this genus after tannins treatment could be evaluated as a benefic effect for the intestinal gut bacterial community, which could lead to a healthier fish gut microbiota after an inflammatory event.

Conclusions

Based on the results of the present study, it is possible to conclude that tannins may play a relevant role in counteracting the negative effects of fishmeal replacement with plant ingredients in fish diets. In fact, positive effects have been observed at several level (gut histology and immunohistochemistry, inflammatory factors, and microbiome). Hence, in the process of improving aquaculture sustainability by reducing the use of fishmeal in aquafeed, the inclusion of tannins in fish diets may be helpful to maintain fish performance, fish health and reducing the cost of aquafeed, so that improving farmers and aquafeed industries economical results.

References

Ahmadifar, E., Yousefi, M., Karimi, M., Raieni, R.F., Dadar, M., Yilmaz, S., Dawood, M.A.O., Abdel-Latif, H.M.R., 2020. Benefits of Dietary Polyphenols and Polyphenol-Rich Additives to Aquatic Animal Health: An Overview. Rev. Fish. Sci. Aquac. 1-34. https://doi.org/10.1080/23308249.2020.1818689.

Ahmadifar, E., Pourmohammadi Fallah, H., Yousefi, M., Dawood, M.A.O., Hoseinifar, S.H., Adineh, H., Yilmaz, S., Paolucci, M., Doan, H.V., 2021. The Gene Regulatory Roles of Herbal Extracts on the Growth, Immune System, and Reproduction of Fish. Animals. 11, 2167. https://doi.org/10.3390/ ani11082167.

Aleström, P., Winther-Larsen, H.C., 2016. Zebrafish offer aquaculture research their services. Genomics in Aquaculture, 165–194. https://doi.org/10.1016/B978-0-12-801418-9.00007-X.

Andoh, A., Bamba, T., Sasaki, M., 1999. Physiological and anti-inflammatory roles of dietary fiber and butyrate in intestinal functions. JPEN J Parenter Enteral Nutr. Sep-Oct;23(5 Suppl):S70-3. doi: 10.1177/014860719902300518.

Arias-Jayo, N., Abecia, L., Alonso-Sáez, L., Ramirez-Garcia, A., Rodriguez, A., Pardo, M.A., 2018. High-Fat Diet Consumption Induces Microbiota Dysbiosis and Intestinal Inflammation in Zebrafish. Microb. Ecol. 76, 1089–1101. https://doi.org/10.1007/s00248-018-1198-9.

Bennett, K.W., Eley, A., 1993. Fusobacteria: New taxonomy and related diseases. J. Med. Microbiol. 39(4):246-54. https://doi.org/10.1099/00222615-39-4-246.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., et al., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37 (8), 852–857, https://doi.org/10.1038/s41587-019-0209-9.

Bravo-Tello, K., Ehrenfeld, N., SolõÂs, C.J., Ulloa, P.E., Hedrera, M., Pizarro-Guajardo, M., 2017. Effect of microalgae on intestinal inflammation triggered by soybean meal and bacterial infection in zebrafish. PLoS ONE 12(11): e0187696. https://doi.org/10.1371/journal.pone.0187696.

Brogi, L., Marchese, M., Cellerino, A., Licitra, R., Naef, V., Mero, S., Bibbiani, C., Fronte, B., 2021. β-Glucans as Dietary Supplement to Improve Locomotion and Mitochondrial Respiration in a Model of Duchenne Muscular Dystrophy. Nutrients. 13, 1619. https://doi.org/10.3390/nu13051619.

Brugman, S., 2016. The zebrafish as a model to study intestinal inflammation, Dev Comp Immunol. http://dx.doi.org/10.1016/j.dci.2016.02.020.

Bule, M., Khan, F., Nisar, M.F., Niaz, K., 2020. Tannins (hydrolysable tannins, condensed tannins, phlorotannins, flavono-ellagitannins). In book: Recent Advances in Natural Products AnalysisPublisher: Elsevier.

Buzzini, P., Arapitsas, P., Goretti, M., Branda, E., Turchetti, B., Pinelli, P., Ieri, F, Romani, A., 2008. Antimicrobial and Antiviral Activity of Hydrolysable Tannins. Mini-Rev. Med. Chem. 8(12), 1179–1187. doi:10.2174/138955708786140990.

Cai, Y., Zhang, J., Chen, N.G., Shi, Z., Qiu, J., He, C., Chen, M., 2016. Recent Advances in Anticancer Activities and Drug Delivery Systems of Tannins. Med. Res. Rev. 37(4), 665–701. https://doi.org/10.1002/med.21422.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample

inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.
Cámara-Ruiz, M., Balebona, M.C., Moriñigo, M.Á., Esteban, M.Á., 2020. Probiotic Shewanella putrefaciens (SpPdp11) as a Fish Health Modulator: A Review. Microorganism. 8, 1990. DOI: 10.3390/microorganisms8121990.
Chen, C.-J., Ou, Y.-C., Lin, S.-Y., Liao, S.-L., Chen, S.-Y., Chen, J.-H., 2006. Manganese modulates pro-inflammatory gene expression in activated glia. Neurochem. Int. 49(1), 62–71. https://doi.org/10.1016/j.neuint.2005.12.020.
Coccia, E., Imperatore, R., Orso, G., Melck, D., Varricchio, E., Volpe, M.G., Paolucci, M., 2019. Explants of *Oncorhynchus mykiss* intestine to detect bioactive molecules uptake and metabolic effects: Applications in aquaculture. Aquaculture. 506:193–204. https://doi.org/10.1016/j.aquaculture.2019.03.041.
Culhane, A.C., Thioulouse, J., Perrière, G., Higgins, D.G., 2005. MADE4: an R package for multivariate analysis of gene expression data. Bioinformatics. 1;21(11):2789-90. https://doi.org/10.1093/bioinformatics/bti394.
Da Silva, L.M.R., Lima, J.D.S.S., Magalhães, F.E.A., Campos, A.R., De Araújo, J.I.F., Batista, F.L.A., De Araújo, S.M.B., De Sousa, P.H.M., Lima, G.C., Holanda, D.K.R., Rolim, R.C., Figueiredo, R.W., Figueiredo, E.A.T., Duarte, A.S.G., Ricardo, N.M.P.S., 2020. Graviola Fruit Bar Added Acerola By-Product Extract Protects Against Inflammation and Nociception in Adult Zebrafish (Danio rerio). J. Med. Food 2020, 23, 173–180. https://doi.org/10.1089/jmf.2019.0078.
Das, A.K., Islam, M. N., Faruk, M. O., Ashaduzzaman, M., Dungani, R., 2020. Review on tannins: Extraction processes, applications and possibilities. South African Journal of Botany, 135, 58–70. https://doi.org/10.1016/j.sajb.2020.08.008.
Eichmiller, J.J., Hamilton, M.J., Staley, C., Sadowsky, M.J., Sorensen, P.W., 2016. Environment shapes the fecal microbiome of invasive carp species. Microbiome. 4:44. doi: 10.1186/s40168-016-0190-1.
Estruch, G., Collado, M.C., Monge-Ortiz, R., Tomás-Vidal, A., Jover-Cerdá, M., Peñaranda, D.S., Pérez Martínez, G., Martínez-Llorens, S., 2018. Long-term feeding with high plant protein based diets in gilthead seabream (Sparus aurata, L.) leads to changes in the inflammatory and immune related gene expression at intestinal level. BMC Vet Res. 14,302. https://doi.org/10.1186/s12917-018-1626-6.
FAO 2020. The State of World Fisheries and Aquaculture 2020. Sustainability in action. Rome.https://doi.org/10.4060/ca9229en.
Fiesel, A., Gessner, D.K., Most, E., Eder K., 2014. Effects of dietary polyphenol-rich plant products from grape or hop on pro-inflammatory gene expression in the intestine, nutrient digestibility and faecal microbiota of weaned pigs. BMC Vet Res. 10, 196. https://doi.org/10.1186/s12917-014-0196-5.
Fronte, B., Licitra, R., Bibbiani, C., Casini, L., De Zoysa, M., Miragliotta, V., Sagona, S., Coppola, F., Brogi, L., Abramo, F., 2021. Fishmeal Replacement with Hermetia illucens Meal in Aquafeeds: Effects on Zebrafish Growth Performances, Intestinal Morphometry, and Enzymology. Fishes. 6, 28. https://doi.org/10.3390/fishes 6030028.
Fronte, B., Abramo, F., Brambilla, F., De Zoysa, M., Miragliotta, V., 2019. Effect of hydrolysed fish protein and autolysed yeast as alternative nitrogen sources on gilthead sea bream (Sparus aurata) growth performances and gut morphology. Ital. J. Anim. Sci. 18, 799–808. https://doi.org/10.1080/1828051X.2019.1581584.
Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., 2007. Expanding the utilization of sustainable plant products in aquafeeds: A review. Aquac. Res. 38, 551–579. https://doi.org/10.1111/j.1365-2109.2007.01704.x.
Goldsmith, J. R., Jobin, C., 2012. Think Small: Zebrafish as a Model System of Human Pathology. J Biomed Biotechnol. 1–12. https://doi.org/10.1155/2012/817341.
Goel, G., Puniya, A.K., Aguilar, C.N., Singh, K., 2005. Interaction of gut microflora with tannins in feeds. Naturwissenschaften, 92(11), 497–503. https://doi.org/10.1007/s00114-005-0040-7.
Gong, X., Jiang, S., Tian, H., Xiang, D., Zhang, J., 2020. Polyphenols in the Fermentation Liquid of Dendrobium candidum Relieve Intestinal Inflammation in Zebrafish Through the Intestinal Microbiome-Mediated Immune Response. Front. Immunol. 11, 1542. doi: 10.3389/fimmu.2020.01542.
Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J., Brummer, R.J., 2008. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther. 15;27(2):104-19. https://doi.org/10.1111/j.1365-2036.2007.03562.x.
Hoseinifar, H., Jahazi, A., Nikdehghan, N., Van Doan, H., Volpe, M.G., Paolucci, M., 2020. Effects of dietary polyphenols from agricultural by-products on mucosal and humoral immune and antioxidant responses of convict cichlid (Amatitlania nigrofasciata). Aquaculture. 517: 734709. https://doi.org/10.1016/j.aquaculture.2019.734790.
Huang, Q., Liu, X., Zhao, G., Hu, T., Wang, Y., 2018. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. Animal Nutrition, 4(2), 137–150. https://doi.org/10.1016/j.aninu.2017.09.004.
Hussain, T., Tan, B., Yin, Y., Blachier, F., Tossou, M.C.B., Rahu, N., 2016. Oxidative Stress and Inflammation: What Polyphenols Can Do for Us? Oxid Med Cell Longev. 1–9. https://doi.org/10.1155/2016/7432797.
Imperatore, R., Tunisi, L., Mavaro, I., D'Angelo, L., Attanasio, C., Safari, O., Motlagh, H.A., De Girolamo, P., Cristino, L., Varricchio, E., Paolucci, M., 2020. Immunohistochemical Analysis of Intestinal and Central Nervous System Morphology in an Obese Animal Model (Danio rerio) Treated with 3,5-T2: A Possible Farm Management Practice? Animals. 10, 1131. https://doi.org/10.3390/ani10071131.
Imperatore, R., Coccia, E., D'Angelo, L., Varricchio, E., De Girolamo, P., Paolucci, M., 2018. Evidence for leptin receptor immunoreactivity in the gastrointestinal tract and gastric leptin regulation in the rainbow trout (Oncorhynchus mykiss). Ann. Anat. 220, 70–78. https://doi.org/10.1016/j.aanat.2018.07.007.
Jahazi, A., Hoseinifar, S.H., Jafari, V., Hajimoradloo, A., Van Doan, H., Paolucci, M., 2020. Dietary supplementation of polyphenols positively affects the innate immune response, oxidative status, and growth performance of common carp, Cyprinus carpio L., Aquaculture. 517: 734709. https://doi.org/10.1016/j.aquaculture.2019.734709.
Kawabata, K., Yoshioka, Y., Terao, J., 2019. Role of Intestinal Microbiota in the Bioavailability and Physiological Functions of Dietary Polyphenols. Molecules. 24(2), 370. doi:10.3390/molecules24020370.
Khanbabaee, K., van Ree, T., 2001. Tannins: classification and definition. Nat Prod Rep. 18(6), 641–649. doi:10.1039/b101061l.
Knoop, K.A., Newberry, R.D., 2018. Goblet cells: multifaceted players in immunity at mucosal surfaces. Mucosal Immunol. 11(6):1551-1557. https://doi.org/10.1038/s41385-018-0039-y.
Krogdahl, Å., Bakke-McKellep, A., Baeverfjord, G., 2003. Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (Salmo salar L.). Aquac. Nutr. 9, 361–371. https://doi.org/10.1046/j.1365-2095.2003.00264.x.
Larsen, A.M., Mohammed, H.H., Arias, C.R., 2014. Characterization of the gut microbiota of three commercially valuable warmwater fish species. J Appl Microbiol. 116(6):1396-404. https://doi.org/10.1111/jam.12475.
Lawrence, C., 2007. The husbandry of zebrafish (Danio rerio): A review. Aquaculture. 269, 1–20. https://doi.org/10.1016/j.aquaculture.2007.04.077.
Li, M., Feng, L., Jiang, W.-D., Wu, P., Liu, Y., Jiang, J., Kuang, S.-Y., Tang, L., Zhou, X.Q., 2020. Condensed tannins decreased the growth performance and impaired intestinal immune function in on-growing grass carp (Ctenopharyngodon idella). Br J Nutr. 123(7), 737–755. https://doi.org/10.1017/S0007114519003295.
Li, Y.-X., Wijesekara, I., Li, Y., Kim, S.-K., 2011. Phlorotannins as bioactive agents from brown algae. Process Biochemistry. 46(12), 2219–2224. https://doi.org/10.1016/j.procbio.2011.09.015.
Licitra, R., Marchese, M., Brogi, L., Fronte, B., Pitto, L., Santorelli, F.M., 2021a. Nutraceutical Screening in a Zebrafish Model of Muscular Dystrophy: Gingerol as a Possible Food Aid. Nutrients. 13, 998. https://doi.org/10.3390/nu13030998.
Licitra, R., Martinelli, M., Petrocchi Jasinski, L., Marchese, M., Kiferle, C., Fronte, B., 2021b. In Vivo Evaluation of Cannabis sativa Full Extract on Zebrafish Larvae Development, Locomotion Behavior and Gene Expression. Pharmaceuticals. 14(12):1224. https://doi.org/10.3390/ph14121224.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Method. Methods. 25(4):402-8. https://doi.org/10.1006/meth.2001.1262. Mueller-Harvey, I., 2006. Unravelling the conundrum of tannins in animal nutrition and health. J. Sci. Food Agric. 86(13), 2010–2037. https://doi.org/10.1002/jsfa.2577.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., Nichols, P.D., 2009. Feeding aquaculture in an era of finite resources. Proc. Natl Acad. Sci USA. 106, 15103–15110. https://doi.org/10.1073/pnas.0905235106.

Naylor, R.L., Hardy, R.W., Buschmann, A.H., Bush, S.R., Cao, L., Klinger, D.H., Little D.C., Lubchenco, J., Shumway, S.E., Troell, M., 2021. A 20-year retrospective review of global aquaculture. Nature. 591(7851), 551–563. doi:10.1038/s41586-021-03308-6.

Okuda, T., 2005. Systematics and health effects of chemically distinct tannins in medicinal plants. Phytochemistry. 66(17), 2012–2031. https://doi.org/10.1016/j.phytochem.2005.04.023.

Ogi, A., Licitra, R., Naef, V., Marchese, M., Fronte, B., Gazzano, A., Santorelli, F. M., 2021. Social Preference Tests in Zebrafish: A Systematic Review. Frontiers in veterinary science, 7, 590057. https://doi.org/10.3389/fvets.2020.590057.

Omnes, M.-H., Le Goasduff, J., Le Delliou, H., Le Bayon, N., Quazuguel, P., Robin, J.H., 2017. Effects of dietary tannin on growth, feed utilization and digestibility, and carcass composition in juvenile European seabass (Dicentrarchus labrax L.). Aquaculture Reports, 6, 21–27. https://doi.org/10.1016/j.aqrep.2017.01.004.

Orso, G., Solovyev, M.M., Facchiano, S., Tyrikova, E., Sateriale, D., Kashinskaya, E., Pagliarulo C., Hoseinifar, H.S., Simonov, E., Varricchio, E., Paolucci, M., Imperatore, R., 2021. Chestnut Shell Tannins: Effects on Intestinal Inflammation and Dysbiosis in Zebrafish. Animals, 11(6), 1538. https://doi.org/10.3390/ani11061538.

Pelusio, N.F., Scicchitano, D., Parma, L., Dondi, F., Brini, E., D'Amico, F., Candela, M., Yúfera, M., Gilannejad, N., Moyano, F.J., Gatta, P.P., 2021. Interaction Between Dietary Lipid Level and Seasonal Temperature Changes in Gilthead Sea Bream Sparus aurata: Effects on Growth, Fat Deposition, Plasma Biochemistry, Digestive Enzyme Activity, and Gut Bacterial Community. Front. Mar. Sci. 31;8:549. https://doi.org/10.3389/fmars.2021.664701.

Peinnequin, A., Mouret, C., Birot, O., Alonso, A., Mathieu, J., Clarençon, D., Agay, D., 2004. Rat pro-inflammatory cytokine and cytokine related mRNA quantification by real-time polymerase chain reaction using SYBR green. BMC Immunol. 5, 3. https://doi.org/10.1186/1471-2172-5-3.

Peng, K., Lv, X., Zhao, H., Chen, B., Chen, X., Huang, W., 2022. Antioxidant and intestinal recovery function of condensed tannins in Lateolabrax maculatus responded to in vivo and in vitro oxidative stress. Aquaculture 2022, 547, 737399. https://doi.org/10.1016/j.aquaculture.2021.737399.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glockner, F.O., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41, 590-596. https://doi.org/10.1093/nar/gks1219.

Randazzo, B., Zarantoniello, M., Cardinaletti, G., Cerri, R., Giorgini, E., Belloni, A., Contò, M., Tibaldi, E., Olivotto, I., 2021. Hermetia illucens and Poultry by-Product Meals as Alternatives to Plant Protein Sources in Gilthead Seabream (Sparus aurata) Diet: A Multidisciplinary Study on Fish Gut Status. Animals (Basel). 4;11(3):677. https://doi.org/10.3390/ani11030677.

Rocha, F., Dias, J., Engrola, S., Gavaia, P., Geurden, I., Dinis, M.T., Panserat, S., 2014. Glucose overload in yolk has little effect on the long-term modulation of carbohydrate metabolic genes in zebrafish (Danio rerio). J. Exp. Biol. 217, 1139–1149. https://doi.org/10.1242/jeb.095463.

Roeselers, G., Mittge, E.K., Stephens,W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K., Rawls, J.F., 2011. Evidence for a core gut microbiota in the zebrafish. ISME J. 5, 1595–1608. https://doi.org/10.1038/ismej.2011.38.

Rognes, T., Flouri, T., Nichols, B., Quince, C., Mahé, F., 2016. VSEARCH: A versatile open source tool for metagenomics. PeerJ 10 (art. no. e2584).

Royes, J.-A.B., Chapman F.A., 2003. "Preparing Your Own Fish Feeds: Cir 97/FA097, 2/2003". EDIS. (6). https://doi.org/10.32473/edis-fa097-2003.

Safari, R., Hoseinifar, S.H., Imanpour, M.R., Mazandarani, M., Sanchouli, H., Volpe, M.G., Paolucci, M., 2020. Effects of dietary polyphenols on mucosal and humoral immune responses, antioxidant defense and growth gene expression in beluga sturgeon (Huso huso). Aquaculture. 735494. https://doi.org/10.1016/j.aquaculture.2020.735494.

Salinas, I., 2015. The Mucosal Immune System of Teleost Fish. Biology. 4(3), 525–539. doi:10.3390/biology4030525.

Santangelo, C., Varì, R., Scazzocchio, B., Di Benedetto, R., Filesi, C., Masella, R., 2007. Polyphenols, intracellular signalling and inflammation. Ann Ist Super Sanità. 43, 4: 394-405.

Shimizu, M., 2017. Multifunctions of dietary polyphenols in the regulation of intestinal inflammation. J Food Drug Anal. 25(1):93-99. https://doi.org/10.1016/j.jfda.2016.12.003.

Sieniawska, E., Baj, T., 2017. Tannins. Pharmacognosy, 199–232. doi:10.1016/b978-0-12-802104-0.00010-x

Sorice, A., Siano, F., Capone, F., Guerriero, E., Picariello, G., Budillon, A., Ciliberto, G., Paolucci, M., Costantini, S., Volpe, M.G., 2016). Potential Anticancer Effects of Polyphenols from Chestnut Shell Extracts: Modulation of Cell Growth, and Cytokinomic and Metabolomic Profiles. Molecules, 21, 1411. https://doi.org/10.3390/molecules21101411.

Stephens, W.Z., Burns, A.R., Stagaman, K., Wong, S., Rawls, J.F., Guillemin, K., Bohannan, B.J.M., 2016. The composition of the zebrafish intestinal microbial community varies across development. ISME J. 10, 644–654. https://doi.org/10.1038/ismej.2015.140.

Torrecillas, S., Montero, D., Izquierdo, M., 2014. Improved health and growth of fish fed mannan oligosaccharides: potential mode of action. Fish Shellfish Immunol. 36:525–544. https://doi.org/10.1016/j.fsi.2013.12.029.

Torrecillas, S., Makol, A., Caballero, M.J., Montero, D., Robaina, L., Real, F., Sweetman, J., Tort, L., Izquierdo, M.S., 2007. Immune stimulation and improved infection resistance in European sea bass (Dicentrarchus labrax) fed mannan oligosaccharides. Fish Shellfish Immunol. 23:969–981. https://doi.org/10.1016/j.fsi.2007.03.007.

Ulloa, P.E., Medrano, J.F., Feijoo, C.G., 2014. Zebrafish as animal model for aquaculture nutrition research. Front Genet. 10;5:313. https://doi.org/10.3389/fgene.2014.00313.

Urán, P., Gonçalves, A., Taverne-Thiele, J., Schrama, J., Verreth, J., Rombout, J., 2008. Soybean meal induces intestinal inflammation in common carp (Cyprinus carpio L.). Fish. Shellfish. Immunol. 25, 751–760. https://doi.org/10.1016/j.fsi.2008.02.013.

Van Doan, H., Hoseinifar, S.H., Hung, T.Q., Lumsangkul, C., Jaturasitha, S., El-Haroun, E., Paolucci, M., 2020. Dietary inclusion of chestnut (Castanea sativa) polyphenols to Nile tilapia reared in biofloc technology: Impacts on growth, immunity, and disease resistance against Streptococcus agalactiae. Fish. Shellfish. Immunol. https://doi.org/10.1016/j.fsi.2020.07.010.

Varricchio, E., Coccia, E., Orso, G., Lombardi, V., Imperatore, R., Vito, P., Paolucci, M., 2019. Influence of polyphenols from olive mill wastewater on the gastrointestinal tract, alveolar macrophages and blood leukocytes of pigs. Ital. J. Anim. Sci. 18, 574–586. https://doi.org/10.1080/1828051X.2018.1548911.

Vladimir-Knežević, S., Blažeković, B., Štefan, M.B., Babac, M., 2012. Plant Polyphenols as Antioxidants Influencing the Human Health, Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health, Dr Venketeshwer Rao (Ed.), doi:10.5772/27843.

Yu, Y.-M., Chao, T.-Y., Chang, W.-C., Chang, M. J., Lee, M.-F., 2016. Thymol reduces oxidative stress, aortic intimal thickening, and inflammation-related gene expression in hyperlipidemic rabbits. J Food Drug Anal, 24(3), 556–563. https://doi.org/10.1016/j.jfda.2016.02.004.

Walburn, J.W., Wemheuer, B., Thomas, T., Copeland, E., O'Connor, W., Booth, M., Fielder, S., Egan, S., 2019. Diet and diet-associated bacteria shape early microbiome development in Yellowtail Kingfish (Seriola lalandi). Microb. Biotechnol. 12, 275–288. https://doi.org/10.1111/1751-7915.13323.

Wang, D., DuBois, R., 2010. The role of COX-2 in intestinal inflammation and colorectal cancer. Oncogene. 29, 781–788. https://doi.org/10.1038/onc.2009.421.

Wang, T., Secombes, C.J., 2013. The cytokine networks of adaptive immunity in fish. Fish. Shellfish. Immunol, 35(6), 1703–1718. https://doi.org/10.1016/j.fsi.2013.08.030.
Wang, Y., Majak, W., Mcallister, T.A., 2012. Frothy bloat in ruminants: cause, occurrence, and mitigation strategies. Anim Feed Sci Technol. 172(1e2):103e14. https://doi.org/10.1016/j.anifeedsci.2011.12.012.
Watts, J.E.M., Schreier, H.J., Lanska, L., Hale, M.S., 2017. The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. Mar. Drugs 15, 158. https://doi.org/10.3390/md15060158.
Westerfield, M., 2007. THE ZEBRAFISH BOOK, 5th Edition; A guide for the laboratory use of zebrafish (Danio rerio).

2.6 Study IV - Impact of marine aquaculture on the microbiome associated with nearby holobionts: the case of *Patella caerulea* living in proximity of sea bream aquaculture cages

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Introduction

Fish farming is rapidly increasing in the Mediterranean Sea in order to respond to the rising demand for products for human consumption. Marine aquaculture (mariculture) is an integral part of growing coastal economy and is mainly carried on by caged open systems, with the farmed species in direct contact with the wild coastal ecosystem [1-3]. Current finfish farming practices influence the marine biota at different trophic levels by changing environmental conditions in the surrounding water column, which undergoes severe eutrophication, as well as by impacting the chemical features of the sediments below the cages [1,4,5]. Indeed, sediments in proximity of the cages show an increase in organic matter, due to the sedimentation of uneaten feed and fish feces, as well as accumulation of heavy metals [6,7]. Consequences are shifts in nutrients and carbon fluxes, pH decline, and oxygen depletion in the sea floor, resulting in ammonia and hydrogen sulphide accumulation [4,8]. Such conditions modify the benthic assemblages of fauna and seagrass, affecting the whole food web [9]. It has been demonstrated that the proximity of fish farming cages affects the survival of grazers and other macro-fauna trophic groups, even further than the mere sedimentation zone [1,10].

All environments on our planet, including macro-organisms themselves (defined as holobionts), are colonized by microorganisms living in complex communities called microbiomes. All key biosphere processes, both terrestrial and aquatic, as well as many physiological aspects of animal and plant biology, deeply rely on microbiomes. Being aware of the microbiome importance as a life support system for the planet biosphere, there is now a huge concern about the impact of local and global anthropic factors on the planet microbiomes [11], making microbiomes assessment a central point for a next-generation and more holistic evaluation of the environmental health. In this scenario, the impact of aquaculture on seafloor microbiomes has recently been explored, reporting decreased bacterial biodiversity in the sediments below the cages, overgrowth of microbial groups able to thrive in anaerobic, carbon-enriched conditions, as well as accumulation of fecal bacteria and/or bacteria linked to the sulphur cycle [6,12-17]. The microbial community associated with the water column also appeared to be different within and outside the cage of farmed sea breams [18]. On the contrary, very little is known on the effect of the presence of farming cages on the microbiome of nearby wild holobionts.

Even if resulting from a host-driven selection process, microbiomes of marine holobionts show a strong metacommunity behaviour, being in close interaction with microbiomes from both the environment (e.g., water column and the sediments) and the other holobionts living in their proximity [19-22]. Thus, it is reasonable to expect that the microbiomes associated with wild marine holobionts living in the proximity of fish farms are somehow affected by the interaction with the microbiomes of farmed fish, both directly, by the transfer of fish microorganisms dispersed to the water column and/or sediments, and indirectly, by changing the surrounding environmental bacterial community. The consequent colonization of the wild holobiont microbiomes with allochthonous microbial components would, eventually, result in compositional changes, with cascade impacts on the health and safety of the marine environment. Confirming these hypotheses, it was recently reported that sponges living in proximity of fish farming sites in Philippines harboured a microbial community enriched in genes involved in ammonia oxidation with respect to sponges of the same species collected in pristine waters [23].

In order to explore how the presence of fish farming cages influences the microbiome of the surrounding wild holobionts, we selected a common grazer gastropod from the genus *Patella* as a representative fouling organism. *Patella caerulea* is a common seaweed grazing marine limpet in all Mediterranean rocky shores [24]. Because of their wide distribution, abundance and sedentary lifestyle, limpets of this species have been proposed as biomonitors for the local water quality, in terms of heavy metal accumulation and organic pollutants. In addition, limpets are keystone species for the coastal ecosystem because they regulate the degree of algal coverage and, consequently, succession processes in rocky intertidal communities [25-27].

In particular, in our work we compared (by Next Generation Sequencing 16S rDNA metabarcoding) the *P. caerulea* digestive gland microbiome structure in individuals collected close to sea breams (*Sparus aurata*) aquaculture cages located in a fish farm in Southern Sicily (Italy), Mediterranean Sea, with the one from individuals collected on a rocky coastal tract located far from the aquaculture facility, as control site. The gut, skin and gills microbiomes from the farmed fishes have been also assessed, together with microbial eassemblages from sediments and water at the aquaculture and control sites, allowing us to explore the variation of *P. caerulea* microbiomes at the aquaculture site in a holistic metacommunity context. Both sampling sites were located in the harbor of Licata, an ideal location to investigate the impact of aquaculture systems on the microbiome composition of nearby wild animals due to the limited hydrodynamic circulation inside the harbor and a shallow depth (~10 m), resulting in a large amount of organic matter accumulating on the sea floor under the cages [14]. This comprehensive study design allowed us to dissect the interaction between the microbiomes from farmed fished and surrounding wild holobionts at the metacommunity level, showing patterns of microbial dispersion from the former to the environment and, finally, to locally dwelling wild organisms.

Materials and Methods

Site description and samples collection

The sampling was performed on September, the 25th, 2019, in a marine fish farm located in the harbor of Licata (Figure 1), in Southern Sicily (Mediterranean Sea, coordinates 37.087713°N, 13.943773°E). The facility is composed of 23 floating cages containing sea breams (*S. aurata*) and sea bass (*Dicentrarchus labrax*); further details on the sampling site are reported in Ape *et al.* [14]. We selected one of the sea breams cages as sampling site (37.086667°N, 13.943611°E) and we collected five *S. aurata* individuals and 12 *P. caerulea* individuals, the latter growing in adhesion to the cage plastic tubes. Surface sediment (0-1 cm) and seawater were collected under the cage as well as at two additional sites (37.089732°N, 13.937469°E and 37.091949°N, 13.933703°E) as controls, either by coring or through a sterile plastic bottle, respectively. Limpets samples were detached using a previously sterilized knife and preserved into sterile plastic containers. We also collected 15 *P. caerulea* individuals from the shallow water rocks located along the pier (37.095000°N, 13.933611°E) as described above. From each fish individual, intestinal content (feces), gills and skin, were collected. In more detail, sea bream individuals (avg. 270 g) were euthanized by anesthesia (MS-222) following the national regulations and set on ice until processing. Within 2-5 hours, gut tissues were obtained by aseptic dissection and the intestinal content was squeezed out as described in Mente *et al.* [28] and stored in sterile tubes. Finally, a 2x2 cm of

skin (left side) and the second gill branch (left side) were aseptically collected with sterile scissors, rinsed with sterile phosphate buffer and stored in sterile tubes. All samples were kept at –20 °C until shipping in the respective labs.

Figure 1 - Sampling site description. Map of the marine fish farm located in the harbor of Licata (37.087713°N, 13.943773°E). The sampling sites are indicated with red balloons and labelled accordingly. Aquaculture site is located at 37.086667°N, 13.943611°E, control sediment and water samples were collected far from the fish cages (37.089732°N, 13.937469°E and 37.091949°N, 13.933703°E) and *P. caerulea* individuals from the shallow water rocks located along the pier were collected at 37.095000°N, 13.933611°E (source: Google Earth, earth.google.com/web/; map data: SIO, NOAA, U.S. Navy, NGA, GEBCO, IBCAO).

Microbial DNA extraction, 16S rRNA gene amplification and sequencing.

For limpet samples, we dissected the digestive gland from each individual under a vertical laminar airflow cabinet using sterile tweezers and scalpels, obtaining a weight range from 0.037 g to 0.460 g for all the glands, depending on each limpet size. Total microbial DNA extraction was performed on limpets digestive glands using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) [29].

All feces collected from each sample were used for DNA extraction. Seawater samples (1 L) were filtered onto 0.22 µm cellulose nitrate membrane filters (Sartorius) and stored at −20 °C until processing. The top 1 cm of each sterile corer used for sediments collection was carefully extruded and stored at −20 °C and 1 g of this sediment was used for DNA extraction. DNA from sea bream feces and tissues, seawater and sediment samples was extracted using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) as previously described [14,30]. All extracted DNA was stored at –80 °C until further processing. PCR amplification of the V3-V4 hypervariable region of the 16S rRNA gene, library preparation and sequencing were carried out as described in paragraph 2.2.

Bioinformatics and statistics.

Raw sequences were processed as described in paragraph 2.2. Two different metrics were used to evaluate alpha diversity - Faith's Phylogenetic Diversity (Faith_pd) (Faith, 1992) and number of observed ASVs. Beta diversity was estimated by computing the unweighted UniFrac distance. To further characterize the compositional specificities of the limpets digestive gland microbiome at aquaculture and control sites in the context of their respective marine metacommunities, PANDAseq assembled paired-end reads were also processed with the QIIME1 (Caporaso et al., 2010) pipeline for OTUs (Operational Taxonomic Units) clustering based on 97% similarity threshold, with taxonomy assignment performed using the SILVA database. As above, all the sequences assigned to eukaryotes or unassigned were discarded.

All statistical analyses were performed using the R software (R Core Team), version 3.6.1., with the packages "Made4" (Culhane et al., 2005) and "vegan" except for environmental parameters that were compared between sites using Mann-Whitney, computed with IBM SPSS Statistics 26.0 (IBM CorporaOon). Ternary plots were prepared using the R packages

"ggtern" (Hamilton and Ferry, 2018), "PMCMR" (Pohlert, 2014) and "vcd" (Meyer et al., 2020). Unweighted UniFrac distances were plotted using the vegan package and the data separation in the Principal Coordinates Analysis (PCoA) was tested using a permutation test with pseudo-F raOos (function "adonis" in the vegan package). Wilcoxon rank-sum test was used to assess differences in alpha diversity and ternary plots, and Kruskal-Wallis test for testing OTUs separation. p-values were corrected for multiple testing with the Benjamini-Hochberg method, with a false discovery rate (FDR) ≤ 0.05 considered statistically significant.

Results

Diversity and compositional structure of the marine microbial communities at aquaculture and control site in the Licata harbor

Sequencing of the V3-V4 hypervariable region of the 16S rRNA gene from the total microbial DNA resulted in 50 samples, from aquaculture and control sites, producing a high-quality output. These included three sediment and three seawater samples, 25 limpet digestive glands (DG) and 19 sea bream samples, of which 12 gut samples, three gill samples and four skin samples. The number of high-quality reads in the samples ranged between 1690 and 20252 reads per sample and they were binned into 6450 ASVs.

According to our data, the overall structure of the limpet DG microbiome segregated from that of seawater and sediments microecosystems, as shown by the Principal Coordinates Analysis (PCoA) based on the unweighted UniFrac distances (Figure 2A) (permutation test with pseudo-F ratio, p-value ≤ 0.001). *P. caerulea* DG microbiome was also characterized by a lower diversity with respect to environmental communities (seawater and sediments) (Figure 2B), although the trend did not reach the statistical significance in all the metrics (Wilcoxon rank-sum test controlled for multiple testing using FDR, p-value 0.4 and 0.02 for Faith PD index and number of observed ASVs, respectively).

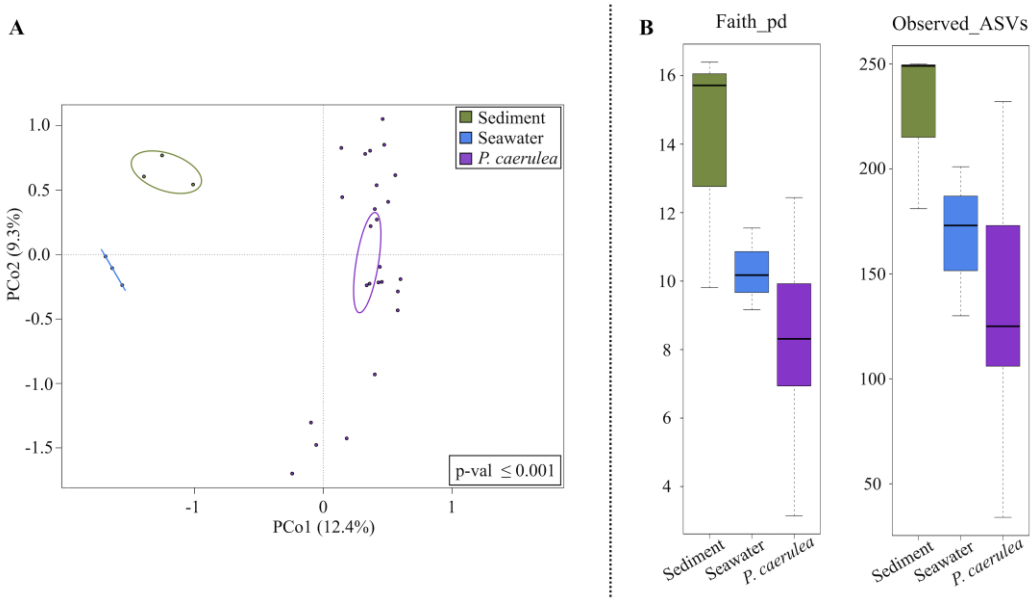


Figure 2 – Alpha and beta diversity comparison between *P. caerulea* and the surrounding environment. (A) Principal Coordinates Analysis (PCoA) based on the unweighted UniFrac distances between microbial profiles of sediments, seawater and limpets, showing a strong separation between the groups (permutation test with pseudo-F ratio, p-value ≤ 0.001). The percentage of variance in the dataset explained by each axis, first and second principal component (PCo1 and PCo2), is 12.4% and 9.3%, respectively. Ellipses include 95% confidence area based on the standard error of the weighted average of sample coordinates. (B) Box-and-whiskers distribution of Faith's Phylogenetic Diversity (Faith_pd) and number of observed ASVs metrics of diversity.

For what concern their phylogenetic composition, the microbial assemblages associated to the three types of samples showed a characteristic layout in terms of dominant and subdominant components, with a specific declination according to the sampling site. Particularly, the microbiome of *P. caerulea* DG was characterized by two dominant phyla, Proteobacteria (mean relative abundance ± SD, 44.1 ± 12.6% and 43.7 ± 12.5% in limpets collected at the control and aquaculture sites, respectively) and Planctomycetes (29.7 ± 13.4% and 28.6 ± 22.4%). Alphaproteobacteria class represented the main fraction of Proteobacteria (72.0 ± 13.2% and 66.5 ± 23.0%). Relevant subdominant phyla (average r.a. > 1%) were Firmicutes (6.4 ± 11.6% and 5.7 ± 4.6%), Actinobacteria (5.5 ± 7.3% and 3.7 ± 7.1%), Bacteroidetes (4.2 ± 8.5% and 2.8 ± 3.2%) and Cyanobacteria (2.1 ± 1.4% and 2.7 ± 3.2%). In the control site, we also found Verrucomicrobia (3.9 ± 3.4%), whereas Tenericutes (9.5 ± 16.5%) and Fusobacteria (1.2 ± 2.7%) were only present in the aquaculture site (Figure 3A). The detailed compositional structure of water and sediments microbiomes at the aquaculture and control sites is available in **Supplementary figures S1 and S2**. Briefly, both sediments and seawater were mainly characterized by Proteobacteria and Bacteroidetes at all sampling sites. In the aquaculture site, the two matrices also included members of the Firmicutes phyla, whereas Verrucomicrobia were only characteristic of seawater. Finally, the microbiomes associated to different tissues of farmed sea breams were also characterized (**Supplementary figure S3**). Our findings showed that all sea bream microbiomes were mainly characterized by Proteobacteria, with Firmicutes and Actinobacteria only represented in the gut samples.

3.2 Changes in the limpet microbiome and surrounding metacommunities at the aquaculture site in the Licata harbor

In order to highlight the impact of aquaculture cages on the limpets DG microbiome, a PCoA of the unweighted UniFrac distances of the microbiomes structure in individuals collected at aquaculture and control sites was performed (Figure 3D). Data indicated a significant segregation between the two ecosystems (permutation test with pseudo-F ratio, p-value ≤ 0.001), demonstrating that phylogenetic composition of *P. caerulea* DG microbiome changed at the aquaculture site. Further, DG from samples collected in the aquaculture site showed a significantly lower internal microbiome diversity, as shown by two different metrics (FDR corrected Wilcoxon rank-sum test, p-value 0.008 and 0.01 for Faith PD index and number of observed ASVs, respectively) (Figure 3C). For what concerns the main DG microbiome compositional specificities, limpets at the aquaculture site were characterized by a higher abundance in members belonging to the family *Mycoplasmataceae* (9.4 ± 16.5% in samples from the cages vs. 0.8 ± 3.3% in controls). Conversely, among the subdominant components, the DG microbiome of individuals at the aquaculture site was significant depleted in members of the uncultured Verrucomicrobiales group DEV007, showing a relative abundance (r.a.) of 0.3 ± 0.5% compared to a r.a. of 2.1 ± 1.9% observed in controls (FDR corrected Wilcoxon rank-sum test, p-value = 0.007) (Figure 3B).

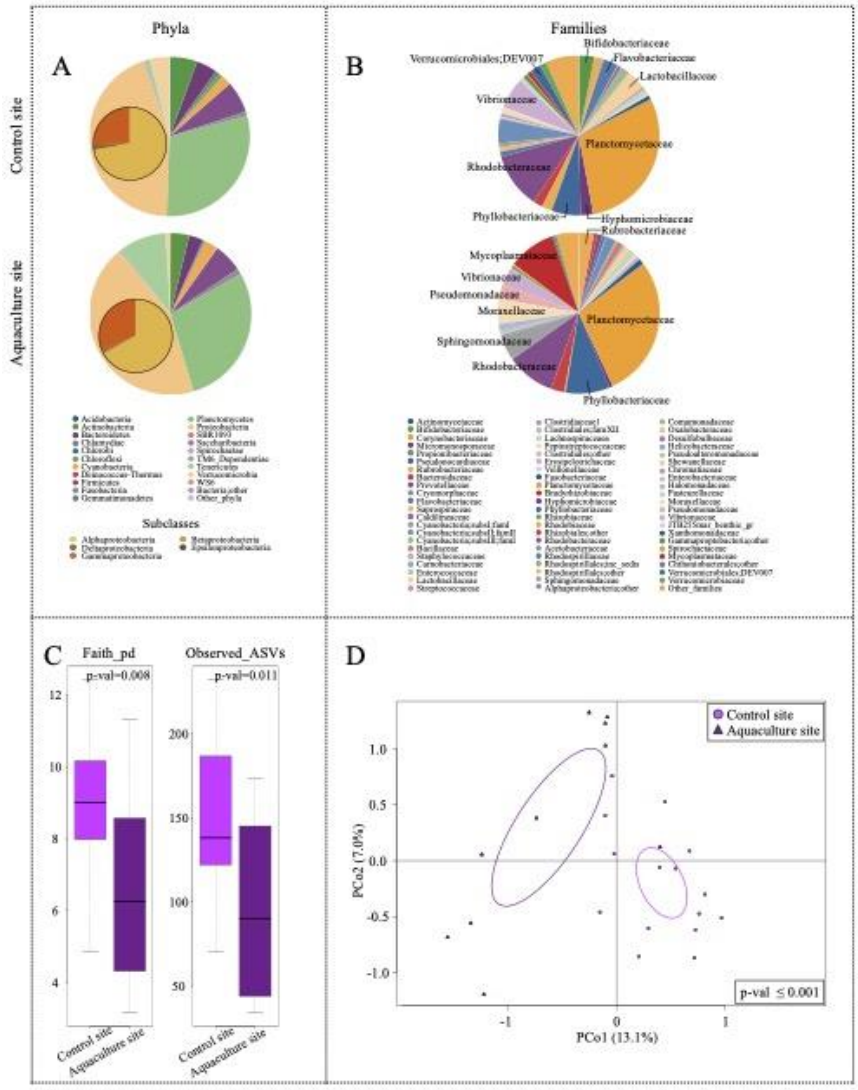


Figure 3 – Overall description of *P. caerulea* microbial communities and alpha and beta diversity comparison between *P. caerulea* of control and aquaculture. Pie charts summarizing the phylum (A) and family (B) level microbiota composition of *P. caerulea* in the two sampling sites. Phyla with relative abundance > 0.5% in at least one sample and families with relative abundance > 2% in at least 10% of samples are represented. Proteobacteria subclasses are expanded on the respective pie chart phylum slice. (C) Box-and-whiskers distribution of Faith's Phylogenetic Diversity (Faith_pd) and number of observed ASVs metrics of diversity. (D) Principal Coordinates Analysis (PCoA) based on the unweighted UniFrac distances between microbial profiles of *P. caerulea* in the two sampling sites shows a strong separation between the groups (permutation test with pseudo-F ratio, p-value ≤ 0.001). The percentage of variance in the dataset explained by each axis, first and second principal component (PCo1 and PCo2), is 13.1% and 7.0%, respectively. Ellipses include 95% confidence area based on the standard error of the weighted average of sample coordinates.

In order to characterize in depth the compositional specificities of the limpets DG microbiome at aquaculture and control sites in the context of their respective marine metacommunities, OTUs were clustered at 97% and the ones showing a relative abundance > 0.5% in at least one microbiome sample type were retained (limpets, seawater, sediments and fish feces, gills and skin). The resulting subset of 192 OTUs was used for the production of ternary plots to highlight the OTUs ecological propensity toward the different local microbial communities (Figure 4A-C). While for the control site a single ternary plot was generated (Figure 4A), considering local water, sediments and the DG from limpets, for the aquaculture site two ternary plots were created, the first matching the one from the control site (Figure 4B) and the second in which seawater was substituted by *S. aurata* as source ecosystem (Figure 4C). Furthermore, 50 OTUs showing a significant different abundance in the DG microbiome from individuals collected at the aquaculture and control sites were identified (FDR corrected Wilcoxon rank-sum test, p-value ≤ 0.05). Among these, 22 OTUs were more abundant in the control site, colored in purple in Figure 4A, whereas 28 OTUs showed a significant opposite trend, plotted in purple in Figures 4B and C. Finally, the highest score alignment against NCBI 16S rRNA database of these OTUs are reported in **Supplementary table T1**.

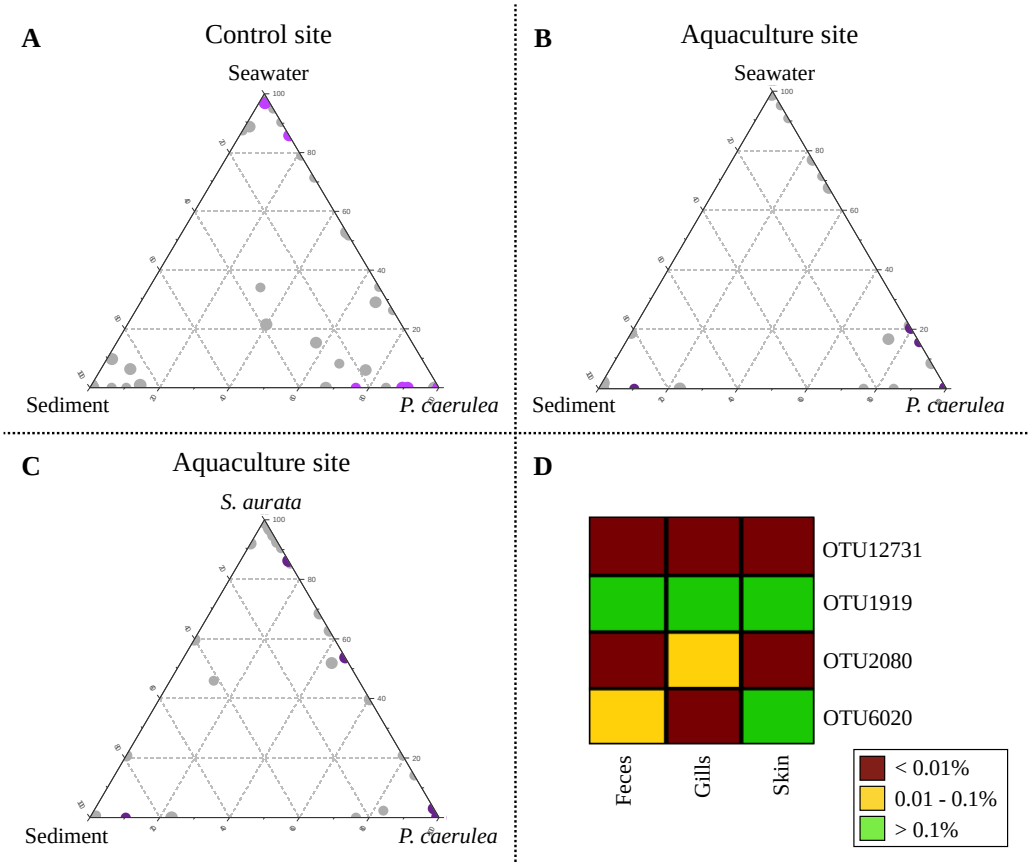


Figure 4 – Impact of aquaculture cage proximity on *P. caerulea* microbiome at 97% similarity OTUs level. (A, B, C) Ternary plots of all OTUs detected in the dataset with relative abundance > 0.5% in at least one samples. Each circle represents one OTU and the size is proportional to the weighted relative abundance. The position of each circle in the graphs represents its propensity toward the different ecosystems at the edges. (A) Purple circles represent the 22 OTUs whose mean relative abundance was significantly higher in the *P. caerulea* control site (FDR-corrected Wilcoxon rank-sum test, p-value ≤ 0.05). (B, C) Purple circles represent the 28 OTUs whose mean relative abundance was significantly higher in the *P. caerulea* aquaculture site (Wilcoxon rank-sum test controlled for multiple testing using FDR, p-value ≤ 0.05). (D) The heatmap represents the differential distribution of the OTUs shared between *P. caerulea* and farmed sea breams in the fish ecosystems (feces, gills and skin).

Focusing on the 50 OTUs differentiating the limpet DG microbiome at aquaculture and control sites, the majority of OTUs enriched in the latter were exclusive of limpets (plotted at the "*P. caerulea*" vertex in Figure 4A) (OTUs 4667, 11135, 4454, 12220, 1496, 4069, 4330, 14127, 5331, 14154, 3304, 11232, 11155, 14091, 11445, 3555 and 4234). These OTUs were assigned to species typically isolated from marine environment (e.g., species belonging to genera *Fodinicurvata*, *Rubinisphaera*, *Roseibacillus*) [44-47] or from marine organisms, such as *Amorphus coralli*, firstly isolated from coral mucus [48]. Other OTUs characterizing the DG from the limpets collected at the control site, such as OTU5034 (genus *Robiginitalea*), OTU2911 (*Actibacter*) and OTU2120 (*Photobacterium*), were shared between limpets and sediments (plotted along the bottom plane of the ternary plot), whereas OTU2289 (*Psychrobacter*) was shared between limpets and water, with higher abundance in the latter (closer to the "Seawater" vertex in the ternary plot). Finally, OTU1355 (*Prochlorococcus*) was shared among all three ecosystems, with a higher abundance in seawater. Similarly to what observed for the control site, the majority of OTUs characteristic of the limpets DG microbiome at the aquaculture site were exclusive of limpets (OTUs 4187, 11247, 11243, 11205, 6912, 5244, 4203, 2259, 4097, 2073, 12731, 11913, 4065, 4965, 2118, 2077, 2154, 11213, 1397, 11152, 4465, 4305, 6020, 3237 and 6006). Amongst these OTUs, four were assigned to the genus *Mycoplasma*, one to *Vibrio* and one to *Acinetobacter*, potential human pathogens found in marine organisms [49-53]. Within the remaining OTUs characterizing the DG microbiome from limpets living in aquaculture proximity, we only found one OTU shared between limpets and sediments (OTU14234, belonging to the

genus *Sulfurovum*), mainly present in sediments. Two OTUs were shared between limpets and seawater in the aquaculture site (OTU1919 and OTU2080, assigned to *Staphylococcus* and *Psychrobacter*, respectively), with a higher abundance in limpets. Finally, limpets and farmed fishes shared four out of the 28 OTUs characteristic of the limpets DG microbiome at the aquaculture site, of which two more present in fish (OTU1919, *Staphylococcus*, and OTU6020, *Pseudomonas*) and two enriched in limpets (OTU12731, *Sphingomonas*, and OTU2080, *Psychrobacter*) (Figure 4B and 4C). Of these four OTUs, OTU1919 and OTU12731 were present at low abundance in all fish tissues, whereas OTU2080 specifically belonged to the gills ecosystem and OTU6020 was more abundant on the skin of fishes (Figure 4D).

Discussion

Monitoring and preservation of coastal marine ecosystems are pivotal for the maintenance of the ecological and economical services that these environments provide, such as habitat provision, nutrient cycling, protection of the coast itself, and food provision through fishery and farming [22,54]. Aquaculture provides a relevant contribution to the food economy of Mediterranean countries. However, similarly to most of the human food production activities, mariculture influences environmental conditions in the surrounding water column, as evidenced by the decline in seawater pH and subsequent shifts in carbonate-bicarbonate equilibria highlighted in the current study (**Supplementary Table S2**), with direct and indirect impacts on marine biota [1]. Particularly, the health and functionality of the marine and coastal ecosystems is tightly linked to the resident environmental microbiomes, as well as to the ones associated to local holobionts. However, research focused on the impact of marine aquaculture on the coastal marine microbiomes is still in its infancy. While a considerable amount of work has been performed to assess the impact of aquaculture practices on the underlying seafloor microbial communities [6,12-17], very few preliminary data have been provided linking the presence of fish farming cages to variations in the microbiome of benthic organisms living in close proximity [23]. In this scenario, we explored the impact of gilthead sea bream cage farming in the Licata harbor, Sicily, Italy, on the microbiome of locally dwelling wild species, by using a commonly found grazer gastropod (the limpet *Patella caerulea*) as representative organism. In particular, we explained the variations of limpets DG microbiomes at the aquaculture and control sites in the context of parallel changes in the local marine metacommunities, including water, sediments and farmed fish microbiomes.

In spite of being a crucial keystone species for coastal environment, very few information is available on the microbiome of limpets up to now, with the exception of a first exploration of the microbiome of *P. pellucida*, a prevalently Atlantic species that mostly parasitize brown algae stems [55]. Coherently, the digestive gland microbiome of *P. caerulea* analyzed in our work was dominated by Proteobacteria, with the most abundant subclass being Alphaproteobacteria instead of Betaproteobacteria, as reported for *P. pellucida*. Also, while *P. pellucida* harbored a large amount of Firmicutes, the second most abundant phylum in *P. caerulea* was Planctomycetes. Differences in proteobacterial classes could be related to several factors, including the different sustaining strategy of the two *Patella* species (with *P. caerulea* grazing on hard materials algal coverage vs. *P. pellucida* parasitizing a single algal species), as well as to different environmental conditions characterizing the sampling areas (warm, shallow, Mediterranean waters vs. Atlantic cold water). The DG microbiome of *P. caerulea* was significantly different from the microbiomes in surrounding environment (water and nearby seafloor samples), confirming previous studies on aquatic holobionts and their ability to operate a non-neutral selection process of microbes from the surrounding environment [21,56-58].

In our work we were successful in demonstrating that limpets dwelling in proximity of aquaculture cages harbored a different digestive gland microbiome with respect to gastropods of the same species collected on distant rocky shores.

Such difference was already evident when looking at the microbiome phylogenetic structure at phylum level, with Tenericutes largely abundant in samples collected on the cages, whereas samples from the rocky shores were enriched in Verrucomicrobia. Frequently in cooperation with Planctomycetes [59], members of Verrucomicrobia are capable of processing decaying organic materials and polysaccharides [60,61]. Several studies have highlighted their symbiotic lifestyle in marine invertebrates with recent findings showing metabolic adaptations enabling a more efficient utilization of specific carbon sources present in the host [21,59,62]. However, we observed that the proximity to the aquaculture site was associated with the reduction of the Verrucomicrobia uncultured family DEV007 in the microbiome of limpets. DEV007 is a marine group of bacteria recently pointed out as particularly sensitive to metal pollution in surface waters and marine sediments [63,64]. Its decrease in limpets growing in adhesion to the aquaculture cage might be related to the increase in heavy metal accumulation that often accompanies aquaculture practices [1], and which can indirectly affect the most sensitive species in the microbiome of nearby wild organisms. Conversely, among the discriminant OTUs enriched in the microbiome of limpets collected on the aquaculture cage, we could find two OTUs putatively assigned to environmental bacteria that are instead reported as able to tolerate heavy metal pollution, namely *Acinetobacter guillouie* and *Mesorhizobium camelthorni* [65,66].

Beside heavy metal contamination, the accumulation of organic matter on the seafloor beneath fish cages is considered one of the major impacts of aquaculture and may lead to a consequent depletion in oxygen availability in sediments, as well as to an increase in toxic products, such as sulphide and ammonium. Several studies have reported on the occurrence of *Sulfidobacteria* in aquaculture, or nearby water, highlighting the potential importance of members of this genus in the sulfur cycling within these systems [67]. Another bacterial species thriving in highly sulfidic fish-farm sediments is *Sulfurovum lithotrophicum*, a chemolithoautotroph ε-Proteobacteria able to use S^0 or $S_2O_3^{2-}$ as electron donors and O_2 or NO^{3-} as electron acceptors. *S. lithotrophicum* bacteria have been isolated in sediments from the oxic-anoxic interface where sulfides meet oxygenated sea water [68]. The retrieval of higher abundances of OTU4065, assigned to *Sulfitobacter*, and OTU14234, belonging to the genus *Sulfurovum*, in *P. caerulea* individuals from the aquaculture site, as well as the observed sharing of the latter OTU with the aquaculture sediments, is in line with these previous findings, suggesting that the microbiome of locally dwelling holobionts might respond to environmental changes caused by the aquaculture practice [21], i.e. heavy metal and sulphide accumulation, through the selection of environmental microorganisms allowing adaptive responses. Moreover, it was also shown that *Sulfitobacter* species might also have an inhibitory activity towards *Vibrio anguillarum*, an important fish pathogen [69]. Thus, it is tempting to hypothesize that limpets might be pushed to select this particular bacterial group within their microbiome as protective agent towards pathogens potentially enriched in the aquaculture site.

In relation to this, the possible pathogen flaw from farmed to wild organisms in mariculture has been pointed out as an unavoidable problem of this particular aquaculture practice. Mollusks and other non-fish scavengers persist in the vicinity of sea cages for longer period than wild fishes, making them a target for pathogens transfer [1]. We found that *Mycoplasmataceae*, the most abundant family within the phylum Tenericutes, tended to be more abundant in the DG microbiome from the limpets dwelling on the cages. Particularly, an OTU assigned to the genus *Mycoplasma* was the most relevant discriminating OTU among *P. cearulea* DG specimens taken at the two sites. Considering that many *Mycoplasma* species are parasite or pathogenic to humans and other animals [49,70], these finding, together with the detection of OTUs assigned to potential fish pathogens from the genera *Vibrio* and *Acinetobacter* [50-53] as significantly most abundant in the DG microbiome from the limpets at the aquaculture cages, confirm that pathogens transfer between farmed fishes and wild limpets is possible. However, it must be also pointed out that Tenericutes, and particularly

Mycoplasma, have been consistently observed as abundant, core members of several aquatic organisms' microbiome, mainly including bivalves, where they exhibit commensalism [71-76]. A possible involvement in mutually beneficial interactions with the host - likely by assisting an efficient processing of complex organic compounds, abundant at aquaculture sites - is being progressively assumed [76-78] and corroborates the idea of a possible role of fouling organisms in reducing the environmental impact of aquaculture [79]. Since the OTUs assigned to the genus *Mycoplasma*, *Vibrio* and *Acinetobacter* enriched in the DG microbiome from *P. caerulea* individuals at the aquaculture site were not detectable in farmed fish samples and no disease was reported at the fish farming plant at the moment of sampling, we could hypothesize that relationships like commensalism, rather than parasitism or pathogenicity, occurred between *P. caerulea* and OTUs belonging to these genera. However, their detection in DG microbiomes from aquaculture still poses questions on their possible spread in the surrounding environment, in the nearby wild organisms as well as to humans, to which they are pathogens.

We also found two OTUs (12731 and 11913) assigned to the *Sphingomonas* genus, shared between limpets and fish, that were significantly more abundant in limpets at the aquaculture site. *Sphingomonas* is a bacterial genus commonly found in fish skin [80] and gut microbiome and in farmed sea breams specifically, as reported by Floris *et al.* [81] and Estruch *et al.* [82]. In particular, *Sphingomonas* has been reported as part of fish beneficial microbiota [83], and strains of this genus isolated from the gut microbiome of gilthead sea bream exhibited antibacterial activity against fish pathogens, such as *Vibrio alginolyticus* and *Photobacterium damselae* [84]. It is thus tempting to speculate that the observed enrichment in these microorganisms in the DG microbiome of *P. caerulea* individuals growing in the aquaculture site represents a protective feature, resulting from an adaptive selection of protective microbiome components from the farmed fish.

Taken together our results support the hypothesis that aquaculture impacts the surrounding microbial communities, not only the ones from underlying sediments, but also the microbiome of locally dwelling wild holobionts. According to our data, this seems to happen either directly, through the transfer of microorganisms from the farmed fish microbiomes to the microbiomes of local wild holobionts, and indirectly, with the aquaculture practice changing the chemical conditions of the environment, resulting in the selection of specific microbiome components in the local marine metacommunities. Changes in *P. caerulea* DG microbiome in individuals growing at the aquaculture site involve the loss of several microorganisms assigned to bacteria commonly found in wild marine organisms, as well as the concomitant acquisition of potential fish and human pathogens and parasites, resulting in an overall significantly lower ecosystem biodiversity. Even if these features generally mirror dysbiotic changes, we were also able to observe possible adaptive microbiome variations, showing the inherent potential of holobiont microbiomes counterpart in allowing the host adaptation to the changing environment, included changes resulting from marine aquaculture practices.

References

Abdel-Aziz, M., Eissa, A. E., Hanna, M., & Abou Okada, M. (2013). Identifying some pathogenic Vibrio/ Photobacterium species during mass mortalities of cultured Gilthead seabream (Sparus aurata) and European seabass (Dicentrarchus labrax) from some Egyptian coastal provinces. *Interna,onal Journal of Veterinary Science and Medicine*, *1*(2), 87-95.

Aceves, A. K., Johnson, P., Bullard, S. A., Lafrentz, S., & Arias, C. R. (2018). Description and characterization of the digestive gland microbiome in the freshwater mussel Villosa nebulosa (Bivalvia: Unionidae). *Journal of Molluscan Studies*, *84*(3), 240-246.

Adair, K. L., & Douglas, A. E. (2017). Making a microbiome: the many determinants of host-associated microbial community composition. *Current Opinion in Microbiology*, *35*, 23-29.

Ape, F., Manini, E., Quero, G. M., Luna, G. M., Sarà, G., Vecchio, P., ... & Mirto, S. (2019). Biostimulation of in situ microbial degradation processes in organically-enriched sediments mitigates the impact of aquaculture. *Chemosphere*, *226*, 715-725.

Baltar, F., Gutiérrez-Rodríguez, A., Meyer, M., Skudelny, I., Sander, S., Thomson, B., ... & Morales, S. E. (2018). Specific effect of trace metals on marine heterotrophic microbial activity and diversity: Key role of iron and zinc and hydrocarbon-degrading bacteria. *Fron,ers in microbiology*, *9*, 3190.

Baquiran, J. I. P., & Conaco, C. (2018). Sponge-microbe partnerships are stable under eutrophication pressure from mariculture. *Marine pollution bulletin*, *136*, 125-134.

Biagi, E., Caroselli, E., Barone, M., Pezzimenti, M., Teixido, N., Soverini, M., ... & Candela, M. (2020). Pa`erns in microbiome composition differ with ocean acidification in anatomic compartments of the Mediterranean coral

Astroides calycularis living at CO2 vents. *Science of The Total Environment*, *724*, 138048.

Bordenstein, S. R., & Theis, K. R. (2015). Host biology in light of the microbiome: ten principles of holobionts and hologenomes. *PLoS biology*, *13*(8), e1002226.

Cabello-Yeves, P. J., Ghai, R., Mehrshad, M., Picazo, A., Camacho, A., & Rodriguez-Valera, F. (2017). Reconstruction of diverse verrucomicrobial genomes from metagenome datasets of freshwater reservoirs. *Frontiers in microbiology*, *8*, 2131.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., BiÑnger, K., Bushman, F. D., Costello, E. K., ... & Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature methods*, *7*(5), 335-336.

Cavicchioli, R., Ripple, W. J., Timmis, K. N., Azam, F., Bakken, L. R., Baylis, M., ... & Webster, N. S. (2019). Scientists' warning to humanity: microorganisms and climate change. *Nature Reviews Microbiology*, *17*(9), 569-586.

Choi, A., Cho, H., Kim, B., Kim, H. C., Jung, R. H., Lee, W. C., & Hyun, J. H. (2018). Effects of finfish aquaculture on biogeochemistry and bacterial communities associated with sulfur cycles in highly sulfidic sediments. *Aquaculture Environment Interactions*, *10*, 413-427.

Cleary, D. F., Swierts, T., Coelho, F. J., Polónia, A. R., Huang, Y. M., Ferreira, M. R., ... & de Voogd, N. J. (2019). The sponge microbiome within the greater coral reef microbial metacommunity. *Nature communications*, *10*(1), 1-12.

Coleman, R. A., Underwood, A. J., BenedeÑ-Cecchi, L., Åberg, P., Arenas, F., Arrontes, J., ... & Hawkins, S. J. (2006). A continental scale evaluation of the role of limpet grazing on rocky shores. *Oecologia*, *147*(3), 556-564.

Cornall, A., Rose, A., Streten, C., McGuinness, K., Parry, D., & Gibb, K. (2016). Molecular screening of microbial communities for candidate indicators of multiple metal impacts in marine sediments from northern Australia. *Environmental toxicology and chemistry*, *35*(2), 468-484.

Culhane, A. C., Thioulouse, J., Perrière, G., & Higgins, D. G. (2005). MADE4: an R package for multivariate analysis of gene expression data. *Bioinformatics*, *21*(11), 2789-2790. Della Santina, P., & Chelazzi, G. (1991). Temporal organization of foraging in two Mediterranean limpets, Patella rustica L. and P. coerulea L. *Journal of experimental marine biology and ecology*, *153*(1), 75-85.

Dickson, A. G., & Millero, F. J. (1987). A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep Sea Research Part A. Oceanographic Research Papers*, *34*(10), 1733-1743. Dickson, A. G. (1990). Thermodynamics of the dissociation of boric acid in synthetic seawater from 273.15 to 318.15 K. *Deep Sea Research Part A. Oceanographic Research Papers*, *37*(5), 755-766.

Duarte, L. N., Coelho, F. J., Oliveira, V., Cleary, D. F., Martins, P., & Gomes , N. C. (2019) . Characterization of bacterioplankton communities from a hatchery recirculating aquaculture system (RAS) for juvenile sole (Solea senegalensis) production. *PloS one*, *14*(1), e0211209.

Dudek, M., Adams, J., Swain, M., Hegarty, M., Huws, S., & Gallagher, J. (2014). Metaphylogenomic and potential functionality of the limpet Patella pellucida's gastrointestinal tract microbiome. *International journal of molecular sciences*, *15*(10), 18819-18839.

Estruch, G., Collado, M. C., Peñaranda, D. S., Tomás Vidal, A., Jover Cerdá, M., Pérez Mar¡nez, G., & Martinez-Llorens, S. (2015). Impact of fishmeal replacement in diets for gilthead sea bream (Sparus aurata) on the gastrointestinal microbiota determined by pyrosequencing the 16S rRNA gene. *PloS one*, *10*(8), e0136389.

Faith, D. P. (1992). Conservation evaluation and phylogenetic diversity. *Biological conservation*, *61*(1), 1-10.

Feldman, S. H., Wimsa`, J., Marchang, R. E., Johnson, A. J., Brown, W., Mitchell, J. C., & Sleeman, J. M. (2006). A novel mycoplasma detected in association with upper respiratory disease syndrome in free-ranging eastern box turtles (Terrapene carolina carolina) in Virginia. *Journal of Wildlife Diseases*, *42*(2), 279-289.

Ferreira, C., Soares, A. R., Lamosa, P., Santos, M. A., & Da Costa, M. S. (2016). Comparison of the compatible solute pool of two slightly halophilic planctomycetes species, Gimesia maris and Rubinisphaera brasiliensis. *Extremophiles*, *20*(6), 811-820.

Floris, R., Manca, S., & Fois, N. (2013). Microbial ecology of intestinal tract of gilthead sea bream (Sparus aurata Linnaeus, 1758) from two coastal lagoons of Sardinia (Italy). *Transitional Waters Bulletin*, *7*(2), 4-12.

Floris, R., Scanu, G., Fois, N., Rizzo, C., Malavenda, R., Spanò, N., & Lo Giudice, A. (2018). Intestinal bacterial flora of Mediterranean gilthead sea bream (Sparus aurata Linnaeus) as a novel source of natural surface-active compounds. *Aquaculture Research*, *49*(3), 1262-1273.

Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., ... & Venter, J. C. (1995). The minimal gene complement of Mycoplasma genitalium. *Science*, *270*(5235), 397-404.

Fraune, S., & Zimmer, M. (2008). Host-specificity of environmentally transmitted Mycoplasma-like isopod symbionts. *Environmental Microbiology*, *10*(10), 2497-2504.

Grigorakis, K., & Rigos, G. (2011). Aquaculture effects on environmental and public welfare–the case of Mediterranean mariculture. *Chemosphere*, *85*(6), 899-919. Hamilton, N. E., & Ferry, M. (2018). ggtern: Ternary diagrams using ggplot2. *Journal of Statistical So0ware*, *87*(1), 1-17.

Haro-Moreno, J. M., Coutinho, F. H., Zaragoza-Solas, A., Picazo, A., Almagro-Moreno, S., & López-Pérez, M. (2020). Dysbiosis in marine aquaculture revealed through microbiome analysis: Reverse ecology for environmental sustainability. *FEMS Microbiology Ecology*, *96*(12), fiaa218.

Holm, J. B., & Heidelberg, K. B. (2016). Microbiomes of Muricea californica and M. fruticosa: comparative analyses of two co-occurring eastern Pacific octocorals. *Frontiers in Microbiology*, *7*, 917.

Holmer, M., Duarte, C. M., Heilskov, A., Olesen, B., & Terrados, J. (2003). Biogeochemical conditions in sediments enriched by organic ma`er from net-pen fish farms in the Bolinao area, Philippines. *Marine Pollution Bulletin*, *46*(11), 1470-1479.

Hornick, K. M., & Buschmann, A. H. (2018). Insights into the diversity and metabolic function of bacterial communities in sediments from Chilean salmon aquaculture sites. *Annals of microbiology*, *68*(2), 63-77.

Infante-Dominguez, C., Lawson, P. A., Johnson, C. N., Sanchez Porro, C., & Ventosa, A. (2015). Fodinicurvata halophila sp. nov., a moderately halophilic bacterium from a marine saltern. *International journal of systematic and evolutionary microbiology*, *65*(Pt_3), 766-771.

Kalantzi, I., Rico, A., Mylona, K., Pergantis, S. A., & Tsapakis, M. (2021). Fish farming, metals and antibiotics in the eastern Mediterranean Sea: Is there a threat to sediment wildlife?. *Science of The Total Environment*, *764*, 142843.

Kallscheuer, N., Jogler, M., Wiegand, S., Peeters, S. H., Heuer, A., Boedeker, C., ... & Jogler, C. (2020). Rubinisphaera italica sp. nov. isolated from a hydrothermal area in the Tyrrhenian Sea close to the volcanic island Panarea. *Antonie Van Leeuwenhoek*, *113*(12), 1727-1736.

Karakassis, I., Tsapakis, M., Hatziyanni, E., Papadopoulou, K. N., & Plaiti, W. (2000). Impact of cage farming of fish on the seabed in three Mediterranean coastal areas. *ICES Journal of Marine Science*, *57*(5), 1462-1471.

King, G. M., Judd, C., Kuske, C. R., & Smith, C. (2012). Analysis of stomach and gut microbiomes of the eastern oyster (Crassostrea virginica) from coastal Louisiana, USA. *PLoS one*, *7*(12), e51475.

Kuebutornye, F. K., Abarike, E. D., Lu, Y., Hlordzi, V., Sakyi, M. E., Afriyie, G., ... & Xie, C. X. (2020). Mechanisms and the role of probiotic Bacillus in mitigating fish pathogens in aquaculture. *Fish physiology and biochemistry*, 1-23.

Lamin, H., Alami, S., Bouhnik, O., Bennis, M., Benkritly, S., Abdelmoumen, H., ... & Missbah-El Idrissi, M. (2021). Identification of the endosymbionts from Sulla spinosissima growing in a lead mine tailings in Eastern Morocco as Mesorhizobium camelthorni sv. aridi. *Journal of Applied Microbiology*, *130*(3), 948-959.

Legrand, T. P., Wynne, J. W., Weyrich, L. S., & Oxley, A. P. (2020). A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. *Reviews in Aquaculture*, *12*(2), 1101-1134.

Leibold, M. A., Holyoak, M., Mouquet, N., Amarasekare, P., Chase, J. M., Hoopes, M. F., ... & Gonzalez, A. (2004). The metacommunity concept: a framework for multi-scale community ecology. *Ecology letters*, *7*(7), 601-613.

Liquete, C., Piroddi, C., Drakou, E. G., Gurney, L., Katsanevakis, S., Charef, A., & Egoh, B. (2013). Current status and future prospects for the assessment of marine and coastal ecosystem services: a systematic review. *PloS one*, *8*(7), e67737.

Luna, G. M., Corinaldesi, C., Dell'Anno, A., Pusceddu, A., & Danovaro, R. (2013). Impact of aquaculture on benthic virus–prokaryote interactions in the Mediterranean Sea. *Water research*, *47*(3), 1156-1168.

Marzocchi, U., Bonaglia, S., Zaiko, A., Quero, G. M., Vybernaite-Lubiene, I., Politi, T., ... & Cardini, U. (2021). Zebra mussel holobionts fix and recycle nitrogen in lagoon sediments. *Frontiers in microbiology*, 3620.

Mathai, P. P., Magnone, P., Dunn, H. M., & Sadowsky, M. J. (2020). Water and sediment act as reservoirs for microbial taxa associated with invasive dreissenid mussels. *Science of the Total Environment*, *703*, 134915.

Mehrbach, C., Culberson, C. H., Hawley, J. E., & Pytkowicx, R. M. (1973). Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure 1. *Limnology and oceanography*, *18*(6), 897-907.

Mente, E., Nikouli, E., Antonopoulou, E., Martin, S. A., & Kormas, K. A. (2018). Core versus diet-associated and postprandial bacterial communities of the rainbow trout (Oncorhynchus mykiss) midgut and faeces. *Biology Open*, *7*(6), bio034397.

Meyer, D.; Zeileis, A.; Hornik, K. vcd: Visualizing Categorical Data. *R package* version 1.4-8, 2020.

Minich, J. J., Poore, G. D., Jantawongsri, K., Johnston, C., Bowie, K., Bowman, J., ... & Allen, E. E. (2020). Microbial ecology of Atlantic salmon (Salmo salar) hatcheries: impacts of the built environment on fish mucosal microbiota. *Applied and environmental microbiology*, *86*(12), e00411-20.

Moncada, C., Hassenrück, C., Gärdes, A., & Conaco, C. (2019). Microbial community composition of sediments influenced by intensive mariculture activity. *FEMS microbiology ecology*, *95*(2), fiz006.

Montalto, V., Rinaldi, A., Ape, F., Mangano, M. C., Gristina, M., Sarà, G., & Mirto, S. (2020). Functional role of biofouling linked to aquaculture facilities in Mediterranean enclosed locations. *Aquaculture Environment Interactions*, *12*, 11-22.

Musella, M., Wathsala, R., Tavella, T., Rampelli, S., Barone, M., Palladino, G., ... & Candela, M. (2020). Tissue-scale microbiota of the Mediterranean mussel (Mytilus galloprovincialis) and its relationship with the environment. *Science of The Total Environment*, *717*, 137209.

Ontañon, O. M., Landi, C., Carleo, A., Gagliardi, A., Bianchi, L., González, P. S., ... & Bini, L. (2018). What makes A. guillouiae SFC 500-1A able to co-metabolize phenol and Cr (VI)? A proteomic approach. *Journal of hazardous materials*, *354*, 215-224. Pierce, M. L. (2016). The Microbiome of the eastern oyster, Crassostrea virginica (Gmelin, 1791): Temporal and spatial variation, environmental influences, and its impact on host physiology.

Pierce, M. L., & Ward, J. E. (2018). Microbial ecology of the Bivalvia, with an emphasis on the family Ostreidae. *Journal of Shellfish Research*, *37*(4), 793-806.

Pierce, M. L., & Ward, J. E. (2019). Gut microbiomes of the eastern oyster (Crassostrea virginica) and the blue mussel (mytilus edulis): Temporal variation and the influence of marine aggregate-associated microbial communities. *Msphere*, *4*(6), e00730-19.

Pita, L., Rix, L., Slaby, B. M., Franke, A., & Hentschel, U. (2018). The sponge holobiont in a changing ocean: from microbes to ecosystems. *Microbiome*, *6*(1), 1-18.

Pohlert, T. (2014). The pairwise multiple comparison of mean ranks package (PMCMR). *R package*, *27*(2019), 9.

Quero, G. M., Perini, L., Pesole, G., Manzari, C., Lione., C., Bastianini, M., ... & Luna, G. M. (2017). Seasonal rather than spatial variability drives planktonic and benthic bacterial diversity in a microtidal lagoon and the adjacent open sea. *Molecular ecology*, *26*(21), 5961-5973.

Reguera, P., Couceiro, L., & Fernández, N. (2018). A review of the empirical literature on the use of limpets Patella spp. (Mollusca: Gastropoda) as bioindicators of environmental quality. *Ecotoxicology and environmental safety*, *148*, 593-600.

Rosa, R., Marques, A., & Nunes, M. L. (2012). Impact of climate change in Mediterranean aquaculture. *Reviews in Aquaculture*, *4*(3), 163-177.

Rubio-Portillo, E., Villamor, A., Fernandez-Gonzalez, V., Antón, J., & Sanchez-Jerez, P. (2019). Exploring changes in bacterial communities to assess the influence of fish farming on marine sediments. *Aquaculture*, *506*, 459-464.

San Diego-McGlone, M. L., Azanza, R. V., Villanoy, C. L., & Jacinto, G. S. (2008). Eutrophic waters, algal bloom and fish kill in fish farming areas in Bolinao, Pangasinan, Philippines. *Marine Pollution Bulletin*, *57*(6-12), 295-301.

Sanz-Lázaro, C., Belando, M. D., Marín-Guirao, L., Navarrete Mier, F., & Marín, A. (2011). Relationship between sedimentation rates and benthic impact on Maërl beds derived from fish farming in the Mediterranean. *Marine Environmental Research*, *71*(1), 22-30.

Sharifah, E. N., & Eguchi, M. (2012). Mixed cultures of the phytoplankton Nannochloropsis oculata and the marine bacterium Sulfitobacter sp. RO3 inhibit the growth of virulent strains of the major fish pathogen Vibrio anguillarum. *Aquaculture Science*, *60*(1), 39-45.

Shi, R., Xu, S., Qi, Z., Zhu, Q., Huang, H., & Weber, F. (2019). Influence of suspended mariculture on vertical distribution profiles of bacteria in sediment from Daya Bay, Southern China. *Marine pollution bulletin*, *146*, 816-826.

Sichert, A., Corze`, C. H., Schechter, M. S., Unfried, F., Markert, S., Becher, D., ... & Hehemann, J. H. (2020). Verrucomicrobia use hundreds of enzymes to digest the algal polysaccharide fucoidan. *Nature microbiology*, *5*(8), 1026-1039.

Sizikov, S., Burgsdorf, I., Handley, K. M., Lahyani, M., Haber, M., & Steindler, L. (2020). Characterization of sponge-associated Verrucomicrobia: microcompartment-based sugar utilization and enhanced toxin–antitoxin modules as features of host-associated Opitutales. *Environmental microbiology*, *22*(11), 4669-4688.

Sohn, H., Kim, J., Jin, C., & Lee, J. (2019). Identification of Vibrio species isolated from cultured olive flounder (Paralichthys olivaceus) in Jeju Island, South Korea. *Fisheries and Aquatic Sciences*, *22*(1), 1-8.

Tičina, V., Katavić, I., & Grubišić, L. (2020). Marine Aquaculture Impacts on Marine Biota in Oligotrophic Environments of the Mediterranean Sea–A Review. *Frontiers in Marine Science*, *7*, 217.

Trevathan-Tacke`, S. M., Sherman, C. D., Hugge`, M. J., Campbell, A. H., Laverock, B., Hurtado-McCormick, V., ... & Macreadie, P. I. (2019). A horizon scan of priorities for coastal marine microbiome research. *Nature ecology & evolution*, *3*(11), 1509-1520.

Viñas, L., Pérez-Fernández, B., Soriano, J. A., López, M., Bargiela, J., & Alves, I. (2018). Limpet (Patella sp) as a biomonitor for organic pollutants. A proxy for mussel?. *Marine pollution bulletin*, *133*, 271-280.

Wang, Y., Zhang, X. H., Yu, M., Wang, H., & Austin, B. (2010). Vibrio atypicus sp. nov., isolated from the digestive tract of the Chinese prawn (Penaeus chinensis O'sbeck). *International journal of systematic and evolutionary microbiology*, *60*(11), 2517-2523.

Wilkins, L. G., Leray, M., O'Dea, A., Yuen, B., Peixoto, R. S., Pereira, T. J., ... & Eisen, J. A. (2019). Host-associated microbiomes drive structure and function of marine ecosystems. *PLoS biology*, *17*(11), e3000533.

Yoon, J. H., Lee, C. H., Kang, S. J., & Oh, T. K. (2005). Psychrobacter celer sp. nov., isolated from sea water of the South Sea in Korea. *International Journal of Systematic and Evolutionary Microbiology*, *55*(5), 1885-1890.

Yosef, D. Z. B., Ben-Dov, E., & Kushmaro, A. (2008). Amorphus coralli gen. nov., sp. nov., a marine bacterium isolated from coral mucus, belonging to the order Rhizobiales. *International journal of systematic and evolutionary microbiology*, *58*(12), 2704-2709.

Zhang, K., Zheng, X., He, Z., Yang, T., Shu, L., Xiao, F., ... & Yan, Q. (2020). Fish growth enhances microbial sulfur cycling in aquaculture pond sediments. *Microbial biotechnology*, *13*(5), 1597-1610. 64

2.6 Study V - Microbiome network in the pelagic and benthic offshore systems of the northern Adriatic Sea (Mediterranean Sea)

Scicchitano, D., Lo Martire, M., Palladino, G., Nanetti, E., Fabbrini, M., Dell'Anno, A., ... & Candela, M. (2022). Microbiome network in the pelagic and benthic offshore systems of the northern Adriatic Sea (Mediterranean Sea). Scientific Reports, 12(1), 16670.

Introduction

In marine-ecosystems microbes represent the most abundant and diverse biological components, and account for up to 10% of the total microbial biomass on our planet [1, 2]. Microbes, including bacteria, are responsible for energy fluxes in the marine food webs [3; 4], and play a central role in the global biogeochemical cycles and ecosystem functioning [5, 6, 7]. Because of their global importance, numerous studies have focused on mapping the diversity of marine microbiomes, to understand their distribution patterns and environmental drivers [8; 9; 10] and to assess their potential response to future climate changes [11, 12, 13]. So far, contrasting patterns have been reported, potentially due to the different spatial scales, habitat characteristics and the level of taxonomic resolution at which studies have been conducted [14, 15, 8, 16]. Relevant insights in this direction have been provided by Tara Ocean in 2015 [17], which collected up to 35.000 samples from surface to mesopelagic waters at the global scale to provide a first inventory of the global diversity of microbiomes and to identify factors shaping their composition. These investigations revealed that longitude and environmental factors (mainly temperature and dissolved oxygen) combine to shape the microbiome composition in the global oceans and are responsible, at least in part, of the observed biogeographical patterns. Conversely, latitude explained only a minimal fraction of the observed diversity [9]. Other key studies based on a systematic and coupled analysis of the pelagic and benthic microbiomes from globally distributed samples, showed structured biogeographical patterns of marine bacterial assemblages, only partially explained by the assessed environmental factors (e.g., temperature, oxygen and pH). Furthermore, a remarkable difference in the composition of pelagic and benthic bacterial assemblages was observed, revealing a pelagic-benthic coupling [18] limited to the 7% of the total communities in open waters.

Studies, which specifically addressed the latitudinal patterns of diversity at the global scale, reported an increase in microbiome dissimilarity with increasing distance from the sampling points up to 5000 km [17]. However, the diversity of microbiomes at regional scale (distance between sampling sites <100 km) was only slightly lower than for larger distances, suggesting the existence of a relevant variability in marine microbial communities even at such spatial scale. This finding was also confirmed by studies on the taxonomic composition of benthic prokaryotic assemblages along bathymetric gradients in Mediterranean Sea, which reported high local variability of microbial assemblages [19], potentially due to intra-specific interactions, limited dispersion, and historical contingencies, which may combine with stochastic physical disturbances [20]. Taken together, these findings suggest the existence of a relevant degree of marine microbiome plasticity at the local scale, both for the pelagic and benthic communities, whose range and degree of variability, as well as functional implications, still need to be dissected. In order to provide some glimpses in this direction, in the present work we conducted a synoptical study on the microbiomes of the water column and surface sediments from 19 sites in a 130 km^2 area located 13.5 Km afar from the Emilia Romagna coast (Italy), in the North-Western Adriatic Sea (Mediterranean Sea).

The Northwestern Adriatic Sea is characterized by shallow waters (maximum depth: ca. 40 m) and, in the coastal area, the ecosystem productivity is mainly sustained by nutrient inputs, especially from the Po river [20, 21]. Two currents dominate the circulation in Adriatic: the Western Adriatic Current (WAC), flowing toward the southeast along the Western Italian coast, and the East Adriatic Current (EAC) which flows from the northwest along the eastern Croatian coast [20,21].

Riverine inflow into the northern Adriatic forms a buoyant coastal layer - the Western Coastal Layer (WCL) - flowing southward along the Italian coast. The principal compensating inflow occurs along the eastern boundary by EAC, where warm, high-salinity Levantine Intermediate Waters (LIW) is advanced to the North [21,22]. During the pre-winter and winter periods, after the development of the coastal thermohaline front, the inflow of fresh waters from the Po river (and other sources along the coast) is prevalently retained inside the coastal zone, establishing a dynamic limitation between inshore and offshore systems were riverine nutrients are mainly kept in the coastal area [21,22]. The spring inversion of the total heat budget leads to a decrease in the density of the surface layer and generates a thermocline. Therefore - during the late spring and summer - the water column is highly stratified [22,23] and 3 different layers separates over the whole northern basin. The low-density surface layer is directly influenced by runoff and distribution of diluted riverine waters, while the bottom layer is initially occupied by cold, dense, non-diluted winter waters, later replaced by deep middle Adriatic waters. In these stratified conditions, surface waters flow from the coastal area and inject into the surface layer to reach toward the center of the basin [22,23,24].

In our work, by applying 16S rRNA Next Generation Sequencing and network-based approach, we have been able to map the variation at the local-scale of the pelagic and sediment microbiomes in the Northwestern Adriatic Sea. The coupled investigation of the pelagic and benthic microbiomes from each sampling site also allowed us to identify connections, exchanges, and isolation of microbial members in the two realms. Together with the dissection of the respective microbiome network structures, the present study allowed us to provide new insights into the structuring of the marine microbial assemblages at the local and regional scales.

Materials and Methods

Study Area and Sampling Procedure

The present study was conducted in September 2021 in 19 sites (whose geographic coordinates, water depths and distance from coast are reported in **Supplementary Table 1)** located in an offshore area of 130 Km^2 in the North-western Adriatic Sea (Latitude: from 44.0686667 to 44.2524444 and Longitude: from 12.72288889 to 12.90647222M **Figure 1**). From each site, one sample of water (10 m depth) and one to 3 samples of sediment were collected, for a total of 19 water samples and 25 sediment samples. Water samples were collected using a Niskin bottle. Immediately after collection, 2L of sea water were poured into a previously sterilized plastic bottle. Surface sediments (the top 10 cm) were also collected, using a Van Veen grab. After homogenization, a portion of 10 grams of them was transferred into sterile plastic containers. Samples were stored in the dark until arrival at the laboratory. While sediments were immediately frozen at -80°C, water samples were filtered onto 47 mm diameter cellulose mixed ester 0.2 µm pore-size filters (MF-Millipore) through vacuum filtration system [25] under laminar flow hood. Filters were stored in sterile Eppendorf at –80 °C until processed.

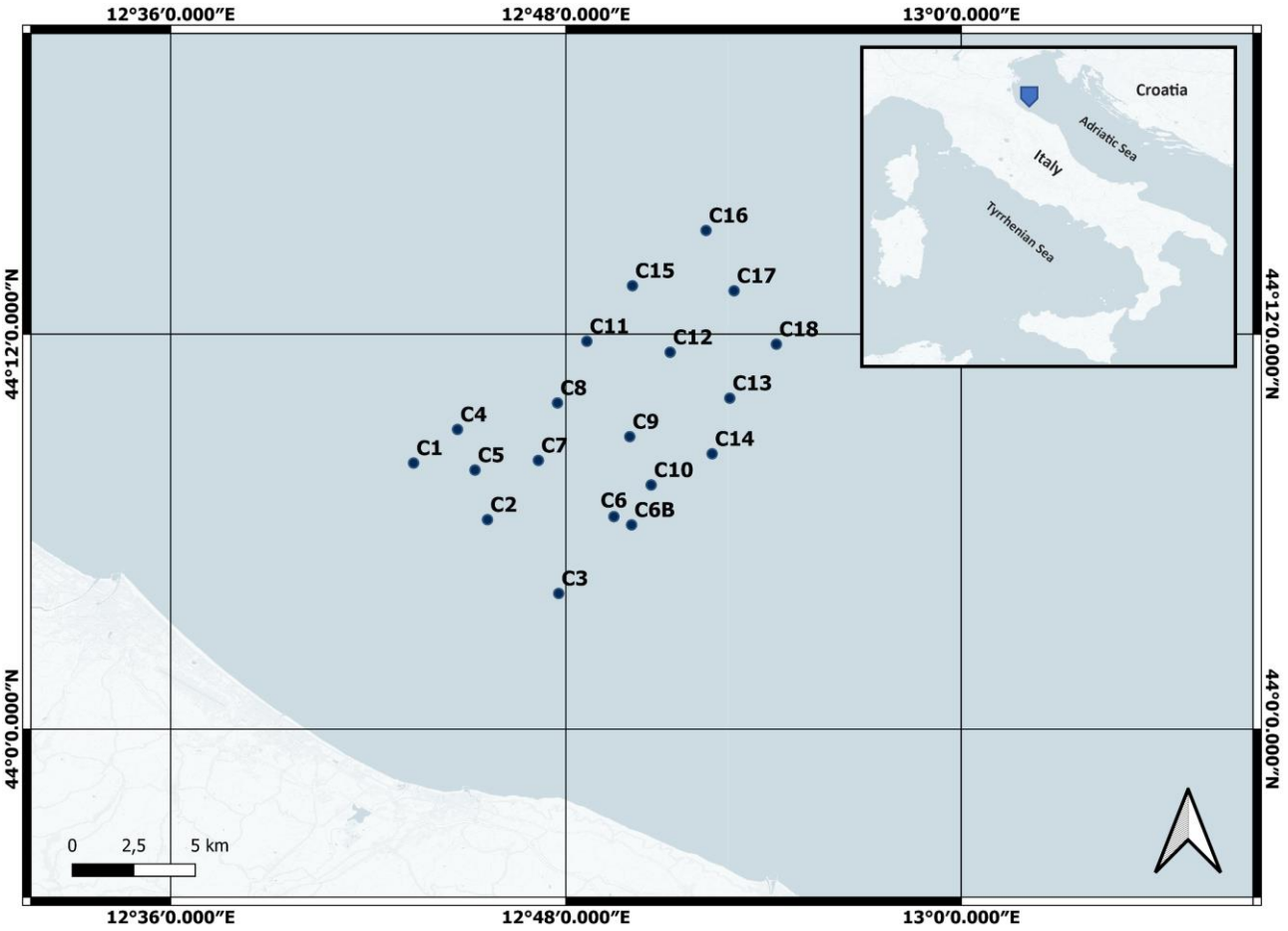


Figure 1: Sampling site and offshore study area. Sampling sites (both for water and sediment) are represented as blue dots.

Biochemical Components of the sedimentary Organic Matter

Chlorophyll-*a*, phaeopigment, protein, carbohydrate and lipid concentrations in sediment samples were analyzed according to Danovaro [26]. Briefly, chlorophyll-*a* and phaepigments were analyzed fluorometrically and total phytopigment concentrations were defined as their sum. Proteins, carbohydrates and lipids were determined spectrophotometrically [26]. Concentrations of proteins, carbohydrates, and lipids were converted into carbon equivalents using 0.49, 0.40 and 0.75 mgC mg^{-1}, as conversion factors, respectively and their sum defined as biopolymeric carbon (BPC, a proxy of available trophic resources, [27]).

Microbial DNA Extraction

Extraction of the total DNA from water samples was performed from the entire membrane filters using the DNAeasy PowerWater extraction kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions [28]. For sediment samples, 250 mg of each sample was weighed, and total DNA was extracted with the DNAeasy PowerSoil kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with minor adjustments. Specifically, all samples were homogenized using the FastPrep instrument (MP Biomedicals, Irvine, CA) at 5.5 movements/s for 1 min, repeated for three cycles, and the elution step was preceded by a 5-min incubation at 4 °C [29]. Extracted DNA was then quantified by using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at -20 °C until further processing.

Sequencing, library preparation and bioinformatic analysis

PCR amplification of the V3-V4 hypervariable region of the 16S rRNA gene was carried out in a 50 µL final volume reaction containing 25 ng of microbial DNA, 2X KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), and 200 nmol/L of 341F and 785R primers carrying Illumina overhang sequencing adapter. For water samples the thermal cycle consisted of 3 minutes at 95 °C, 25 cycles of 30 s at 95 °C – 30 s at 55 °C and 30 s at 72 °C, and a final elongation step of 5 min at 72 °C [28]. Sediment samples followed the same PCR amplification protocol with a total of 30 amplification cycles [29]. PCR products were purified with Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA). Indexed libraries were prepared by limited-cycle PCR with Nextera technology and cleaned-up as described above. Libraries were normalized to 4 nM and pooled. The sample pool was denatured with 0.2 N NaOH and diluted to a final concentration of 6 pM with a 20% PhiX control. Sequencing was performed on an Illumina MiSeq platform using a 2 × 250 bp paired-end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA, USA). A pipeline combining PANDAseq [30] and QIIME2 [31] was used to process raw sequences. High-quality reads (min/max length = 350/550 bp) were retained using the "fastq filters" function of Usearch11 [32]. Specifically, reads with an expected error per base E = 0.03 (i.e., 3 expected errors every 100 bases) were discarded, based on the phred Q score probabilities. The resulting reads from the length and quality filtering were binned into amplicon sequence variants (ASVs) using DADA2 [33], the Taxonomy was assigned using the VSEARCH algorithm [34] against SILVA database (December 2017 release) [35]. All the sequences assigned to eukaryotes (i.e., chloroplasts and mitochondria) or unassigned were discarded. Sequencing reads were deposited in ENA (project number PRJEB52873).

Definition of the alpha-diversity sectors

The QGIS software [36] was used to construct the maps of the study area and to construct the maps based on the Shannon alpha diversity values of each water and sediment sample. The longitude and latitude geographical coordinates (**Supplementary Table 1**) were used to plot the precise sampling locations into the software. The distribution of the Shannon alpha diversity values across the samples was obtained through the *Triangulated Irregular Network* interpolation method on QGIS (TIN interpolation). In order to define the alpha-diversity sectors, for both the water and the sediment microbiomes, samples distribution according to the Shannon alpha-diversity values were first obtained. The obtained ranks have been than utilized for the identification of correspondent alpha-diversity sectors in the area under study. More specifically, for the water microbiome, the following alpha-diversity sectors have been identified: (i) South-sector, where > than 70% of the correspondent samples were included in the 3° and 4° alpha-diversity quartiles; (ii) Central-sector, where 100% of the correspondent samples were included in the 1° and 2° alpha-diversity quartiles; (iii) North-sector, where > than 65 % of the of the correspondent samples were included in the 3° and 4° alpha-diversity quartiles. Analogously, for the sediment microbiome, the following sectors have been identified: (i) South sector, where > than 80% of the correspondent samples were included in the 1° and 2° alpha-diversity quartiles; (ii) North-east sector, where 90% of the correspondent samples were included in the 3° and 4° alpha-diversity quartiles; (iii) North-west sector, where > than 80 % of the of the correspondent samples were distributed between in the 2° and 4°alpha-diversity quartiles. The quartile distribution of the water and sediment samples and the corresponding sector are reported In **Supplementary Table 2**.

Biostatistical Analysis and networks construction

All statistical analyses were performed using the R software [37], using the packages "Made4" [38] and "vegan" [39]. Unweighted UniFrac distances were plotted using the vegan package, and the data separation in the Principal Coordinates Analysis (PCoA) was tested using a permutation test with pseudo-F ratios (function "adonis" in the vegan package). Wilcoxon rank-sum test and Kruskal-Wallis test were used to assess significant differences in alpha diversity and taxon relative abundance between groups. *P*-values were corrected for multiple testing with "p.adjust" function in R, with a false discovery rate (FDR) ≤ 0.05 considered statistically significant. Bacterial co-abundance groups (CAGs) were identified as previously described [40; 41; 42]. Briefly, the associations among the bacterial orders were evaluated using the Kendall correlation test visualized using hierarchical Ward clustering with a Spearman correlation distance metrics. The Wiggum plot network analysis was created using Cytoscape [43]. Circle sizes were proportional to orders abundance or over-abundance, and connections between nodes were represented as "gray line" or "red line" for positive or negative correlation, respectively. Over-abundance values were calculated using the ratio between the mean relative abundance in a specific area and the average relative abundance in the whole area of the study (meanArea/meanTot). Hub nodes, cohesion and modularity identification/calculation were based on area-specific networks obtained by FlashWeave [44] and the correspondent samples for each area. Specifically, hub nodes were identified for each microbial network by looking to the combination of the highest values of closeness centrality, betweenness centrality and degree on Cytoscape [43] as previously described [45]. Cohesion and modularity were calculated with the "igraph" package in R following the same procedures proposed by Hernandez and colleagues [46].

Results

Assessment of environmental parameters in the study area

Sampling sites and the studied area are represented in **Figure 1**. During the sampling campaign, the temperature of superficial seawater was 23 °C whereas at 10-m depth of 10 °C. Data on the concentrations of proteins (PRT), carbohydrates (CHO) and lipids (LIP) as well as chlorophyll-a (Chl-a), phaeopigments (PHEO) and biopolymeric C (PBP) in the sediment samples are reported in **Supplementary Table 3**. In the study area, PRT were the dominant class of the investigated organic compounds, ranging from 1.4 to 7.96 mg/g (mean value of 4.18 ± 0.39 mg/g). CHO concentrations varied from 0.27 to 1.24 mg/g (mean value: 0.66 ± 0.06 mg/g), while LIP ranged from 0.25 to 1.62 mg/g (mean value: 0.74 ± 0.07 mg/g). Chl-a and PHEO concentrations in the sediments were, on average, 1.22 ± 0.22 µg/g and 14.66 ± 1.04 µg/g, respectively (range: 0.35-5.37 µg/g for Chl- and 6.49 to 26.91 µg/g for PHEO). Finally, the range of variability of BPC concentrations was comprised between 0.92 and 5.14 mg/g, with a mean value of 2.86 ± 0.26 mg/g.

Composition of pelagic and sediments microbiomes

The V3/V4 regions from the 16s rRNA gene was successfully sequenced from a total of 44 samples (19 waters and 25 sediments), providing 549'318 high quality reads (12'485 ± 3'235 per sample) clustered in 8'271 Amplicon Sequence Variants (ASVs) (206.8 ± 94.4 per sample). None of the detected ASVs have been assigned at the species level, while the assignment rates at the genus, family and order levels were 42, 48 and 48%, respectively. The total diversity at ASVs level was 150 for the water microbiome and 218 for the sediment one. When we assessed for the total assigned diversity at the different phylogenetic ranks, the order level showed the highest value (27.61), respect to family and genus levels scoring 23.8 and 12.96, respectively. In **Supplementary Figure 1** the general compositional structure of the water and

sediment microbiomes at the order level is provided. For the pelagic microbiome, the dominant orders were: *Synechoccus-like Cyanobacteria Subsection I* (relative abundance, rel.ab., 13.4%), *Flavobacteriales* (rel. ab. 12.3%), *Rickettsiales* (rel. ab. 8.2 %), *Oceanospirillales* (rel. ab 7.1%), *Cellvibrionales* (rel. ab. 6.5%) and *Rhodobacterales* (rel. ab. 5.3%). Among the subdominant fraction, the most represented orders were: *SAR11 clade* (rel. ab. 4.5%), *Vibrionales* (rel. ab. 4.2 %), *Planctomycetales* (rel. ab. 3.4%), *Rhodospirillales* (rel. ab. 3.3 %), *Sphingobacteriales* (rel. ab 2.9%) and *Verrucomicrobiales* (rel. ab. 2.3%). Differently, sediments were dominated by *Campylobacterales*, *Clostridiales*, *Desulfobacterales*, *Bacillales* and *Holophagae-Subgroup 10*, showing relative abundances of 10.3, 7.8, 7.4, 5.0 and 5.4%, respectively. For the benthic, *Clostridiales, Desulfobacterales* and *Campylobacterales* and *Xanthomonadales* were almost exclusive for the sediment one. However, several orders were shared between the two ecosystems. In particular, *Flavobacteriales*, *Rickettsiales*, *Cellvibrionales*, *Oceanospirillales*, *Alteromonadales, Rhodobacteriales*, and *Vibrionales* were most abundant in water samples, and *Verrucomicrobiales*, *Rhizobiales*, *Planctomycetales* and *Acidomicrobiales* were almost equally represented in water and sediment samples.

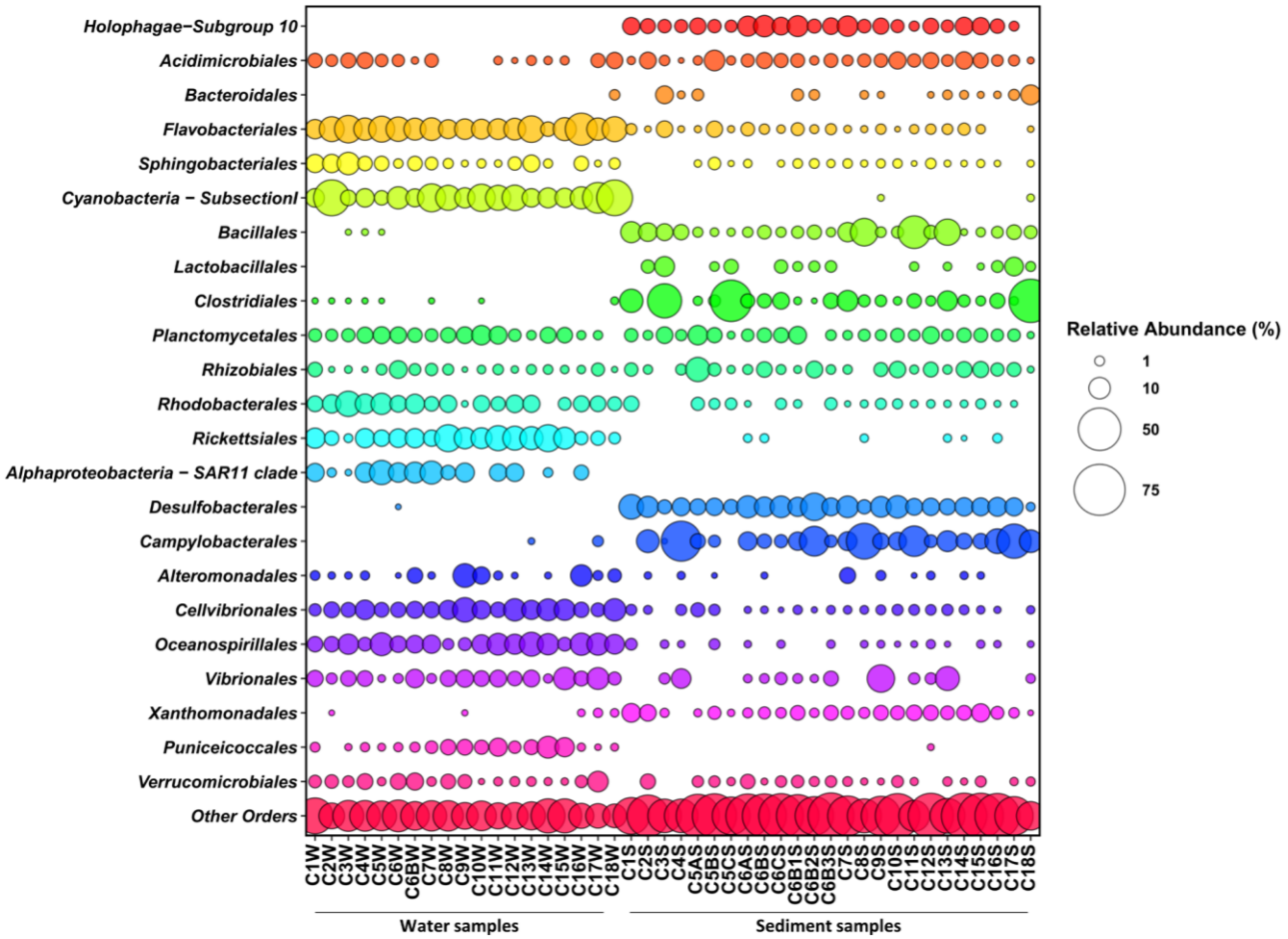


Figure 2: Bubble chart showing the relative abundance of major orders (r.ab. > 5% in at least two samples) in the water samples (left part of the graph) and sediment samples (right part of the graph).

Changes in abundance and diversity of the pelagic and sediment microbiomes at the local scale

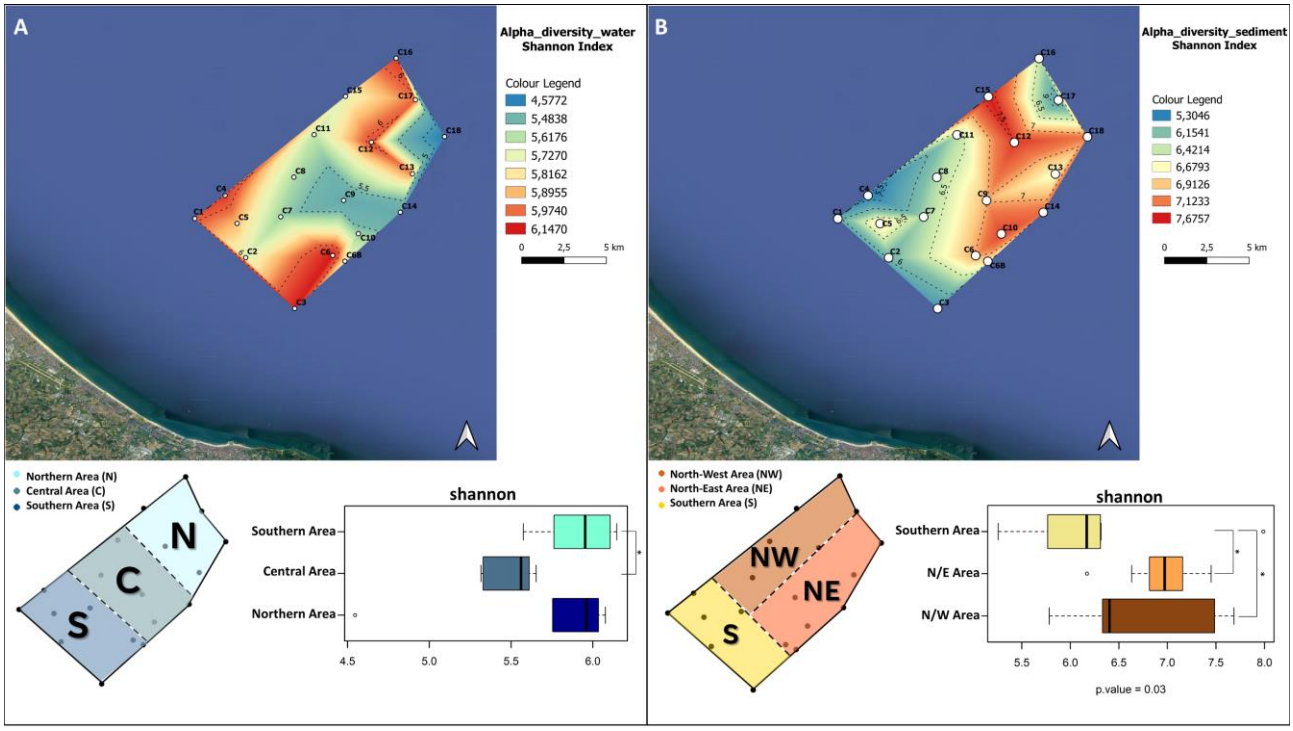


Figure 3: Representation of the alpha-diversity patterns in the area for both water and sediment samples. A) (Upper part) Distribution of the alpha diversity values among water samples, generated with TIN interpolation of the single values. Colour scale from red to blue represents a decrease in alpha diversity; black lines represent contour lines of the interpolation. (Bottom part) Subset of samples divided into 3 areas based on Shannon diversity; for water samples, the Northern Area, the Central Area and the Southern Area were identified. Box plot of Shannon index calculated for the 3 identified areas of water samples. B) (Upper part) Distribution of the alpha diversity values among sediment samples, generated with TIN interpolation of the single values. Colour scale from red to blue represents a decrease in alpha diversity; black lines represent contour lines of the interpolation. (Bottom part) Subset of samples divided into 3 areas based on Shannon diversity; for sediment samples, the North-West Area, the North-East Area and the Southern Area were identified. Box plot of Shannon index calculated for the 3 identified areas of sediment samples (Wilcoxon rank-sum test; $p < 0.05$ *).

To identify change in the microbiome patterns in the study area, we first accounted for changes in alpha and beta-diversity across the 3 transects. Specifically, to highlight alpha-diversity patterns, the area plots of the Shannon index variation in water and sediments samples were computed (**Figure 3**). For both the pelagic and sediment microbiomes high and low alpha-diversity sectors have been identified, showing significant differences in Shannon diversity values. For the pelagic ecosystem, the North and the South sectors were characterized by microbiomes with higher alpha-diversity, compared with the Central sector (**Figure 3A**). Similarly, for the sediment microbiome, two high alfa-diversity sectors were identified in the North-East and North-West sectors, while a lower diversity area was detected in the Southern sector (**Figure 3B**). We subsequently assessed beta-diversity patterns in the study area. To this aim, the PCoA of the ASVs variation in water and sediment microbiome samples was carried out. According to our findings, for the pelagic microbiome, samples belonging to the previously identified alpha-diversity sectors - South, Central and North - significantly segregated in the PCoA plot (Adonis; p = 0.001; **Figure 4A)**. Similarly, for the sediment microbiome, samples segregated according to the corresponding alpha-diversity sectors (North-Est, North-West and South) (Adonis, p = 0.006) (**Figure 4B**). When we searched for the correlations between PCoA coordinates and water column depth or distance from the coast, significant relationships were detected for both pelagic and sediment microbiomes (**Supplementary Figure S2**). For the pelagic microbiomes, the MDS1 significantly correlated with depth (R = 0.9, $p <$

0.005) and distance from the coast (R = 0.9, $p < 0.005$), while, for the sediment microbiome, we obtained analogous significant correlations but with MDS2, R = 0.25, $p < 0.01$. Further, when we assessed correlations among samples alpha-diversity and PCoA coordinates, a positive correlation with MDS1 was observed for sediment microbiomes (R = 0.6, p < 0.005), while only tendencies were obtained for the pelagic microbiome. When we accounted for differences in the biochemical composition in sediments corresponding to the sectors, we observed a higher concentration of all biochemical components of sedimentary organic matter (proteins, carbohydrates, lipids and total phytopigments) in the Northern sectors (**Figure 5)**. Finally, the correlation between the UniFrac distances matrix of sediment microbiome samples and the correspondent distance matrices of the biochemical composition was significant (**Table 1)** (Mantel Test in R). When we assessed the linear regression between the different microbial orders detected in the sediment microbiome and the concentrations of LIP, PRT and CHO, no biological relevant correlations were detected ($R^2 > 0.25$) (**Supplementary Figure S3**).

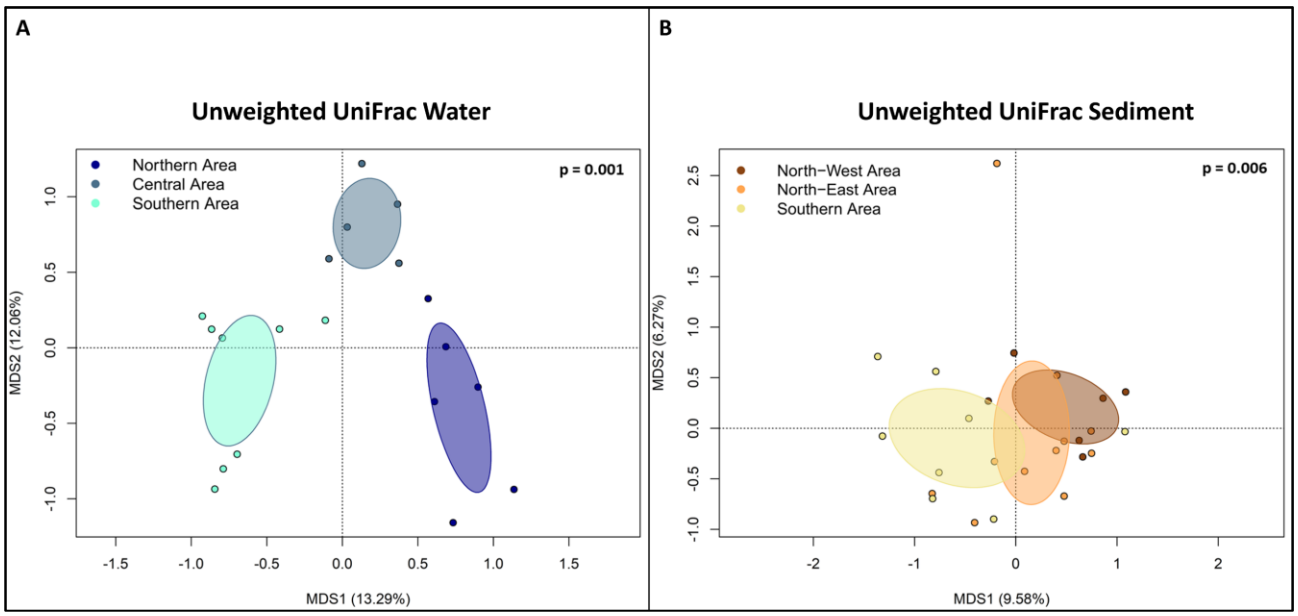


Figure 4: Beta diversity of the bacterial community of the water (A) and sediment (B) samples in the studied area. A) PCoA based on unweighted UniFrac distances between pelagic microbiome of the 3 areas, samples are significantly separated (Adonis; $p = 0.001$). B) PCoA based on unweighted UniFrac distances between sediment microbiome of the 3 areas, samples are significantly separated (Adonis; $p = 0.006$).

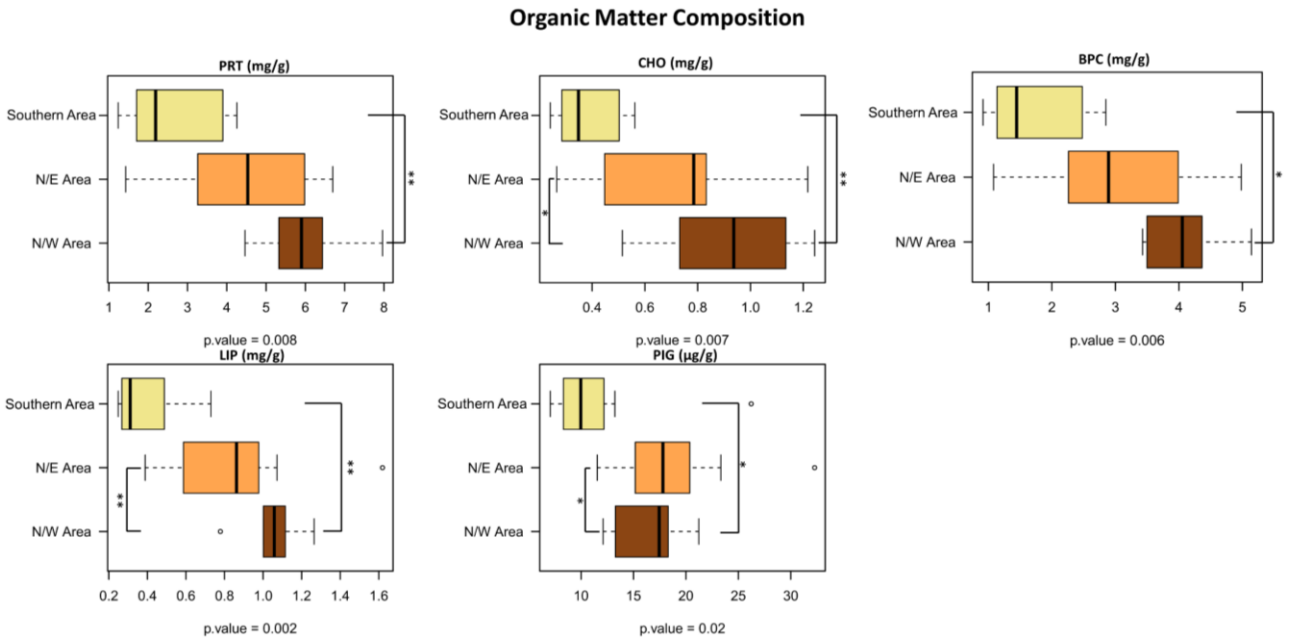


Figure 5: Boxplots showing the variation of the biochemical components across the 3 sectors of the area under study, in terms of concentration (mg/g or µg/g). The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line. Significant variations across groups are highlighted in the figure (Wilcoxon rank-sum test; $p \leq 0.05$ *, $p \leq 0.01$ **). **PRT**: Total Proteins; **CHO**: Total Carbohydrates; **LIP**: Total Lipids; **PIG**: Total Phytopigments; **BPC**: Biopolymeric Carbon.

Table 1: Output of Mantel test analyses on the Spearman correlation of the unweighted UniFrac distances of microbiome structure and distance matrix of biochemical components generated with *dist* function in R (method = "*Euclidean*"), number of permutations: 9999.

	Mantel Statistic r	Significance (p value)
Carbohydrates x UniFrac distances	0.2552	2.7e-03
Proteins correlation x UniFrac distances	0.2701	6e-04
Lipids correlation x UniFrac distances	0.3118	7e-04
Phytopigments correlation x UniFrac distances	0.3275	1.6e-3

Variation in the pelagic and sediment microbiomes network structure at the local scale

With the attempt to better identify the community-level implications of diversity patterns observed for the pelagic and sediments microbiomes in the study area, a network-based approach was applied. To this aim, the overall network structure of the pelagic and sediment microbiomes was obtained and then the correspondent declinations in the different sectors were assessed. For the creation of the overall microbiome networks, the co-abundance associations between orders were computed, then orders were clustered in co-abundance groups CAGs (**Supplementary Figure S4**). For both ecosystems, 3 different CAGs were detected and named according to the dominant order. The CAGs composition is provided in the **Supplementary Table 4**. For the pelagic microbiome, the detected CAGs were *Rhodobacteriales* CAG, the *Vibrionales* CAG and the *Falavobacteriales* CAG, while for the sediment microbiomes the correspondent CAGs were *Desulfobacterales* CAG, *Clostridiales* CAG and *Campylobacterales* CAG. In **Figure 6**, we provided the Wiggum plots of the overall network structure of the pelagic and sediment microbiomes, where the compositional relationships between the correspondent CAGs are represented.

The variation of the pelagic and sediment networks in the different sectors were than explored. To this aim, for both ecosystems, the sector specific patterns of over-abundance modules (CAGs) and nodes (orders) were computed, and the respective over-abundant network plots were created (**Figure 7 and 8**). The box plot showing the variation in relative abundance of the over-abundant CAGs and orders in each sector are provided in **Supplementary Figure S5**.

According to our findings, for the pelagic microbiomes, the 3 sectors showed a specific pattern of over-abundance CAGs. Particularly, the *Falvobacteriales* CAG was most abundant in the North sector, while the *Vibrionales* CAG and *Rhodobacteriales* CAG were most represented in the Central and South area, respectively. Focusing on the single orders, each sector showed a specific set of over-abundant components: (i) for the North sector: *Xanthomonadales* and *E01-9C-26 marine group* (for the *Flavobacteriales* CAG), *MB11C04 marine group*, *Vibrionales* and *KI89A clade* (for the *Vibrionales* CAG) and *Clostridiales* (for the *Rhodobacteriales* CAG) resulted over-abundant; (ii) for the Center sector the over-abundant orders were *Alteromonadales* (for the *Flavobacteriales* CAG), *Planctomycetales*, *Phycisphaerales*, *Sphingomonadales*, *Micrococcales*, *Puniceicoccales*, *Burkholderiales* and *Rickettsiales* (for the *Vibrionales* CAG), *Bradymonadales* and *Bacteroidota Order* II (for the *Rhodobacteriales* CAG); (iii) for the South sector were over-abundant the following orders all belonging to the *Rhodobacteriales* CAG: *Chlamydiales*, *Rhizobiales*, *Sphingobacteriales*, *Bacteroidota Orders II and III*, *Bdellovibrionales*, *Legionellales*, *Acidimicrobiales*, *Parvularculales*, *SAR11 clade*, *Myxococcales* and *Rhodobacterales*. For what concern the sediment microbiome, an analogous situation was observed. In particular, at the CAGs level, the North-West sector was enriched in the *Campylobateriales* CAGs, while depleted in *Desulfobacterales* CAGs and *Clostridiales* CAGs compared to North-East and South sectors. For what concerns the orders, the following site-specific over-abundant pattern was observed: (i) *NB1-j*, *Thiotrichales*, *Myxococcales*, *Anaerolineales*, *Gaiellales*, *Chlamydiales*, *Holophagae Subgroup 23* (for the *Desulfobacterales* CAGs), *Pseudomonadales* and *Fusobacteriales* (for the *Clostridiales* CAGs) and *Nitrospirales*, *Gemmatimonadales*, *Bacillales*, *Campylobacterales*, *Phycisphaerales* (for the *Campylobateriales* CAGs) were over-abundant in the North West sector; (ii) *Bacteroidota Order II*, *Cytophagales* , *HTA4* (for the *Desulfobacterales* CAGs), *Pseudomonadales Vibrionales* (for the *Clostridiales* CAGs) and *Desulfurellales* (for the *Campylobateriales* CAGs) were over abundant in the North East sector; (iii) *Alteromonadales*, *KI89A clade*, *Rhodobacterales*, *Spirochaetales*, *Solirubrobacterales*, *SAR324 clade(Marine group B)*, *Cellvibrionales*, *Burkholderiales* and *NB1-j* (for the *Desulfobacterales* CAG), *Oceanospirillales*, *Lactobacillales*, *Corynebacteriales*, *Clostridiales*, *Micrococcales*, *Flavobacteriales* (for the *Clostridiales* CAGs) and *Desulfarculales (*for the *Campylobateriales* CAGs) were over abundant in the South sector. Finally, for both the water and sediment microbiome, site-specific community networks were created for each of the 3 alpha diversity sectors. For each local network, correspondent key parameters in term of modularity, total connectivity, negative to positive cohesions and hubs orders are provided in **Table 2.**

Table 2: The table represents the parameters of Networks of the single Area, in terms of Negative to Positive Cohesion Ratio (N:P), Modules, Total connectivity and hubbs order of the Network, both for pelagic microbiome (first three rows of the table) and sediment microbiome (last three rows of the table).

Single Networks Parameters					
	N:P Cohesion Ratio	**Modules**	**Total Connectivity**	**Hubbs Species**	
Northern Area -Water	1.39	22	1.06	Sphingobacteriales	Cytophagales
Central Area -Water	1.63	25	0.69	Planctomycetales	
Southern Area -Water	0.39	20	0.77	Acidomicrobiales	Rhizobiales
N/W Area - Sediment	0.95	25	0.58	Rhizobiales	
N/E Area - Sediment	1.5	39	0.71	Planctomycetales	
Southern Area - Sediment	0.88	26	0.80	Micrococcales	Desulfobacterales

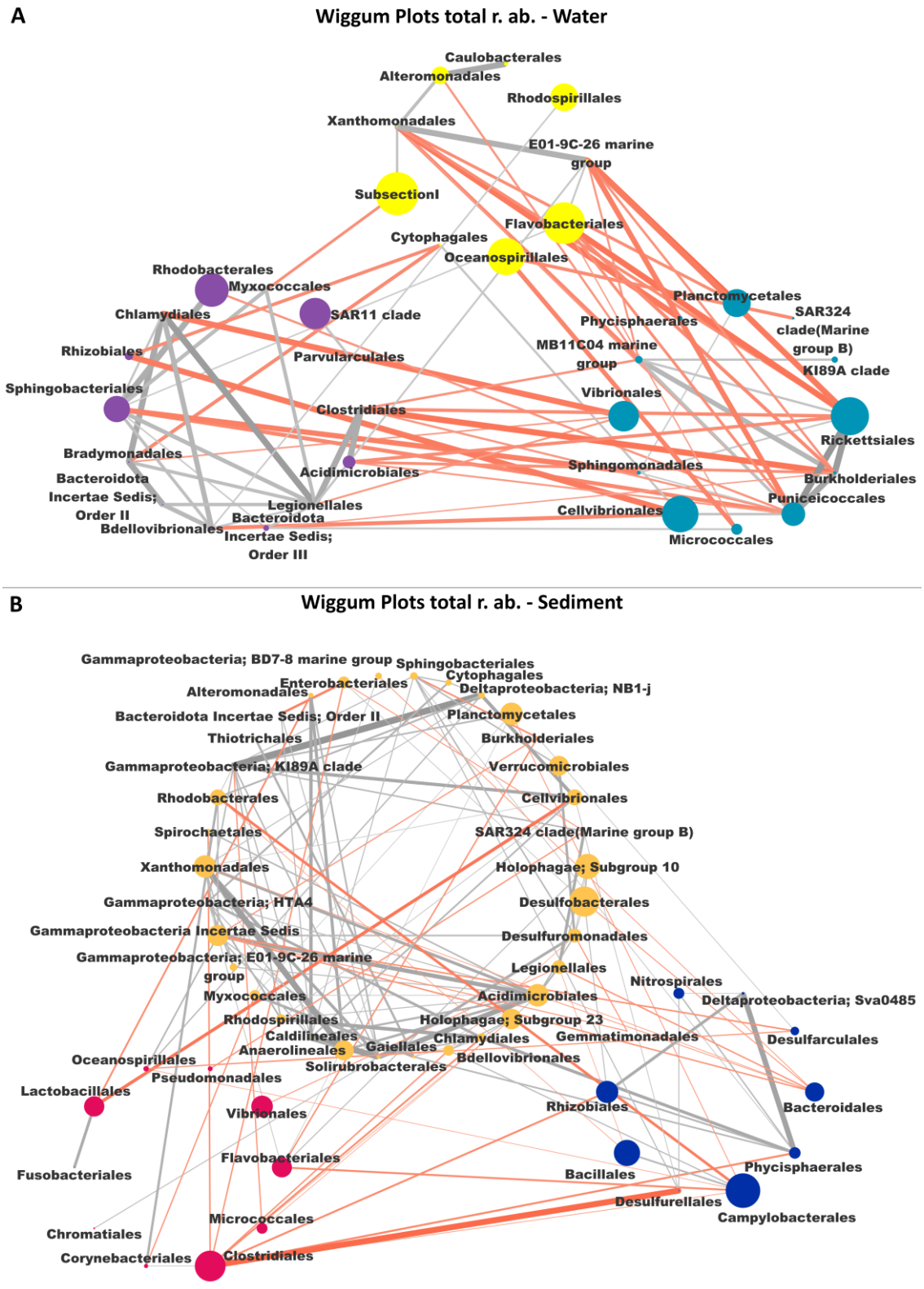


Figure 6: Wiggum plots representing the overall relative abundance of each bacterial order in the 3 CAGs for water (A) and sediment (B) microbiome. CAGs are named according to the most abundant order and are colour coded as follows: A) *Rhodobacterales* (Violet), *Vibrionales* (Bondi Blue) and *Flavobacteriales* (Yellow) for water microbiome; B) *Desulfobacterales* (Ocher), *Clostridiales* (Pink) and *Campylobacterales* (Blue) for sediment microbiome. Each node represents a bacterial order, and its dimension is proportional to its mean relative abundance in all samples. Connections between nodes represent positive (gray) and negative correlation (red) between order.

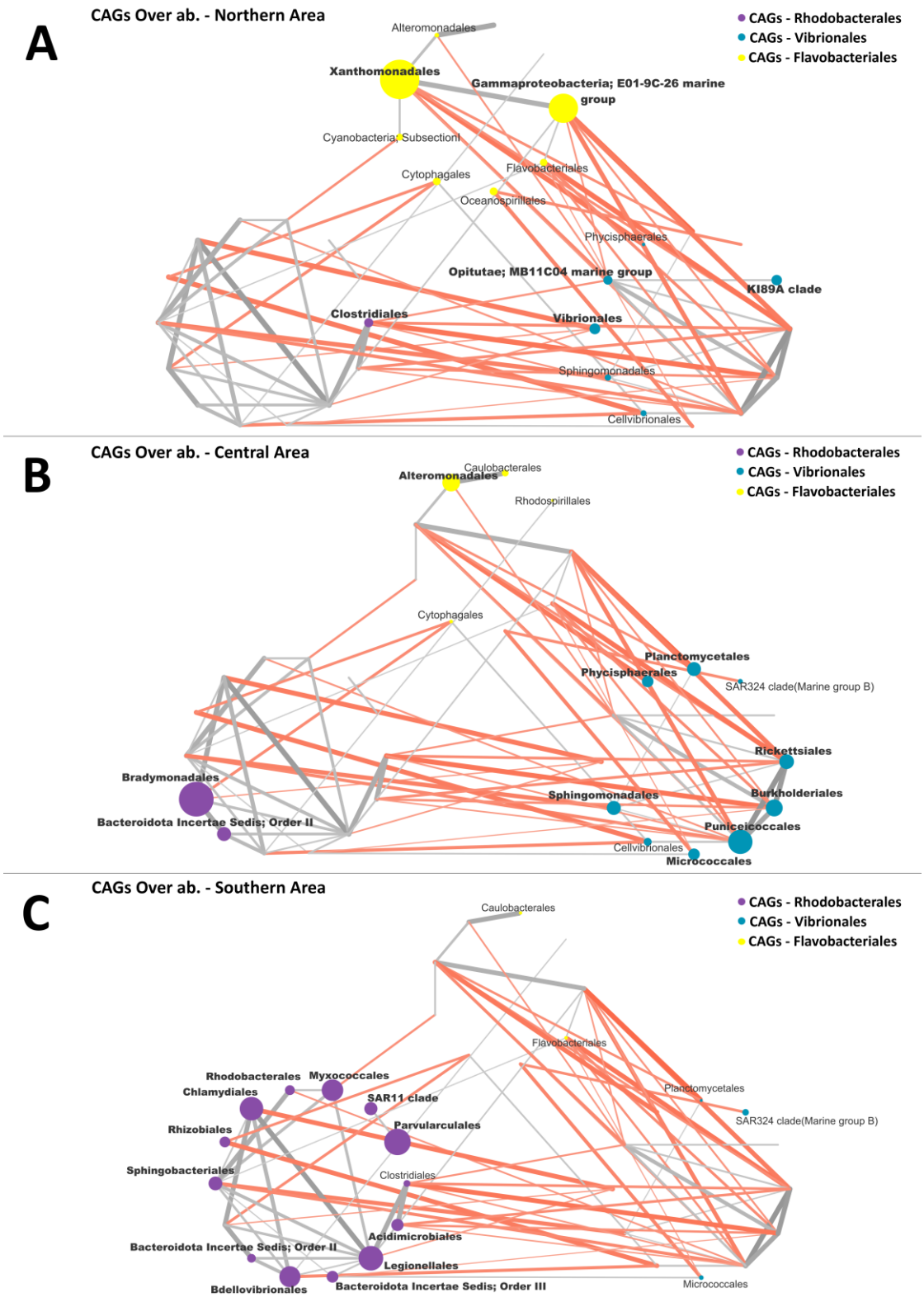


Figure 7: Wiggum plots representing the over-abundance of each bacterial order in the 3 CAGs in the water ecosystem in each Area: A) Northern Area; B) Central Area and C) Southern Area. CAGs are named according to the most abundant order in each CAG and are colour coded as follows: *Rhodobacterales* (Violet), *Vibrionales* (Bondi Blue) and *Flavobacteriales* (Yellow). Each bacterial order is depicted as a node whose size is proportional to its over-abundance. Node and name of bacterial order with an over-abundance < 1 are not represented, and those with an over-abundance ≥ 1.3 are bolded.

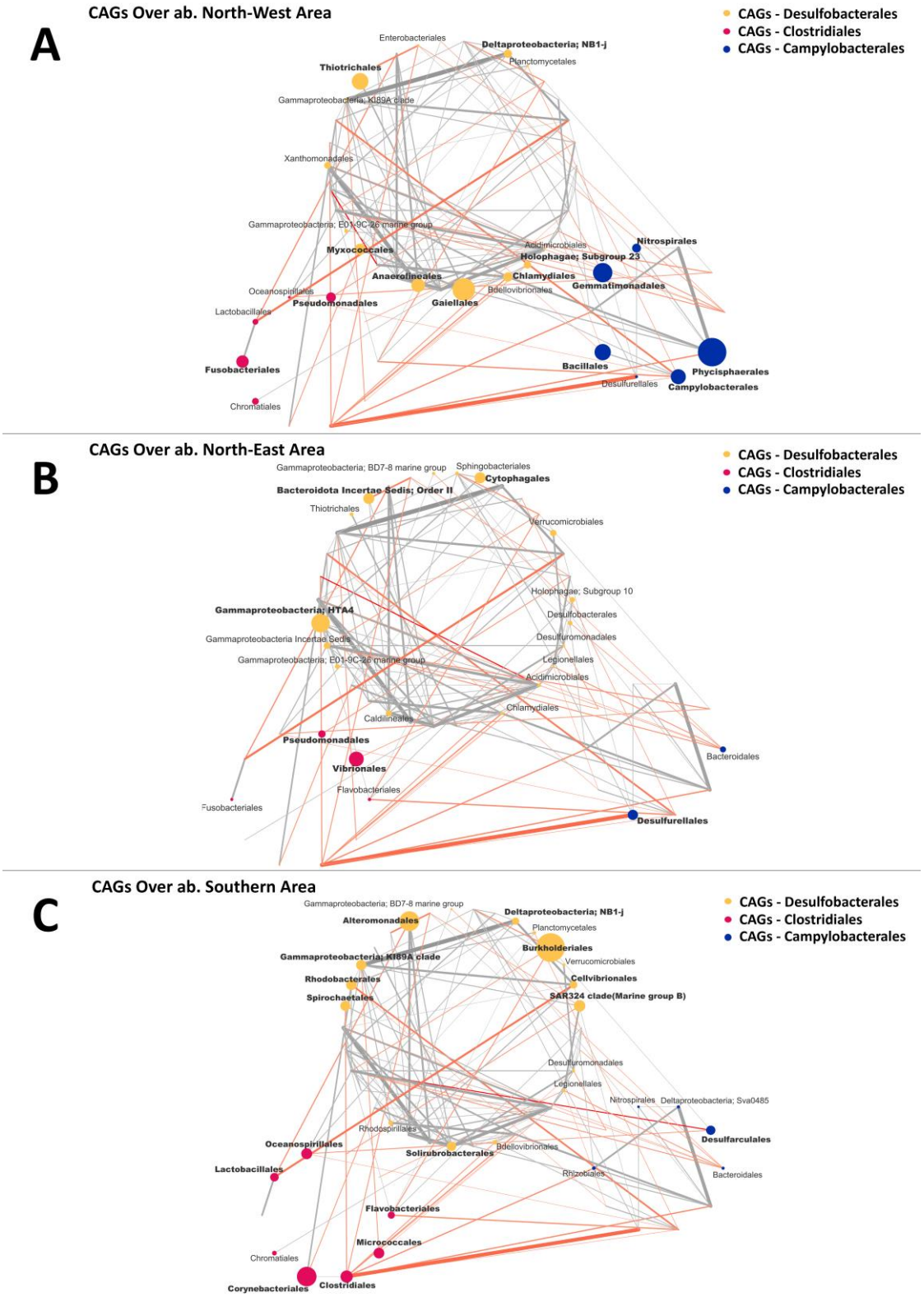


Figure 8: Wiggum plots representing the over-abundance of each bacterial order in the 3 CAGs in the sediment ecosystem in each Area: A) North-West Area; B) North-East Area and C) Southern Area. CAGs are named according to the most abundant order in each CAG and are colour coded as follows: *Desulfobacterales* (Ocher), *Clostridiales* (Pink) and *Campylobacterales* (Blue). Each bacterial order is depicted as a node whose size is proportional to its over-abundance. Node and name of a bacterial order with an over-abundance < 1 are not represented, and those with an over-abundance ≥ 1.3 are bolded.

Discussion

In the present study we conducted a synoptical analysis of the assemblage composition of the pelagic and sediment microbiomes in a 130 km^2 offshore area of the Northern-western Adriatic Sea (Mediterranean Sea). The protein contents and proteins to carbohydrates ratio (as a proxy of the nutritional quality of the organic matter) detected in sediment samples allowed to rank the area under study from meso-oligotrophic to eutrophic [47], generally showing a higher concentration for all the assessed biochemical components with respect what reported in other studies from same geographical area [48] or other coastal benthic ecosystems worldwide [27].

According to our findings, the pelagic ecosystem of the investigated area was dominated by *Synechoccus-like Cyanobacteria Subsection I*, a photosynthetic primary producer characteristic of nutrient-rich coastal ecosystems [48; 49], and by *Flavobacteriales*, *Oceanospirillales* and *Rhodobacteriales*. These latter microorganisms represent aerobic heterotrophs with an important role in the degradation of the dissolved organic matter (DOM) pool, known to prosper as r-strategist in copiotrophic environments such as the Adriatic Sea [50]. Conversely, *SAR11 clade* and *Cellvibrionales*, were only represented as minor components in our samples, being k-strategist cosmopolitan marine heterotrophs which typically dominate oligotrophic waters [51]. Primary producer bacteria and organic carbon degraders were complemented with members involved in sulfur cycling such as *Rhodobacteriales* and *Rhodospirillales*, suggesting a certain balance in nutrient cycling in the pelagic ecosystem of the North Adriatic [52; 53]. Finally, in the pelagic microbiome we detected *Rickettsiales*, as a dominant component, that generally is a host-associated microorganism present in nutrient-enriched ecosystems [54; 55; 56].

For what concerns the sediment microbiome, it was largely dominated by organic carbon fermenters - even with known possible terrestrial origins – such as members of *Clostridiales*, *Bacillales*, *Vibrionales* and *Lactobacillales* [57]. In particular, these microorganisms are known for their importance in the degradation of the organic carbon in anaerobic eutrophic sediments of coastal ecosystems [58; 7; 59]. Furthermore, the sediment microbiome was dominated by microbial components able to reduce sulfate (*Desulfobacterales)* and nitrite (*Acidomicrobiales*) in anaerobic conditions, with an important role in biogeochemical cycling [58; 7; 60]. These components also include *Planctomycetales*, as anaerobes able to perform Anammox [61]. The synoptical investigation of microbiomes in seawaters and sediments allowed us to explore their connections between the two ecosystems. As typical for shallow waters [9] different microbiome components were shared between pelagic and sediments assemblages - indicating a benthic-pelagic coupling. The shared groups included copiotrophic microorganisms assimilating DOM at low O_2 levels, such as *Oceanospirillales*, *Alteromonadales*, *Vibrionales*, *Planctomycetales* and *Verrucomicrobiales* [62] and the anoxygenic phototroph *Rhodobacterales*, known to inhabit shallow sediments [63].

For both the pelagic and sediment microbiomes, the corresponding networks structures were obtained, allowing for dissecting modules of co-occurring orders as CAGs. Each CAG showed a specific pattern of functional propensity. Particularly, for the pelagic microbiome, the *Flavobacteriales* CAG was characterized by oxygenic phototrophs (*Synechoccus-like Cyanobacteria Subsection I*), DOM assimilating aerobes (*Oceanospirillales* and *Flavobacteriales*), and sulfide oxidizers (*Rhodospirillales*). Differently, the *Vibrionales* CAG was dominated by copiotrophic (*Vibrionales* and *Planctomycetales*) and oligotrophic (*Cellvibrionales* and *K189A* clade) heterotrophs, with the host-associated marine groups *Rickettsiales* as other major components. Marine microorganisms known for their capacity to degrade mono and polycyclic aromatic compounds (*Sphingomonadales* and *Burkholderiales*) were characteristic components of this CAG [64; 65]. Finally, the *Rhodobacteriales* CAG was dominated by host-associated microbial groups, including components of the rhizosphere and plant microbiomes *Sphingobacteriales* and *Rhizobiales* [66; 67], and predatory microorganisms

such as *Bradymonadales*, *Bdellovibrionales* and *Myxococcales* [68; 69; 70,71]. Important members of this CAG were also *SAR11* bacteria, which are among the most abundant carbon-oxidizing bacteria in pelagic systems [72].

For the sediment microbiome, the *Desulfobacterales* CAG was characterized by anaerobes involved in N and S cycling, such as *Planctomycetales* and *Acidomicrobiales* (N reducers) and *Desulfobacterales* (S reducers) [58; 7; 60]. Conversely, the *Campylobacterales* CAG was characterized by several anaerobic heterotrophs such as *Bacteroidales*, *Bacillales* and *Campylobacterales*, the latter being shown to increase in nutrient-enriched waters during microalgal blooms [73]. Finally, the *Clostridiales* CAG was also characterized by carbon fermenters thriving in carbon-rich trophic sediments, but including microorganisms with known terrestrial origin, such as *Clostridiales* and *Lactobacillales* [58; 7; 59]. The hydrodynamic and trophic conditions in the investigated period may explain the largely heterotrophic nature of the pelagic and sediment microbiomes observed in the study area. Indeed, during the summer period, when waters are highly stratified, the limited river inputs (generally characterized by low phytoplankton biomass) reach the offshore systems, where microbial-mediated degradation of organic matter prevails on primary production processes [74].

Based on our analyses we observed that the pelagic and benthic microbiomes in the study area showed sector-specific patterns and distinct assemblage structures. In particular, the pelagic microbiome was characterized by three compositional clusters corresponding to the South, Central, and North sectors, the second characterized by the lowest alpha-diversity. Analogously, for the sediment microbiome, 3 different configurations were observed, corresponding to the North-eastern, North-western, and the South sectors, the latter showing the lowest alpha-diversity. Interestingly, this observed heterogenicity of the pelagic and sediment microbiomes at the local scale corresponded to detectable variations in the respective microbiome networks. Indeed, sector-specific patterns of over-abundance modules (CAGs) and nodes (orders) were defined. The pelagic microbiome, in the North and the Central sectors, was characterized by the over-abundance of heterotrophic groups belonging to the *Flavobacteriales* and *Vibrionales* CAGs, such as *Flavobacteriales*, *KI89A clad*e, *MB11C04 marine group*, *Clostridiales* and *Vibrionales*, capable to prosper in nutrient-rich waters assimilating DOM [75; 76; 57; 77]. The central sector was also characterized by microbial groups known as hydrocarbon degraders, such as *Sphingomonadales* and *Burkholderiales* [65; 64]. Conversely, the over abundant nodes in the South sector mainly belonged to host-associated microbes of possible terrestrial origins (*Chlamydiales*, *Rhizobiales* and *Legionellales* [78; 67; 79] and predatory orders (*Myxococcales* and *Bdellovibrionales*) [67; 68], which contributed to the *Rhodobacteriales* CAG, also including marine heterotroph prospering in oligotrophic waters as K strategists as *SAR11 clade* [57].

Both the North (North-eastern and -western) and the South sites of the sediment microbiome were characterized by copiotrophic carbon fermenters, but possibly showing different origins. Indeed, while fermenters from the North sites mainly belonged to marine heterotrophs such as *Thiotrichales*, *Gaiellales*, *Pseudomonadales*, *Bacillales*, *Campylobacterales*, *Phycisphaerales* and *Vibrionales* [80; 81; 82; 83; 53], in the South area fermenters belonged to microbial orders of possible terrestrial origin, such as *Corynebacteriales*, *Clostridiales*, *Lactobacillales* and *Spirochaetales*. However, despite the heterogenicity in terms of network over-abundant orders at the different sectors identified in the pelagic and benthic systems of the investigated area, important functional categories, such as organic carbon degraders, nitrogen cyclers, sulfur cyclers and, for the pelagic microbiome, carbon fixing microorganism, were always represented, supporting the well-balanced structures of the observed microbiome networks in term of potentiality for global cycling in a copiotroph coastal marine ecosystem.

According to our findings, the concentrations of biochemical components of the sedimentary organic matter in the three sectors were different, with higher values in the Northern Sector. The differences in trophic availability observed between

Northern and Southern sectors may explain - at least in part - the different compositional structures of the corresponding sediment microbiome, as shown by the correlation of the correspondent samples distance matrices. These findings support the importance of organic matter as a key driver of microbiome diversity in benthic marine ecosystems [85]. At the same time, the higher relevance of terrestrial microorganisms in the south sectors can be explained by the peculiar hydrodynamic conditions of the Northwestern Adriatic Sea during the summer season when the plume of the Po and other local rivers are mainly transported eastwards, toward the center of the basin, rather than being exported southwards as occur in the winter [22,23,24].

Conclusions

Our findings provide new insights into the local changes of the pelagic and sediments microbiomes in an offshore area of the North-western Adriatic Sea. Based on our results, despite the pelagic and benthic microbial assemblages showed a certain heterogenicity in the investigated area they maintained a well-balanced structure, being always structured for the provision of key ecosystem services (e.g., primary production, nutrient cycling, hydrocarbon degradation). Interestingly, Microbiomes at the different sites showed comparable ecological roles but a different origin, such as those of the South site (i.e., the closest to the coast) where both the pelagic and benthic ecosystems were characterized by microbial groups of terrestrial origin. Interestingly, these terrestrial microorganisms seem to become integral to the marine microbiome networks, as indicated by the comparable degree of modularity and connectivity of the local network at the South sites with respect to the other subarea [42]. Even if our study has a limited phylogenetic resolution and does not allow us to assess temporal microbiome changes, our findings rise possible concerns about the biological threshold, in terms of relative abundance, for terrestrial microorganisms - including the ones of fecal origin – to be included in the marine microbiome networks, without altering the ecological balance. However, in this perspective, more research is needed, with an improved phylogenetic resolution, also expanding the observation to other geographical sites and assessing for seasonal changes.

References

1. Flemming HC, Wuertz S. Bacteria and archaea on Earth and their abundance in biofilms. Nat Rev Microbiol. 2019;17(4):247-260.
2. de la Calle F. Marine microbiome as source of natural products. Microb Biotechnol. 2017;10(6):1293.
3. Moran MA. The global ocean microbiome. Sci. 2015;350(6266).
4. Herndl GJ, Reinthaler T. Microbial control of the dark end of the biological pump. Nat Geosci. 2013;6(9):718-724.
5. Arrigo KR. Marine microorganisms and global nutrient cycles. Nature. 2005;437(7057):349-355.
6. Azam F, Malfatti F. Microbial structuring of marine ecosystems. Nat Rev Microbiol. 2007;5(10);782-791.
7. Luna GM. Diversity of marine microbes in a changing Mediterranean Sea. Rend Lincei. 2015;26:49–58.
8. Green J, Bohannan BJ. Spatial scaling of microbial biodiversity. Trends Ecol Evol. 2006;21(9):501-507.
9. Zinger L, Amaral-Zettler LA, Fuhrman JA, Horner-Devine,MC, Huse SM, Welch DBM, et al. Global patterns of bacterial beta-diversity in seafloor and seawater ecosystems. PloS one. 2011;6(9).
10. Salazar G, Sunagawa S. Marine microbial diversity. Curr Biol. 2017;27(11):R489-R494.
11. Das S, Mangwani N. Ocean acidification and marine microorganisms: responses and consequences. Oceanologia. 2015;57(4):349-361.
12. Verde C, Giordano D, Bellas CM, di Prisco G, Anesio AM. Polar marine microorganisms and climate change. Adv Microb Physiol. 2016;69:187-215.
13. Cavicchioli R, Ripple WJ, Timmis KN, Azam F, Bakken LR, Baylis, M, et al. Scientists' warning to humanity: microorganisms and climate change. Nat Rev Micriobiol. 2019;17(9):569-586.
14. Horner-Devine MC, Lage M, Hughes JB, Bohannan BJ. A taxa–area relationship for bacteria. Nature. 2004;432(7018):750-753.
15. Martiny JBH, Bohannan BJ, Brown JH, Colwell RK, Fuhrman JA, Green JL, et al. Microbial biogeography: putting microorganisms on the map. Nat Rev Microbiol. 2006;4(2):102-112.
16. Soininen J, Lennon JJ, Hillebrand H. A multivariate analysis of beta diversity across organisms and environments. Ecology. 2007;88(11):2830-2838.
17. Sunagawa S, Coelho LP, Chaffron S, Kultima JR, Labadie K, Salazar G, et al. Structure and function of the global ocean microbiome. Science. (2015);348(6237).

18. Graf G. Benthic-pelagic coupling: a benthic view. Oceanogr. Mar. Biol.: an annual review. 1992.
19. Corinaldesi C, Tangherlini M, Rastelli E, Buschi E, Martire ML, Danovaro R, Dell'Anno A. High diversity of benthic bacterial and archaeal assemblages in deep-Mediterranean canyons and adjacent slopes. Prog Oceanogr. 2019;171:154-161.
20. Findlay RH, Trexler MB, Guckert JB, White DC. Laboratory study of disturbance in marine sediments: response of a microbial community. Mar Ecol Prog Ser. 1990;62(1):121-133.
21. Grilli F, Accoroni S, Acri F, Aubry FB, Bergami C, Cabrini M, et al. Seasonal and Interannual Trends of Oceanographic Parameters over 40 Years in the Northern Adriatic Sea in Relation to Nutrient Loadings Using the EMODnet Chemistry Data Portal. Water. 2020:12(8), 2280.
22. Marini M, Jones BH, Campanelli A, Grilli F, Lee CM. Seasonal variability and Po River plume influence on biochemical properties along western Adriatic coast. Journal of Geophysical Research: Oceans. 2008, 113(C5).
23. Campanelli A, Grilli F, Paschini E, Marini M. The influence of an exceptional Po River flood on the physical and chemical oceanographic properties of the Adriatic Sea. Dynamics of Atmospheres and Oceans. 2011, 52(1-2):284-297.
24. Franco P, Michelato A. Northern Adriatic Sea: oceanography of the basin proper and of the western coastal zone. Elsevier, In Marine coastal eutrophication. 1992, 35-62.
25. Behzad H, Ohyanagi H, Alharbi B, Ibarra M, Alarawi M, Saito Y, et al. A cautionary signal from the Red Sea on the impact of increased dust activity on marine microbiota. BMC genomics. 2022:*23*(1):1-12.
26. Danovaro R. Methods for the study of deep-sea sediments, their functioning and biodiversity. CRC press. 2010.
27. Pusceddu A, Dell'Anno A, Fabiano M, Danovaro R. Quantity and bioavailability of sediment organic matter as signatures of benthic trophic status. Mar Ecol Prog Ser. 2009;375:41-52.
28. Musella M, Wathsala R, Tavella T, Rampelli S, Barone M, Palladino G, et al. Tissue-scale microbiota of the Mediterranean mussel (Mytilus galloprovincialis) and its relationship with the environment. Sci Total Environ. 2020;717.
29. Palladino G, Rampelli S, Scicchitano D, Musella M, Quero GM, Prada F, et al. Impact of marine aquaculture on the microbiome associated with nearby holobionts: the case of Patella caerulea living in proximity of Sea Bream aquaculture cages. Microorganisms. 2021;9(2):455.
30. Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinform. 2012;13(1):1-7.
31. Bolyen E, Rideou, JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol. 2019;37(8):852-857.
32. Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 2010;26(19):2460-2461.
33. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat methods. 2016;13(7):581-583.
34. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: a versatile open source tool for metagenomics. PeerJ. 2016;4.
35. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic acids Res. 2012;41(D1):D590-D596.
36. https://qgis.org/it/site/
37. **https://www.R-project.org**
38. Culhane AC, Thioulouse J, Perrière G, Higgins DG. MADE4: an R package for multivariate analysis of gene expression data. Bioinformatics. 2005;21(11):2789-2790.
39. **https://cran.r-project.org/web/packages/vegan/index.html**
40. Rampelli S, Guenther K, Turroni S, Wolters M, Veidebaum T, Kourides Y, et al. Pre-obese children's dysbiotic gut microbiome and unhealthy diets may predict the development of obesity. Commun Biol. 2018;1(1):1-11.
41. Claesson MJ, Jeffery IB, Conde S, Power SE, O'connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature. 2012;488(7410):178-184.
42. Schnorr SL, Candela M, Rampelli S, Centanni M, Consolandi C, Basaglia G, et al. Gut microbiome of the Hadza hunter-gatherers. Nat Commun. 2014;5(1):1-12.
43. http://www.cytoscape.org/
44. Tackmann J, Rodrigues JFM, von Mering C. Rapid inference of direct interactions in large-scale ecological networks from heterogeneous microbial sequencing data. Cell Syst. 2019;9(3):286-296.
45. Agler MT, Ruhe J, Kroll S, Morhenn C, Kim ST, Weigel D, Kemen EM. Microbial hub taxa link host and abiotic factors to plant microbiome variation. PLoS Biol. (2016;14(1).
46. Hernandez DJ, David AS, Menges ES, Searcy CA, Afkhami ME. Environmental stress destabilizes microbial networks. ISME J. 2021;15(6):1722-1734.
47. Dell'Anno A, Mei ML, Pusceddu A, Danovaro R. Assessing the trophic state and eutrophication of coastal marine systems: a new approach based on the biochemical composition of sediment organic matter. Mar Pollut Bull. 2002;44(7):611-622.
48. Flombaum P, Gallegos JL, Gordillo RA, Rincón J, Zabala LL, Jiao N, et al. Present and future global distributions of the marine Cyanobacteria Prochlorococcus and Synechococcus. Proc Natl Acad Sci. 2013;110(24):9824-9829.
49. Scanlan DJ, West NJ. Molecular ecology of the marine cyanobacterial genera Prochlorococcus and Synechococcus. FEMS Microbiol Ecol. 2002;40(1);1-12.
50. Lauro FM, McDougald D, Thomas T, Williams TJ, Egan S, Rice S, et al. The genomic basis of trophic strategy in marine bacteria. Proc Natl Acad Sci. 2009;106(37):15527-15533
51. Giovannoni SJ, Tripp HJ, Givan S, Podar M, Vergin KL, Baptista D, et al. Genome streamlining in a cosmopolitan oceanic bacterium. Science. 2005;309(5738):1242-1245.
52. Pohlner M, Dlugosch L, Wemheuer B, Mills H, Engelen B, Reese BK. The majority of active Rhodobacteraceae in marine sediments belong to uncultured genera: A molecular approach to link their distribution to environmental conditions. Front Microbiol. 2019;10:659.
53. Sun F, Wu M, Wang Y, Sun C, Xu Z. Diversity and potential function of bacterial communities in different upwelling systems. Estuar Coast Shelf Sci. 2020;237.
54. Yu X, Walker DH. The order rickettsiales. Prokaryotes. 2006;5:493-528.
55. Darby AC, Cho NH, Fuxelius HH, Westberg J, Andersson SG. Intracellular pathogens go extreme: genome evolution in the Rickettsiales. Trends Genet. 2007;23(10):511-520.
56. Klinges JG, Rosales SM, McMinds R, Shaver EC, Shantz AA, Peters EC, et al. Correction: Phylogenetic, genomic, and biogeographic characterization of a novel and ubiquitous marine invertebrate-associated Rickettsiales parasite, Candidatus Aquarickettsia rohweri, gen. nov., sp. nov. ISME J. 2020;14(1):322.
57. Tinta T, Vojvoda J, Mozetič P, Talaber I, Vodopivec M, Malfatti F, Turk V. Bacterial community shift is induced by dynamic environmental parameters in a changing coastal ecosystem (northern A driatic, northeastern M editerranean S ea)–a 2-year time-series study. Environ Microbiol. 2015;17(10):3581-3596.
58. Borin S, Brusetti L, Daffonchio D, Delaney E, Baldi F. Biodiversity of prokaryotic communities in sediments of different sub-basins of the Venice lagoon. Microbiol Res. 2009;160(5):307-314.

59. Gutiérrez-Barral A, Teira E, Hernández-Ruiz M, Fernández E. Response of prokaryote community composition to riverine and atmospheric nutrients in a coastal embayment: Role of organic matter on Vibrionales. Estuar Coast Shel Sci. 2021;251.
60. Fazi S, Baldassarre L, Cassin D, Quero GM, Pizzetti I, Cibic T, Del Negro P. Prokaryotic community composition and distribution in coastal sediments following a Po river flood event (northern Adriatic Sea, Italy). Estuar Coast Shel Sci. 2020;233.
61. Fuerst JA, Sagulenko E. Beyond the bacterium: planctomycetes challenge our concepts of microbial structure and function. Nat Rev Microbiol. 2011;9(6):403-413.
62. Bunse C, Pinhassi J. Marine bacterioplankton seasonal succession dynamics. Trends Microbiol. 2017;25(6):494-505.
63. Taylor JD, Cottingham SD, Billinge J, Cunliffe M. Seasonal microbial community dynamics correlate with phytoplankton-derived polysaccharides in surface coastal waters. ISME J. 2014;8(1):245-248.
64. Pérez-Pantoja D, Donoso R, Agulló L, Córdova M, Seeger M, Pieper DH, González B. Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environ Microbiol. 2012;14(5):1091-1117.
65. Kertesz MA, Kawasaki A, Stolz A. Aerobic hydrocarbon-degrading alphaproteobacteria: Sphingomonadales. Taxonomy, genomics and ecophysiology of hydrocarbon-degrading microbes. 2019;105-124.
66. Lei S, Xu X, Cheng Z, Xiong J, Ma R, Zhang L, et al. Analysis of the community composition and bacterial diversity of the rhizosphere microbiome across different plant taxa. Microbiologyopen. 2019;8(6).
67. Carvalho FM, Souza RC, Barcellos FG, Hungria M, Vasconcelos ATR. Genomic and evolutionary comparisons of diazotrophic and pathogenic bacteria of the order Rhizobiales. BMC Microbiol. 2010;10(1):1-15.
68. Wang S, Mu DS, Zheng WS, Du ZJ. Complete genome sequence of Bradymonas sediminis FA350T, the first representative of the order Bradymonadales. Mar Genom. 2019;46: 62-65.
69. Sockett RE. Predatory lifestyle of Bdellovibrio bacteriovorus. Annu Rev Microbiol. 2009;63:523-539.
70. Mu DS, Wang S, Liang QY, Du ZZ, Tian R, Ouyang Y, et al. Bradymonabacteria, a novel bacterial predator group with versatile survival strategies in saline environments. Microbiome. 2020;8(1):1-15.
71. Muñoz-Dorado J, Marcos-Torres FJ, García-Bravo E, Moraleda-Muñoz A, Pérez J. Myxobacteria: moving, killing, feeding, and surviving together. Front Microbiol. 2016;7:781.
72. Giovannoni SJ. SAR11 bacteria: the most abundant plankton in the oceans. Ann Rev Mar Sci. 2017;9:231-255.
73. Liu M, Dong Y, Zhao Y, Zhang G, Zhang W, Xiao T. Structures of bacterial communities on the surface of Ulva prolifera and in seawaters in an Ulva blooming region in Jiaozhou Bay, China. World J Microbiol Biotechnol. 2011;27(7):1703-1712.
74. Danovaro, R., Armeni, M., Luna, G. M., Corinaldesi, C., Dell'Anno, A., Ferrari, C. R., ... & Giani, M. (2005). Exo-enzymatic activities and dissolved organic pools in relation with mucilage development in the Northern Adriatic Sea. Science of the total environment, 353(1-3), 189-203.
75. Osterholz H, Singer G, Wemheuer B, Daniel R, Simon M, Niggemann J, Dittmar T. Deciphering associations between dissolved organic molecules and bacterial communities in a pelagic marine system. ISME J. 2016;10(7):1717-1730.
76. Cho JC, Giovannoni SJ. Cultivation and growth characteristics of a diverse group of oligotrophic marine Gammaproteobacteria. Appl Environ Microbiol. 2004;70(1):432-440.
77. Sizikov S, Burgsdorf I, Handley KM, Lahyani M, Haber M, Steindler L. Characterization of sponge-associated Verrucomicrobia: microcompartment-based sugar utilization and enhanced toxin–antitoxin modules as features of host-associated Opitutales. Environ Microbiol. 2020;22(11):4669-4688.
78. Polkinghorne A, Schmidt-Posthaus H, Meijer A, Lehner A, Vaughan L. Novel Chlamydiales associated with epitheliocystis in a leopard shark Triakis semifasciata. Dis Aquat Org. 2010;91(1):75-81.
79. Graells T, Ishak H, Larsson M, Guy L. The all-intracellular order Legionellales is unexpectedly diverse, globally distributed and lowly abundant. FEMS Microbiol Ecol. 2018;94(12).
80. Buongiorno J, Herbert LC, Wehrmann LM, Michaud AB, Laufer K, Røy H, et al. Complex microbial communities drive iron and sulfur cycling in Arctic fjord sediments. Appl Environ Microbiol. 2019;85(14):e00949-19.
81. Chen RW, He YQ, Cui LQ, Li C, Shi SB, Long LJ, Tian XP. Diversity and Distribution of Uncultured and Cultured Gaiellales and Rubrobacterales in South China Sea Sediments. *Front Microbiol*. 2021;12.
82. Eppinger M, Baar C, Raddatz G, Huson DH, Schuster SC. Comparative analysis of four Campylobacterales. Nat Rev Microbiol. 2004;2(11):872-885.
83. Fukunaga Y, Kurahashi M, Sakiyama Y, Ohuchi M, Yokota A, Harayama S. Phycisphaera mikurensis gen. nov., sp. nov., isolated from a marine alga, and proposal of Phycisphaeraceae fam. nov., Phycisphaerales ord. nov. and Phycisphaerae classis nov. in the phylum Planctomycetes. J Gen Appl Microbiol. 2009;55(4):267-275.
84. Hoshino T, Doi H, Uramoto GI, Wörmer L, Adhikari RR, Xiao N, et al. Global diversity of microbial communities in marine sediment. Proceedings of the national academy of sciences. 2020; 117(44), 27587-27597.

CHAPTER 3

3. CONCLUDING REMARKS

The presented thesis work provides more knowledge on how several feed additives and/or supplementation could affect fish health status with a specific focus on their gut microbiome. In addition, we wanted to understand how different anthropogenic activities, such as aquaculture, could affect the surroundings in terms of pollution, exploitation of marine resources and habitat alteration, and how all these aspects could affect the marine host-associated microbes and marine environmental microbiome.

In the first study, we observed that a blend of Organic Acids (OA) and Natural Identical Compounds (NIC) are able to improve growth and feed utilization of rainbow trout keeping homeostasis at the GM and cytokine gene expression, pointing out the lack of inflammatory activity in the intestinal mucosa of rainbow trout fed increasing dietary blend, during normal growing temperature conditions. However, OA and NIC were not able to revert the negative effects of fish growing performance due to the increased water temperature.

In the second work conducted in this thesis, we observed the inclusion of different tannin doses play a relevant role in counteracting intestinal inflammation caused by fishmeal feed replacement with plant ingredients in zebrafish, highlighting an anti-inflammatory effect of these natural additives against intestinal inflammation.

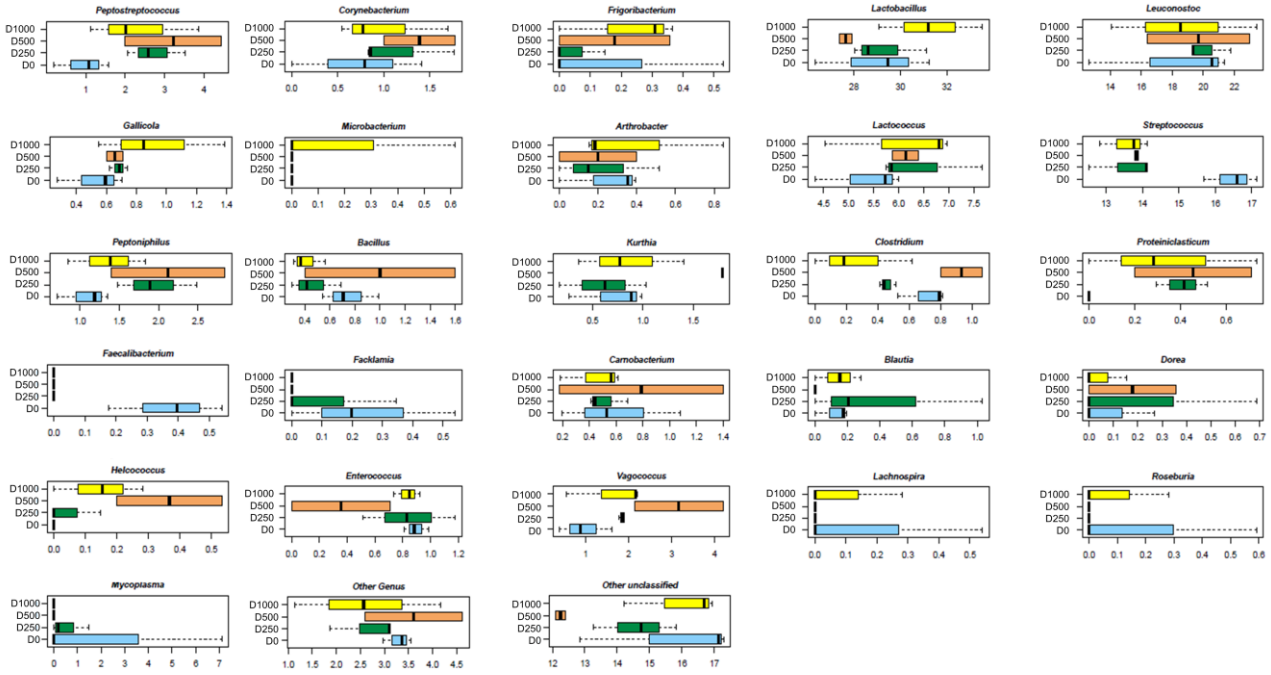
The last diet test study focused on the dietary lipid level during seasonal temperature changes in gilthead sea bream, highlighted that a higher lipid level did not improve growth and feed efficiency during the seasonal temperature changes compared to low dietary lipid level. However, low lipid level improved feed intake, growth and nutrient utilization after seasonal temperature changes. According to these results, the utilization of lower dietary lipid levels in gilthead sea bream should be preferred when fish are exposed to temperature changes.

Regarding the monitoring and preservation of coastal marine ecosystems, we dissected the interactions between the microbiomes from farmed fishes and the surrounding wild holobionts at the metacommunity level, in consideration of the strong impact of mariculture on environmental conditions in the surrounding water column. Particularly, we showed patterns of microbial dispersion from the farmed fishes to the environment and, finally, to locally dwelling wild organisms, through the use of the sedentary organism *Patella caerulea* as a proxy for metacommunity changes.

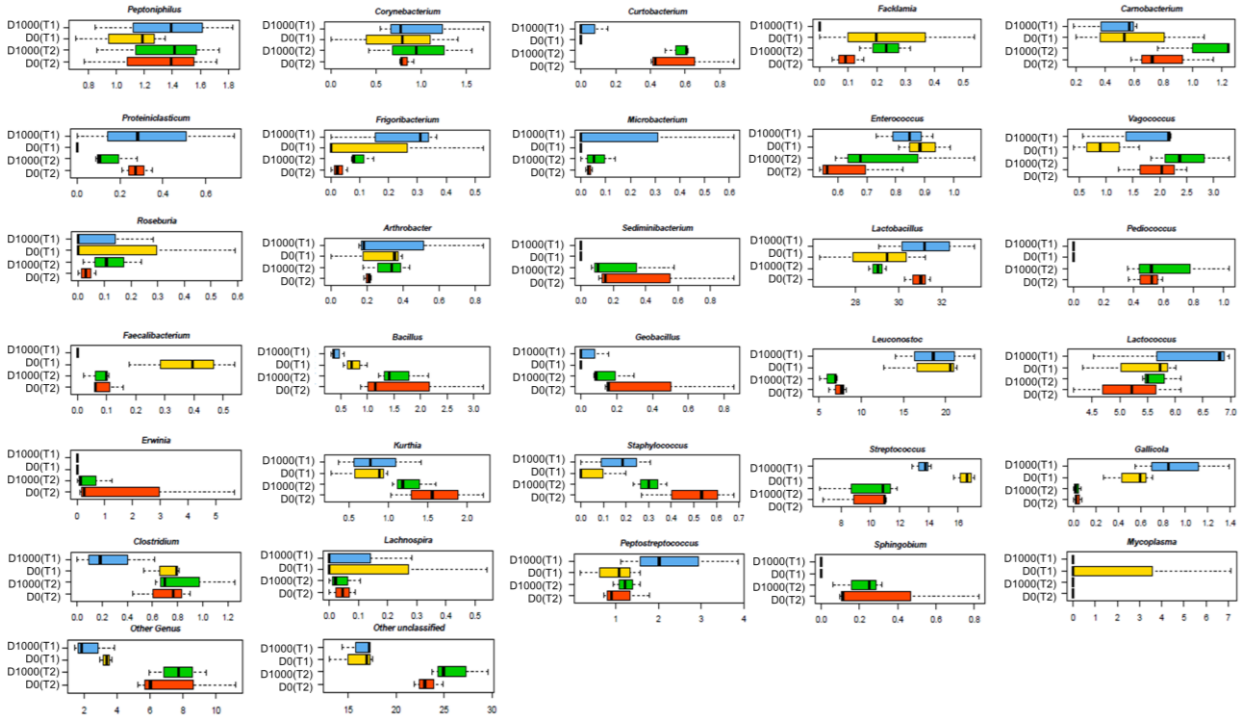
As basis of marine microbiome ecology in a highly productive area, our last study provides new insights into the local changes of the pelagic and sediments microbiomes in an offshore area, highlighting a well-balanced structure capable to provide key ecosystem services (e.g., primary production, nutrient cycling, hydrocarbon degradation). However, microbiomes from different sites have different origin, such close to shore, both the pelagic and benthic microbial ecosystems, were characterized by microbial groups of terrestrial origin, which seems to be become integral to the marine microbiome networks. However, in this perspective, more research is needed with an improved phylogenetic resolution, expanding the observation to other geographical sites, and assessing seasonal variations.

4. SUPPLEMENTARY MATERIAL

Study I - Effects of increasing dietary level of organic acids and nature-identical compounds on growth, intestinal cytokine gene expression and gut microbiota of rainbow trout (Oncorhynchus mykiss) reared at normal and high temperature



Supplementary Figure 1. Boxplots showing comparison of the microbiota relative abundances among different groups at genus level (paired Wilcoxon rank-sum test, $p > 0.05$) of rainbow trout fed for 82 days with increasing dietary blend levels (organic acids and natural identical compounds) (i.e. D0, D250, D500, D1000).



Supplementary Figure 2. Boxplots showing comparison of the microbiota relative abundances among different groups at genus level (paired Wilcoxon rank-sum test, $p > 0.05$) of rainbow trout fed with D0 and D1000 diets before (day 82, T1) and after (day 89, T2) exposure to suboptimal water rearing temperature of 23°C for 7 days.

Study II - Interaction between dietary lipid level and seasonal temperature changes in gilthead sea bream *Sparus aurata*: effects on growth, fat deposition, plasma biochemistry, digestive enzyme activity and gut bacterial community

Supplementary Table 1. Samples size for each related analyses performed during the trial

Samples	Analyses	day 0	day 58	day 58-59	day 121
Liver weight	HSI		n=18	Thermal change at a rate of 3 °C day^{-1}	n=15
Viscera weight	VSI		n=18		n=15
Mesenteric fat weight	MFI		n=18		n=15
Liver	Fat lipid content				n=18
Blood	Plasma biochemistry		n=9		n=15
Whole gut	Gut enzymes		n=9		n=15
Digesta content	Gut microbiome		n=9		n=9
Carcass	Proximate composition	overall n=10	n=9		n=15

Samples are given as number (n) of samples per treatment.

Supplementary Table 2. Relative increment of growth performance and nutritional indices measured in gilthead seabream fed different lipid level before and after temperature shift.

	L16		L21		*P-value*		
	HL	LH	HL	LH	*Inte*	*Temp*	*Diet*
Growth performances							
FBW	0.30 ± 0.07^{a}	0.90 ± 0.04^{b}	0.25 ± 0.02^{a}	0.71 ± 0.16^{b}	*0.221*	*< 0.0001*	*0.052*
WG	-0.38 ± 0.20^{a}	2.20 ± 0.22^{c}	-0.49 ± 0.01^{a}	1.26 ± 0.57^{b}	*0.057*	*< 0.0001*	*0.023*
SGR	-0.64 ± 0.12^{a}	0.79 ± 0.17^{c}	-0.70 ± 0.02^{a}	0.30 ± 0.30^{ab}	*0.079*	*< 0.0001*	*0.033*
FCR	0.24 ± 0.14	-0.03 ± 0.07	0.24 ± 0.15	0.01 ± 0.05	*0.740*	*0.004*	*0.760*
FI	-0.54 ± 0.11^{a}	0.68 ± 0.07^{c}	-0.61 ± 0.04^{a}	0.29 ± 0.18^{ab}	*0.045*	*< 0.0001*	*0.009*
Survival	-0.01 ± 0.02	-0.01 ± 0.02	-0.02 ± 0.03	0.00 ± 0.01	*0.597*	*0.597*	*0.837*
Nutritional indices							
PER	-0.20 ± 0.08^{a}	0.04 ± 0.09^{b}	-0.20 ± 0.08^{a}	-0.01 ± 0.09^{ab}	*0.585*	*0.002*	*0.629*
GPE	-0.26 ± 0.17^{a}	0.13 ± 0.01^{b}	-0.07 ± 0.15^{ab}	0.02 ± 0.17^{ab}	*0.109*	*0.021*	*0.619*
GLE	-0.14 ± 0.31	0.01 ± 0.24	-0.43 ± 0.32	0.25 ± 0.72	*0.328*	*0.143*	*0.939*
LER	-0.20 ± 0.08^{a}	0.04 ± 0.09^{b}	-0.20 ± 0.08^{a}	-0.01 ± 0.09^{ab}	*0.594*	*0.002*	*0.623*

Data are given as the tanks mean (n=3) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$). L16 = low lipid 16% diet ; L21 = high lipid 21% diet ; HL = constant temperature exposure to high (H) 23°C until temperature switch (day 0-58), then to constant low (L) 17°C until end of trial (day 59 -121); LH = constant temperature exposure to low (L) 17°C until temperature switch (day 0-58th), then to constant high (H) 23°C until end of trial (day 59th -121st).
IBW= Initial body weight (g).
FBW = Final body weight (g).
WG = Weight gain (g).
SGR = Specific growth rate (% day-1) = 100 * (ln FBW- ln IBW) / days.
FCR = Feed conversion rate = feed intake / weight gain.
FI = Feed intake (g kg ABW−1 day−1) = ((1000*total ingestion)/(ABW))/days)).
ABW = average body weight = (IBW + FBW)/2.
Survival = Survival (%).
PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).
GPE = Gross protein efficiency = 100*[(%final body protein*FBW) - (%initial body protein*IBW)]/total protein intake fish.
GLE = Gross lipid efficiency = 100*[(%final body lipid*FBW) - (%initial body lipid*IBW)]/total lipid intake fish.
LER = Lipid efficiency ratio = ((FBW-IBW)/lipid intake).
SD = standard deviation.

Supplementary Table 3. Relative increment of body composition and somatic indices measured in gilthead seabream fed different lipid level before and after temperature shift.

	L16		L21		*P-value*		
	HL	LH	HL	LH	*Inte*	*Temp*	*Diet*
Whole body composition, %							
Protein	-0.01 ± 0.03	0.03 ± 0.02	0.03 ± 0.02	0.01 ± 0.05	*0.113*	*0.608*	*0.863*
Lipid	0.05 ± 0.07	0.12 ± 0.10	-0.03 ± 0.09	0.12 ± 0.15	*0.561*	*0.111*	*0.590*
Ash	0.03 ± 0.10	-0.06 ± 0.03	0.04 ± 0.10	0.07 ± 0.09	*0.264*	*0.468*	*0.191*
Moisture	0.00 ± 0.02	-0.02 ± 0.01	0.00 ± 0.03	-0.02 ± 0.02	*0.924*	*0.210*	*0.762*
Somatic indices							
HSI	0.22 ± 0.18^{b}	-0.27 ± 0.09^{a}	0.35 ± 0.09^{b}	-0.31 ± 0.11^{a}	*0.278*	*< 0.0001*	*0.527*
MFI	-0.16 ± 0.34	-0.15 ± 0.08	-0.07 ± 0.24	0.02 ± 0.34	*0.822*	*0.766*	*0.440*
VSI	-0.07 ± 0.11	-0.01 ± 0.13	0.01 ± 0.26	-0.03 ± 0.13	*0.593*	*0.943*	*0.772*

Data are given as the mean (n=3 ± SD). In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$). L16 = low lipid 16% diet ; L21 = high lipid 21% diet ; HL = constant temperature exposure to high (H) 23°C until temperature switch (day 0-58th), then to constant low (L) 17°C until end of trial (day 59th -121st); LH =. constant temperature exposure to low (L) 17°C until temperature switch (day 0-58th), then to constant high (H) 23°C until end of trial (day 59th -121st).
HSI = Hepatosomatic index (%) = 100*(liver weight/FBW).
MFI = Mesenteric Fat Index (%) = 100*(mesenteric fat weight/FBW).
VSI = Viscerosomatic index (%) = 100*(viscera weight/FBW).
SD = Standard deviation.

Supplementary Table 4. Relative increment of gut digestive enzyme activity measured in gilthead seabream fed different lipid level before and after temperature shift.

	L16		L21		*P-value*		
	HL	LH	HL	LH	*Inte*	*Temp*	*Diet*
Pepsin	0.63 ± 0.91	0.04 ± 0.21	-0.45 ± 0.08	-0.16 ± 0.55	*0.195*	*0.646*	*0.077*
Trypsin	-0.54 ± 0.29	-0.58 ± 0.04	-0.59 ± 0.08	-0.40 ± 0.15	*0.288*	*0.502*	*0.538*
Chymotrypsin	-0.30 ± 0.39	-0.25 ± 0.17	-0.46 ± 0.05	-0.24 ± 0.16	*0.525*	*0.333*	*0.592*
Amylase	-0.77 ± 0.13^{a}	-0.71 ± 0.10^{ab}	-0.79 ± 0.09^{a}	-0.45 ± 0.15^{b}	*0.066*	*0.020*	*0.129*
Lipase	-0.52 ± 0.22	-0.43 ± 0.06	-0.35 ± 0.06	-0.19 ± 0.23	*0.700*	*0.228*	*0.063*

Data are given as the mean (n=3 diet ± SD). Different letters indicate significant difference (Two-way ANOVA $P \leq 0.05$) between treatments. L16 = low lipid 16% diet ; L21 = high lipid 21% diet ; HL = constant temperature exposure to high (H) 23°C until temperature switch (day 0-58th), then to constant low (L) 17°C until end of trial (day 59th -121st); LH =. constant temperature exposure to low (L) 17°C until temperature switch (day 0-58), then to constant high (H) 23°C until end of trial (day 59 -121).

Supplementary Table 5. Relative increment of plasma biochemistry values measured in gilthead seabream fed different lipid level before and after temperature switch.

	L16		L21		*P - value*		
	HL	LH	HL	LH	*Inte*	*Temp*	*Diet*
GLU	-0.17 ± 0.12^{a}	0.03 ± 0.17^{ab}	-0.11 ± 0.03^{a}	0.23 ± 0.11^{b}	*0.324*	*0.004*	*0.080*
Urea	0.33 ± 0.19	0.27 ± 0.30	0.25 ± 0.10	0.29 ± 0.22	*0.688*	*0.945*	*0.811*
CREA	-0.14 ± 0.18	-0.13 ± 0.13	-0.32 ± 0.08	-0.04 ± 0.25	*0.218*	*0.192*	*0.648*
Uric Ac	1.18 ± 2.89^{a}	1.44 ± 2.25^{a}	1.93 ± 0.95^{ab}	6.88 ± 1.34^{b}	*0.078*	*0.055*	*0.028*
Tot Bil	-0.16 ± 0.83	0.21 ± 0.60	-0.65 ± 0.14	-0.54 ± 0.13	*0.683*	*0.454*	*0.071*
Bil Ac	-0.69 ± 0.09	0.54 ± 0.72	-0.71 ± 0.11	0.30 ± 1.00	*0.785*	*0.020*	*0.736*
CHOL	-0.06 ± 0.16	-0.12 ± 0.04	-0.07 ± 0.23	-0.16 ± 0.05	*0.848*	*0.373*	*0.722*
TRIG	-0.56 ± 0.28	-0.55 ± 0.20	-0.41 ± 0.03	-0.71 ± 0.06	*0.171*	*0.192*	*0.943*
HDL	0.56 ± 0.15^{b}	0.07 ± 0.13^{a}	0.30 ± 0.16^{ab}	0.13 ± 0.08^{a}	*0.078*	*0.003*	*0.224*
TP	0.08 ± 0.06^{b}	-0.04 ± 0.07^{ab}	0.00 ± 0.06^{ab}	-0.10 ± 0.05^{a}	*0.831*	*0.013*	*0.094*
ALB	0.03 ± 0.02	-0.01 ± 0.10	-0.05 ± 0.04	-0.07 ± 0.08	*0.865*	*0.535*	*0.108*
AST	0.19 ± 0.73^{ab}	-0.65 ± 0.17^{a}	0.98 ± 0.38^{b}	-0.26 ± 0.24^{a}	*0.452*	*0.003*	*0.046*
ALP	-0.86 ± 0.08	-0.05 ± 0.67	-0.82 ± 0.02	-0.39 ± 0.26	*0.384*	*0.018*	*0.488*
CK	0.66 ± 0.56^{b}	-0.73 ± 0.27^{a}	1.89 ± 0.15^{c}	-0.68 ± 0.14^{a}	*0.014*	*< 0.0001*	*0.010*
LDH	1.20 ± 2.43	-0.68 ± 0.30	2.64 ± 1.58	-0.34 ± 0.27	*0.532*	*0.021*	*0.320*
Ca^{+2}	0.03 ± 0.04	0.03 ± 0.05	-0.07 ± 0.04	-0.01 ± 0.04	*0.292*	*0.201*	*0.018*
P	-0.03 ± 0.07	0.05 ± 0.08	-0.11 ± 0.20	-0.12 ± 0.14	*0.555*	*0.694*	*0.138*
K^{+}	0.05 ± 0.24	0.05 ± 0.19	0.31 ± 0.11	0.11 ± 0.04	*0.324*	*0.316*	*0.124*
Na^{+}	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.02	0.02 ± 0.04	*0.902*	*0.123*	*0.101*
Fe	-0.20 ± 0.17	0.01 ± 0.17	-0.34 ± 0.07	-0.21 ± 0.23	*0.676*	*0.123*	*0.101*
Cl	0.01 ± 0.02	0.01 ± 0.01	0.01 ± 0.02	-0.01 ± 0.04	*0.567*	*0.414*	*0.785*
Mg	0.03 ± 0.08	-0.01 ± 0.03	-0.01 ± 0.05	0.05 ± 0.01	*0.123*	*0.720*	*0.715*
CORT	0.16 ± 0.70	-0.43 ± 0.02	0.56 ± 1.33	2.44 ± 3.30	*0.215*	*0.500*	*0.115*
ALB/GLO	-0.07 ± 0.06	0.05 ± 0.04	-0.07 ± 0.04	0.04 ± 0.05	*0.998*	*0.003*	*0.944*
CaxP	0.00 ± 0.03	0.09 ± 0.11	-0.16 ± 0.22	-0.13 ± 0.17	*0.764*	*0.507*	*0.065*
Na/K	0.00 ± 0.22	0.01 ± 0.19	-0.22 ± 0.05	-0.09 ± 0.03	*0.508*	*0.426*	*0.099*

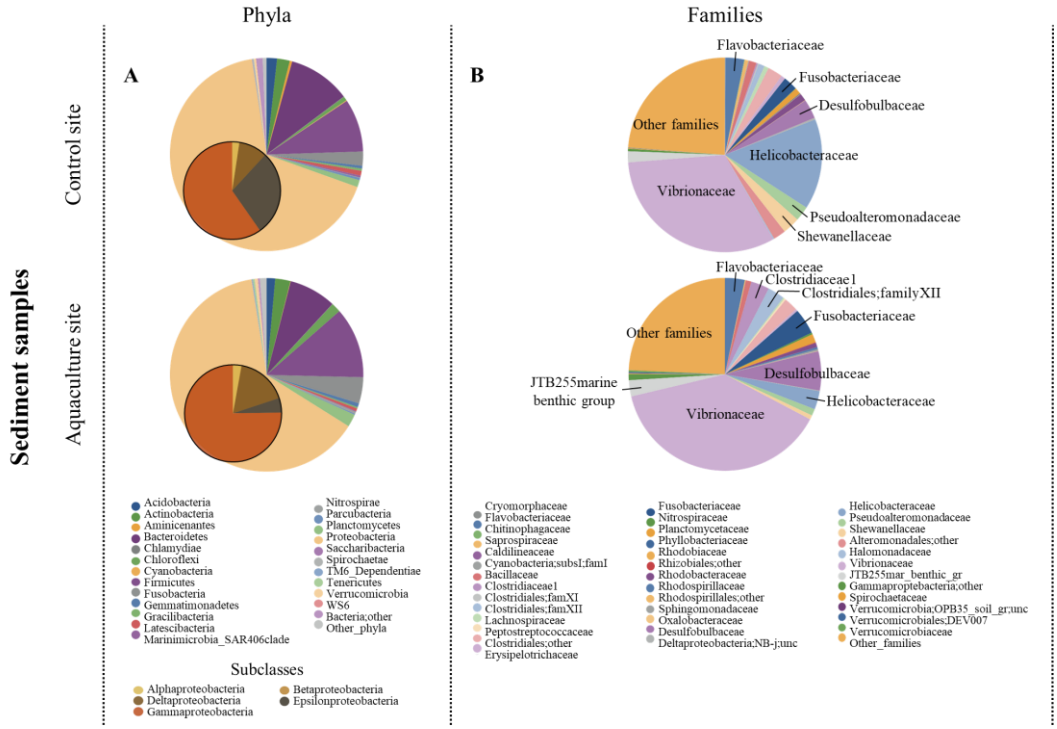
Data are given as the mean (n=9 diet^{-1} on day 58th ; n=15 diet^{-1} on day 121st) ± SD. Different letters indicate significant difference (Two-way ANOVA $P \leq 0.05$) between treatments. L16 = low lipid 16% diet ; L21 = high lipid 21% diet ; HL = constant temperature exposure to high (H) 23°C until temperature switch (day 0-58th), then to constant low (L) 17°C until end of trial (day 59th -121st); LH =. constant temperature exposure to low (L) 17°C until temperature switch (day 0-58th), then to constant high (H) 23°C until end of trial (day 59th -121st).
GLU, glucose , (mg dL^{-1}) ; Urea , (mg dL^{-1}) ; CREA, creatinine , (mg dL^{-1}) ; Uric Ac, uric acid , (mg dL^{-1}) ; Tot Bil, total bilirubin , (mg dL^{-1}) ; Bil Ac, bile acid , (µmol dL^{-1}) ; CHOL, cholesterol , (mg dL^{-1}) ; TRIG, triglycerides , (mg dL^{-1}) ; HDL, high density lipoprotein; TP, total protein , (mg dL^{-1}) ; Alb, albumin , (g dL^{-1}) ; Ast, aspartate aminotransferase , (U L^{-1}); Alp, alkaline phosphatase , (U L^{-1}) ; CK, creatine kinase , (U L^{-1}) ; LDH, lactate dehydrogenase , (U L^{-1}) ; Ca^{+2} , calcium , (mg dL^{-1}) ; P, inorganic phosphorus , (mg dL^{-1}) ; K^{+}, potassium , (mEq L^{-1}) ; Na^{+}, sodium , (mEq L^{-1}) ; Fe, iron , (µg dL^{-1}) ; Cl, chloride , (mEq L^{-1}) ; Mg, magnesium , (mg dL^{-1}) ; CORT, cortisol , (µg dL^{-1}) ; ALB/GLO, albumin/globulin; CaxP, calcium*phosphorus ; Na/K, sodium/potassium ; SD, standard deviation.

Study III - Dietary supplementation with a blend of chestnut and quebracho extracts improves intestinal morphology, microbiota, inflammatory status, and innate immune response in zebrafish (*Danio rerio*)

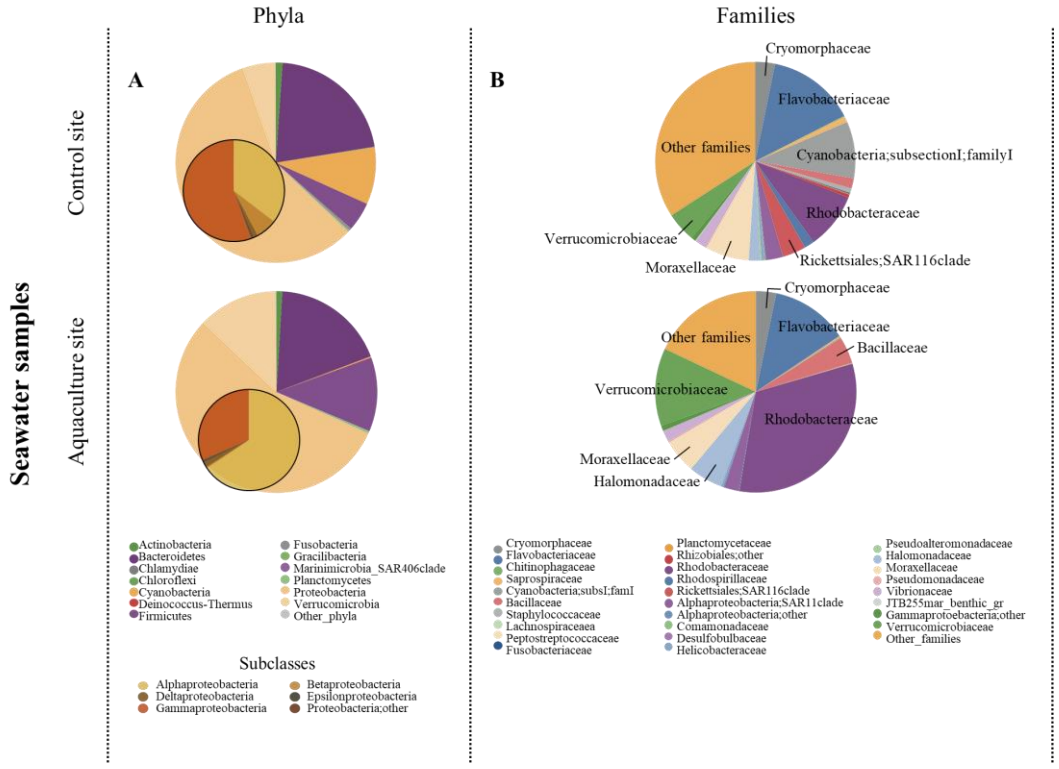
Table S1: Primer sequences for qRT-PCR

Gene	Sequence 5'-3'	Reference
beta actin	F: GCAGAAGGAGATCACATCCCTGGC	https://doi.org/10.3390/nu13030998
	R: CATTGCCGTCACCTTCACCGTTC	
cox2	F: CCCTGTCAGAATCGAGGTGT	https://doi.org/10.1038/nchembio.147
	R: TTGGGAGAAGGCTTCAGAGA	
il1b	F: GGACTTCGCAGCACAAAATGAA	https://doi.org/10.1101/2020.04.09.033837
	R: TTCACTTCACGCTCTTGGATGA	
cxcl8-l1	F: GTCGCTGCATTGAAACAGAA	https://doi.org/10.4049/jimmunol.1203266
	R: CTTAACCCATGGAGCAGAGG	
tnfα	F: GGGCAATCAACAAGATGGAAG	https://doi.org/10.1038/s41598-018-28511-w
	R: GCAGCTGATGTGCAAAGACAC	

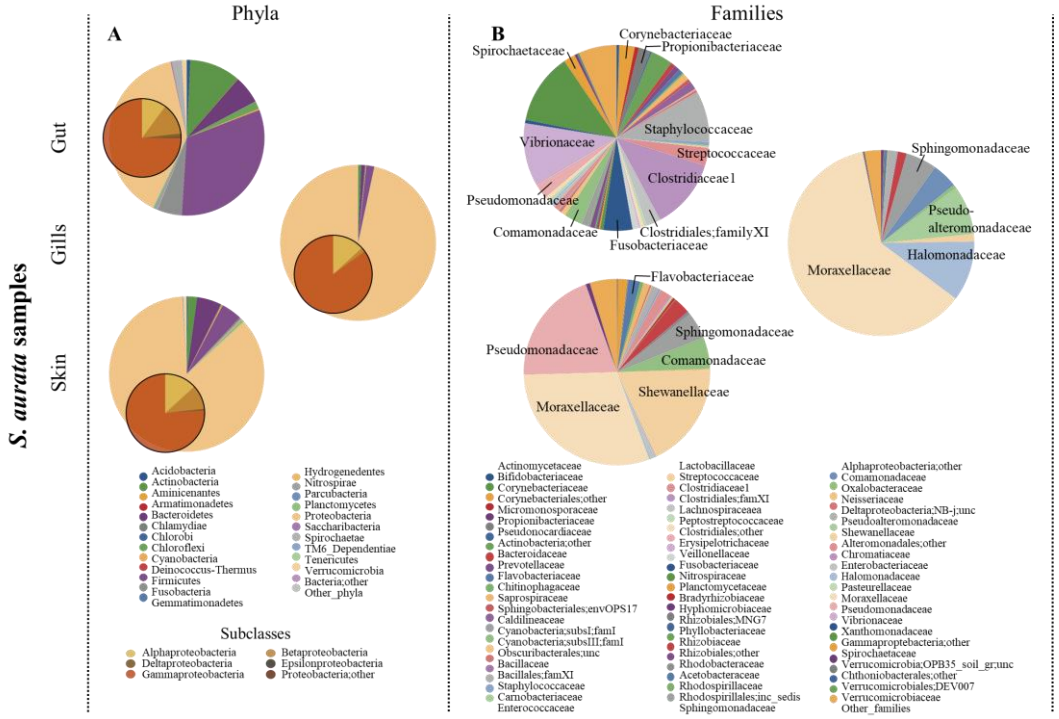
Study IV - Impact of marine aquaculture on the microbiome associated with nearby holobionts: the case of *Patella caerulea* living in proximity of sea bream aquaculture cages



Supplementary figure S1 - Pie charts summarizing the phylum (A) and family (B) level microbiota composition of sediment samples in the two sampling sites. Phyla with relative abundance > 0.5% in at least one sample and families with relative abundance > 2% in at least 10% of samples are represented. Proteobacteria classes are expanded on the respective pie chart phylum slice. subs=subset; fam=family; unc=uncultured; inc=incertae; mar=marine; gr=group.



Supplementary figure S2 - Pie charts summarizing the phylum (A) and family (B) level microbiota composition of seawater samples in the two sampling sites. Phyla with relative abundance > 0.5% in at least one sample and families with relative abundance > 2% in at least 10% of samples are represented. Proteobacteria classes are expanded on the respective pie chart phylum slice. subs=subset; fam=family; unc=uncultured; inc=incertae; mar=marine; gr=group.



Supplementary figure S3 - Pie charts summarizing the phylum (A) and family (B) level microbiota composition of *S. aurata* samples in the three fish districts (feces, gills and skin). Phyla with relative abundance > 0.5% in at least one sample and families with relative abundance > 2% in at least 10% of samples are represented. Proteobacteria classes are expanded on the respective pie chart phylum slice. subs=subset; fam=family; unc=uncultured; inc=incertae; mar=marine; gr=group.

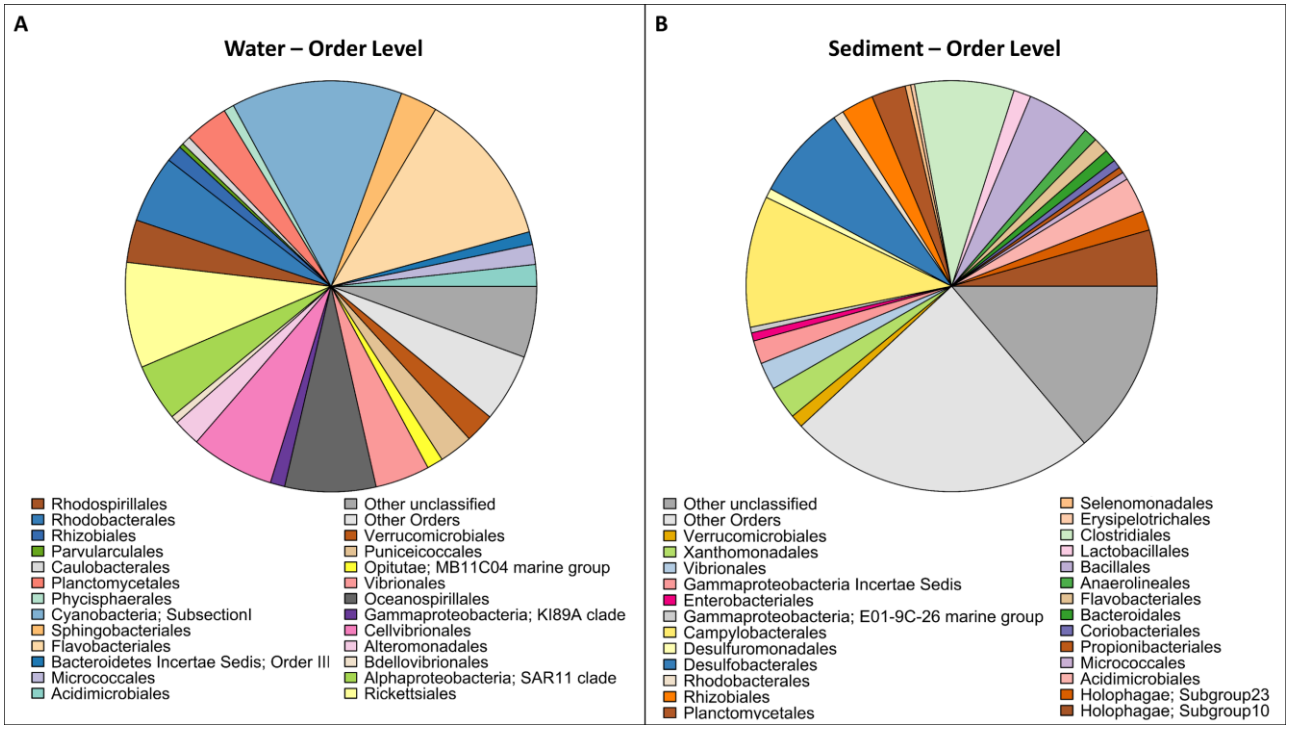
	OTU_ID	BLAST	Control site r.a. (%)			Aquaculture site r.a. (%)				p-value
			P. caerulea	Sediment	Seawater	*P. caerulea*	Sediment	Seawater	Fish	
Control site	4667	*Fodinicurvata halophila*	0.19	0.00	0.00	0.00	0.00	0.00	0.00	0.004
	11135	*Lactobacillus johnsonii*	0.10	0.00	0.00	0.04	0.00	0.00	0.00	0.05
	4454	*Fluviibacterium aquatile*	0.22	0.00	0.00	0.00	0.00	0.00	0.00	0.0004
	5034	*Robiginitalea biformata*	0.06	0.02	0.00	0.00	0.00	0.00	0.00	0.03
	12220	*Rhodobacteraceae*	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.0001
	1496	*Agaricicola taiwanensis*	0.48	0.00	0.00	0.02	0.00	0.00	0.00	0.01
	4069	*Amorphus coralli*	0.10	0.00	0.00	0.01	0.00	0.00	0.00	0.02
	4330	*Ochrobactrum oryzae*	0.11	0.00	0.00	0.00	0.00	0.00	0.00	0.02
	14127	*Rubinisphaera brasiliensis*	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.02
	5331	*Roseibacillus ponti*	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.0008
	2911	*Actibacter sediminis*	0.29	0.03	0.00	0.00	0.13	0.00	0.00	0.0004
	2289	*Psychrobacter celer*	0.03	0.00	0.17	0.00	0.00	0.07	0.06	0.008
	14154	*Phyllobacterium*	0.20	0.00	0.00	0.06	0.00	0.00	0.00	0.04
	3304	*Stappia taiwanensis*	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00002
	11232	*Rubinisphaera italica*	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.03
	1355	*Prochlorococcus marinus*	0.02	0.02	1.18	0.00	0.01	1.88	0.01	0.007
	11155	*Lactobacillus rhamnosus*	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.006
	14091	*Bifidobacterium bifidum*	0.83	0.00	0.00	0.02	0.00	0.00	0.00	0.003
	11445	*Ahrensia kielensis*	0.13	0.00	0.00	0.00	0.00	0.00	0.00	0.0007
	3555	*Bifidobacterium longum*	0.50	0.00	0.00	0.01	0.00	0.00	0.00	0.0004
	2120	*Photobacterium swingsii*	0.23	0.02	0.00	0.01	0.00	0.00	0.00	0.003
	4234	*Hyphomicrobium*	0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.03
Aquaculture site	4187	*Phyllobacteriaceae*	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.0003
	11247	*Rhodopirellula*	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.03
	11243	*Alienimonas californiensis*	0.00	0.00	0.00	0.35	0.00	0.00	0.00	0.001
	11205	*Wenxinia marina*	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.003
	6912	*Mycoplasma mobile*	0.45	0.00	0.00	2.92	0.00	0.00	0.00	0.0004
	5244	*Acinetobacter guillouiae*	0.03	0.00	0.00	0.16	0.00	0.00	0.00	0.04
	4203	*Mesorhizobium thiogangeticum*	0.00	0.00	0.00	0.36	0.00	0.00	0.00	0.003
	2259	*Mycoplasma mobile*	0.02	0.00	0.00	0.19	0.00	0.00	0.00	0.002
	4097	*Mesorhizobium camelthorni*	0.00	0.00	0.00	0.37	0.00	0.00	0.00	0.0002
	2073	*Halomonas*	0.05	0.00	0.00	0.18	0.00	0.00	0.00	0.05
	12731	*Sphingomonas*	0.37	0.00	0.00	1.96	0.00	0.00	0.00	0.001
	11913	*Sphingomonas*	0.06	0.00	0.00	0.40	0.00	0.00	0.00	0.0006
	4065	*Sulfitobacter pontiacus*	0.00	0.00	0.03	0.20	0.00	0.00	0.00	0.03
	4965	*Rhodopirellula baltica*	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.03
	2118	*Vibrio atypicus*	0.42	0.01	0.00	0.63	0.00	0.00	0.00	0.02
	1919	*Staphylococcus*	0.01	0.01	0.01	0.06	0.00	0.01	0.39	0.04
	2077	*Mycoplasma mobile*	0.05	0.00	0.00	0.33	0.00	0.00	0.00	0.005
	2154	*Mycoplasma mobile*	0.03	0.00	0.00	0.16	0.00	0.00	0.00	0.002
	11213	*Rubinisphaera italica*	0.01	0.00	0.00	0.27	0.00	0.00	0.00	0.005
	1397	*Foliisarcina bertiogensis*	0.00	0.00	0.00	0.10	0.00	0.00	0.00	0.002
	11152	*Bacteroides*	0.00	0.00	0.00	0.08	0.00	0.00	0.00	0.006
	2080	*Psychrobacter marincola*	0.00	0.00	0.19	0.13	0.00	0.03	0.00	0.02
	14234	*Sulfurovum lithotrophicum*	0.00	0.82	0.00	0.01	0.08	0.00	0.00	0.01
	4465	*Phyllobacteriaceae*	0.00	0.00	0.00	0.27	0.00	0.00	0.00	0.0001
	4305	*Marimonas arenosa*	0.00	0.00	0.00	0.14	0.00	0.00	0.00	0.04
	6020	*Pseudomonas*	0.00	0.00	0.00	0.07	0.00	0.00	0.08	0.01
	3237	*Bythopirellula goksoyri*	0.00	0.00	0.00	0.13	0.00	0.00	0.00	0.03
	6006	*Gimesia maris*	0.02	0.00	0.00	0.14	0.00	0.00	0.00	0.05

Supplementary table S1 - Ecological distribution and highest score alignment against NCBI 16S rRNA database of OTUs showing a significantly higher mean relative abundance in the limpets collected from control site with respect to those from the aquaculture cage and vice versa. p-values were calculated for the two *P. caerulea* groups (control vs. aquaculture, FDR-corrected Wilcoxon rank-sum test, p-value ≤ 0.05). Species, genera or families are retrieved on the BLAST column based on the last common taxonomic level shared between all BLAST best hits.

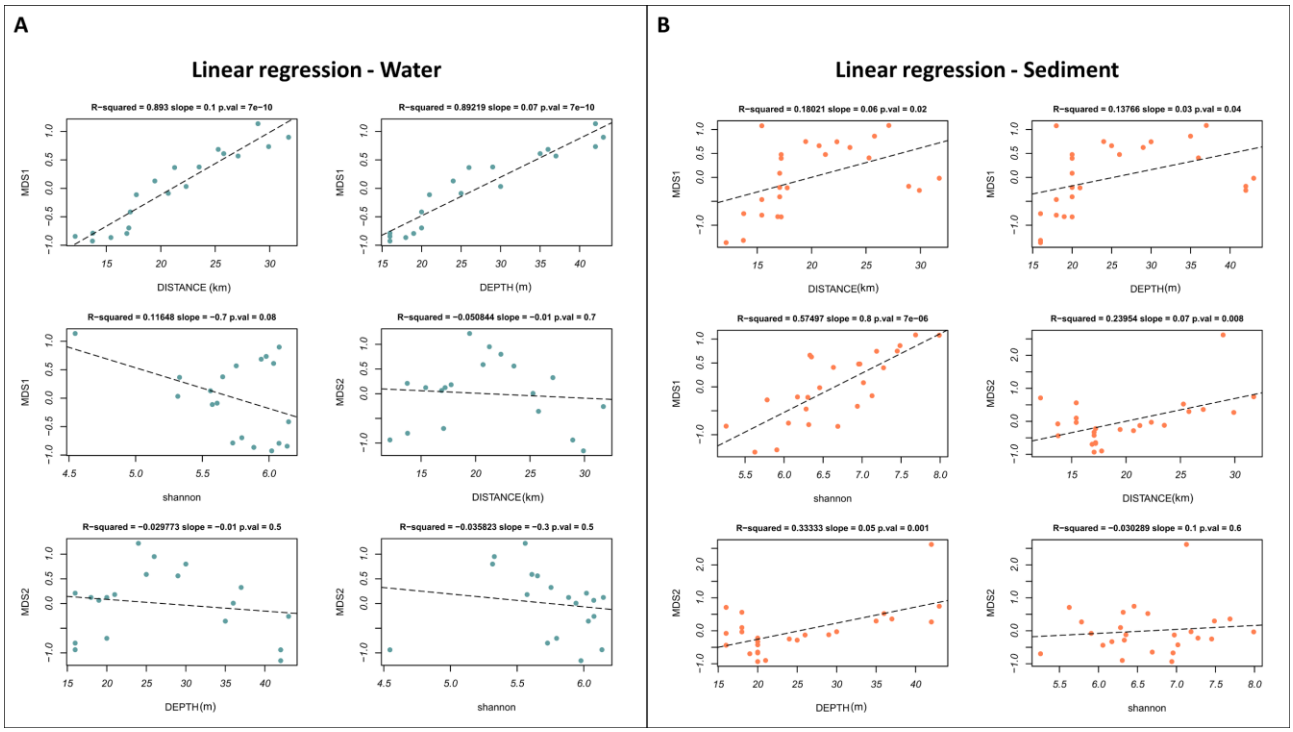
Supplementary table S2 - Seawater environmental data. Measurements (N = 6 per site) are shown for the control and aquaculture sites. Measured parameters, namely T, pH, TA and salinity (38‰ in control and 34‰ in the in the aquaculture site) were used to calculate the carbonate chemistry parameters through CO2SYS Software. T = Temperature; TA = Total Alkalinity; pCO2 = carbon dioxide partial pressure; HCO_3^- = bicarbonate; CO_3^{2-} = carbonate; DIC = dissolved inorganic carbon; Ωarag = aragonite saturation; NS = not significant; **p<0.01, Mann-Whitney test. In brackets the 95% confidence interval.

	T (°C)	pH_{NBS}	TA ($\mu mol\ kg^{-1}$)	pCO_2 (μatm)	HCO_3^- ($\mu mol\ kg^{-1}$)	CO_3^{2-} ($\mu mol\ kg^{-1}$)	DIC ($\mu mol\ kg^{-1}$)	Ω_{arag}
Control	27.3	8.02	2319	667	1898	170	2086	2.67
	(26.9-27.6)	(8.00-8.04)	(2315-2324)	(620-714)	(1882-1914)	(164-177)	(2075-2097)	(2.57-2.76)
Aquaculture	24.8	7.96	2203	700	1877	132.1	2030	2.11
	(24.7-24.9)	(7.95-7.97)	(2191-2213)	(682-717)	(1872-1883)	(130-134)	(2026-2033)	(2.07-2.14)
Mann-Whitney	**	**	**	NS	**	**	**	**

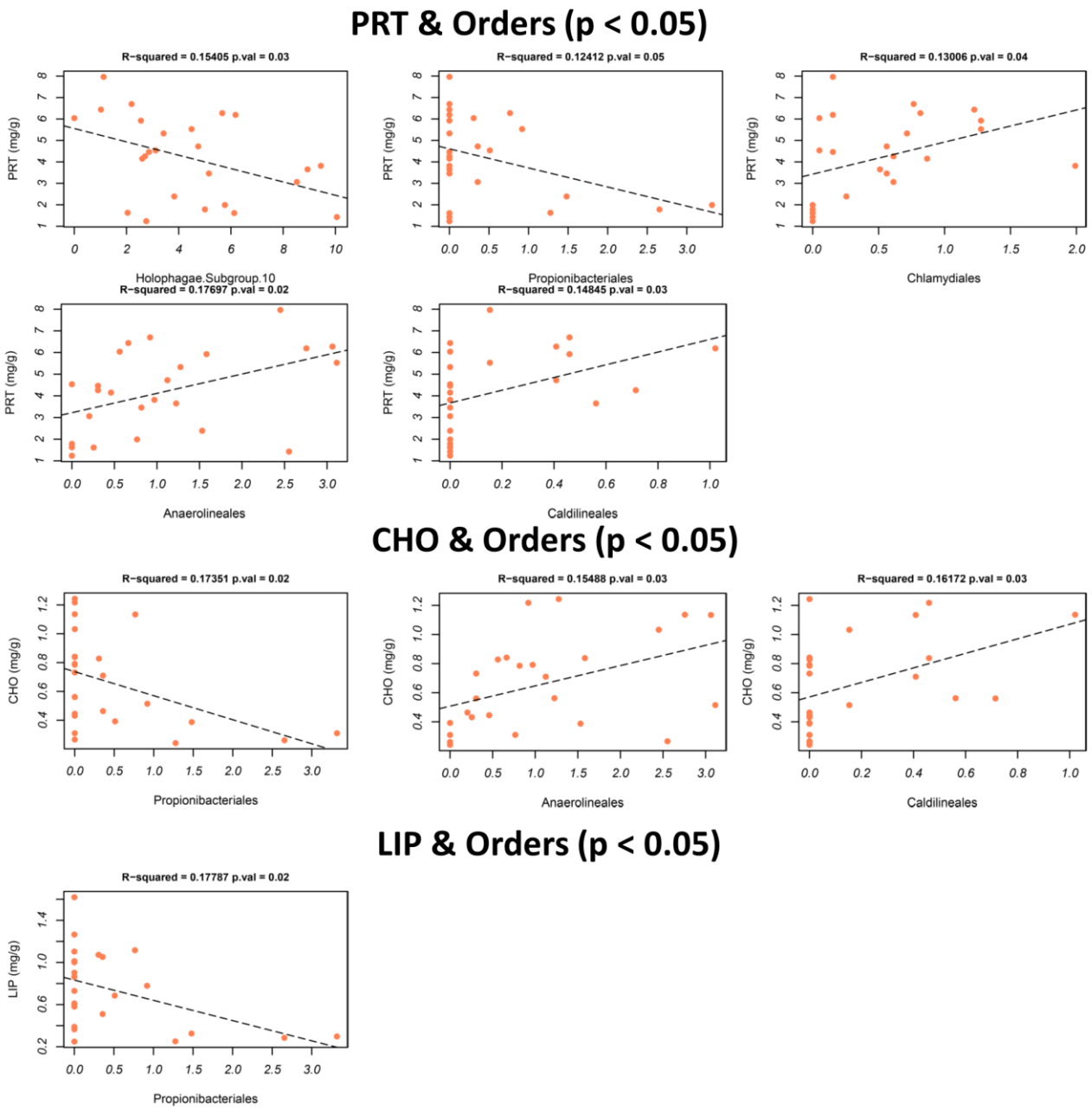
Study V - Microbiome network in the pelagic and benthic offshore systems of the northern Adriatic Sea (Mediterranean Sea)



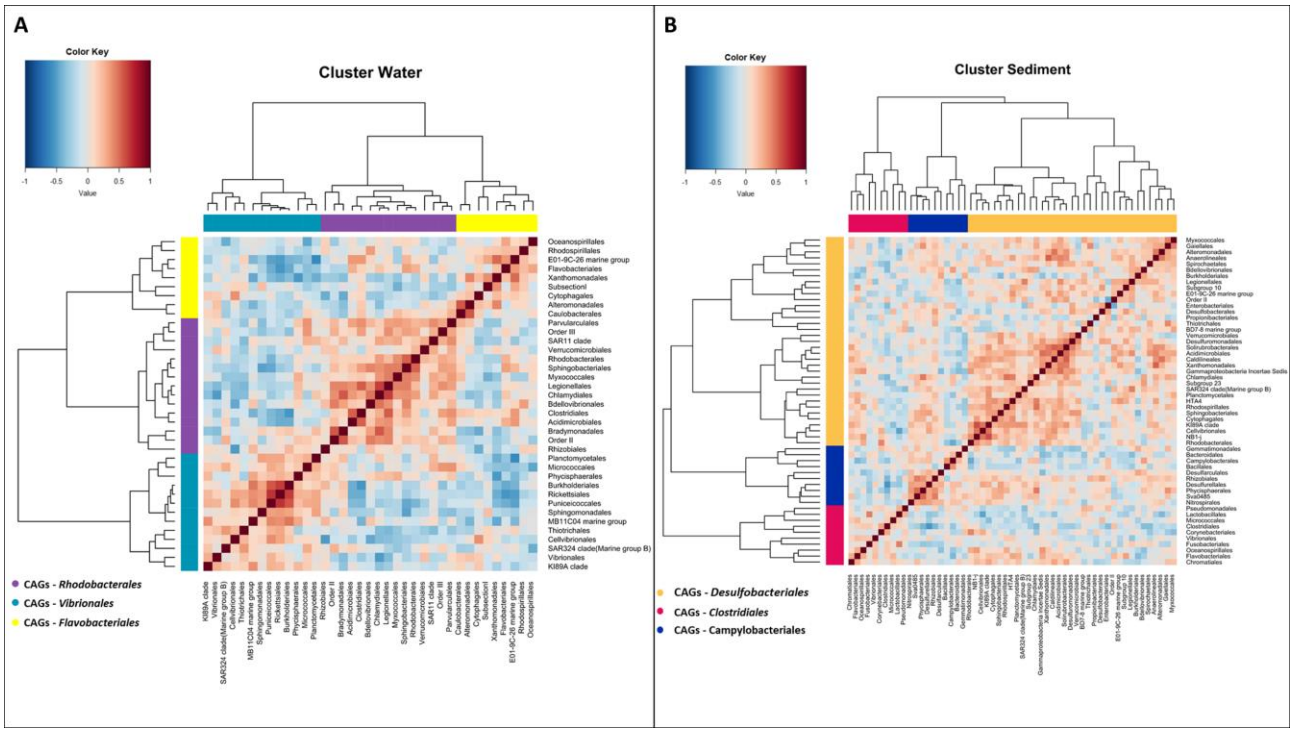
Supplementary Figure 1: Pie charts summarizing the compositional structure of the water (A) and sediment (B) microbiomes at order level. Only orders with relative abundance > 2.0% in at least 2 samples are represented.



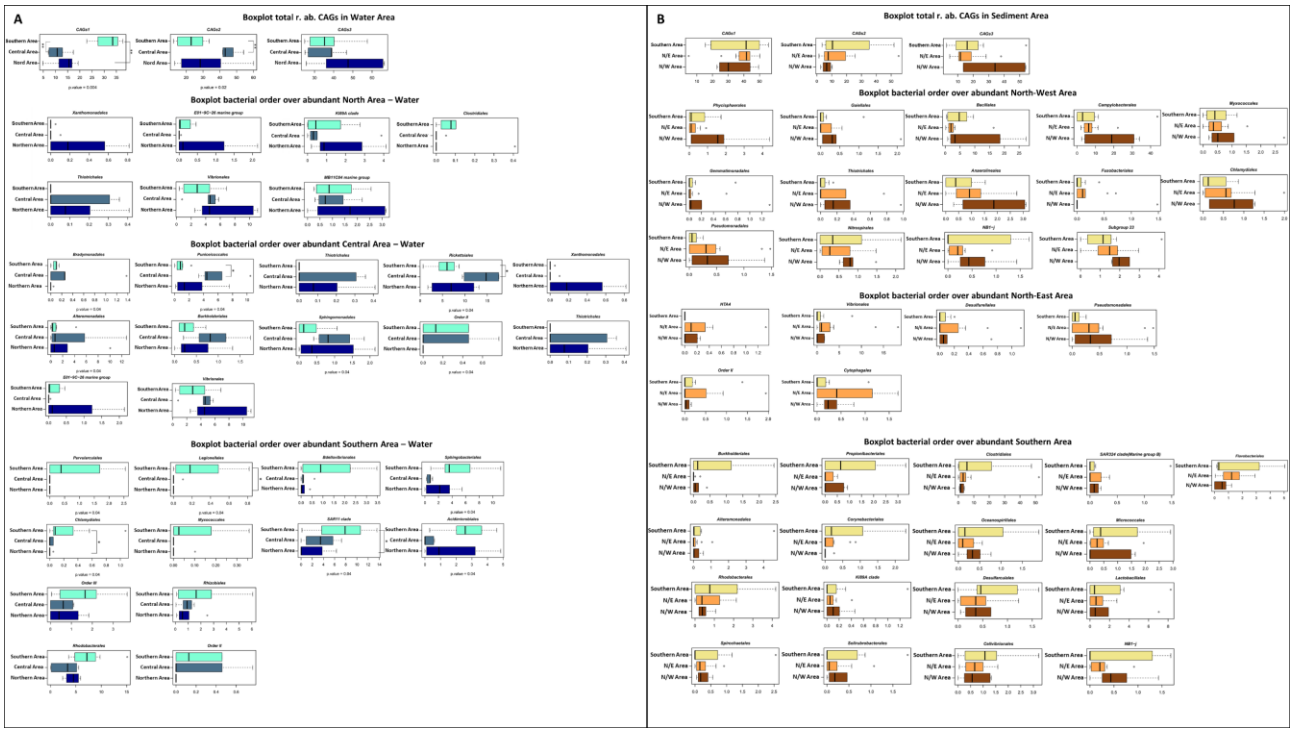
Supplementary Figure 2: Linear regression plots between the two axes (MDS1 and MDS2) of the PCoA of water (A) and sediment (B) samples and three parameters: Distance from coast, Depth of sampling point and Shannon index.



Supplementary Figure 3: Linear regression plots between bacterial Order abundance and biochemical components (PRT, CHO, LIP) in the sediment samples. Only relationship with a $p < 0.05$ are showed.



Supplementary Figure 4: Heatmaps of co-abundant groups (CAGs) identified by hierarchical clustering with the Spearman correlation. A) Heatmap for water microbiome at the order levels, B) Heatmap for sediment microbiome at the order levels. Colour scale for Spearman correlation is provided on the left of each panel, where red represents the highest and dark blue the lowest correlation. Bacterial orders abundance profile was clustered using hierarchical clustering approach, using the profile Spearman correlation and Ward's linkage method. The obtained clusters were represented by dendrograms at the top and left margins of each Heatmap. Within each cluster, bacterial orders were coloured according to the colour legend in the bottom left of each panel.



Supplementary Figure 5: Boxplots showing the variation of the relative abundance of CAGs (Upper part) and of each over abundant order in the same CAGs (over-abundance > 1.3, bottom part) across all sectors under study both for pelagic microbiome (Panel A) and sediment microbiome (Panel B). The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line. Significant variation across groups was highlighted in the figure (Wilcoxon rank-sum test; $p \leq 0.05$ *, $p \leq 0.01$ **).

Supplementary Table 1: Table reporting exact geographical coordinates of the 19 sampling sites with additional information of *distance from coast* (km) and *depth* (m).

	DISTANCE FROM COAST (km)	DEPTH (m)	LATITUDE	LONGITUDE
Sampling Site 1	13.728	16	44.1346944	12.72288889
Sampling Site 2	13.757	16	44.1060278	12.76027778
Sampling Site 3	12.14	16	44.0686667	12.79630556
Sampling Site 4	16.884	19	44.1516944	12.74511111
Sampling Site 5	15.421	18	44.1311389	12.75394444
Sampling Site 6	17.065	20	44.1075833	12.82425
Sampling Site 6B	17.202	20	44.1033889	12.83319444
Sampling Site 7	17.757	21	44.1360278	12.78602778
Sampling Site 8	20.688	25	44.1651111	12.79569444
Sampling Site 9	21.269	26	44.1480556	12.83227778
Sampling Site 10	19.454	24	44.1235833	12.84311111
Sampling Site 11	23.521	29	44.1963333	12.81055556
Sampling Site 12	25.78	35	44.1907778	12.85272222
Sampling Site 13	25.275	36	44.1675278	12.88288889
Sampling Site 14	22.325	30	44.1393611	12.87397222
Sampling Site 15	27.099	37	44.2244444	12.83363889
Sampling Site 16	31.735	43	44.2524444	12.87088861
Sampling Site 17	29.905	42	44.2218889	12.88508333
Sampling Site 18	28.924	42	44.1948611	12.90647222

Sediment Area			Water Area		
Southern Sector			Southern Sector		
Northeast Sector			Central Sector		
Northwest Sector			Northern Sector		
Samples	**Shannon alpha-diversity**	**Quartile distribution**	**Samples**	**Shannon alpha-diversity**	**Quartile distribution**
C4S	5.257365	1°	C18W	4.546159	1°
C3S	5.62557	1°	C14W	5.316015	1°
C17S	5.782988	1°	C9W	5.329229	1°
C1S	5.907133	1°	C10W	5.561033	1°
C2S	6.056522	1°	C7W	5.574893	1°
C6B2S	6.171907	1°	C8W	5.611047	2°
C5AS	6.282452	1°	C11W	5.652874	2°
C7S	6.304958	2°	C2W	5.728202	2°
C5CS	6.316561	2°	C15W	5.754454	2°
C8S	6.331336	2°	C6BW	5.796217	3°
C11S	6.351584	2°	C5W	5.886421	3°
C16S	6.456489	2°	C13W	5.941774	3°
C13S	6.632595	3°	C17W	5.979078	3°
C6BS	6.689232	3°	C1W	6.020465	3°
C6B1S	6.939868	3°	C12W	6.034664	4°
C6CS	6.955684	3°	C4W	6.075191	4°
C9S	6.971714	3°	C16W	6.076636	4°
C6AS	7.017395	3°	C3W	6.136331	4°
C18S	7.127725	4°	C6W	6.147152	4°
C14S	7.186221	4°			
C6B3S	7.27495	4°			
C10S	7.450581	4°			
C12S	7.48579	4°			
C15S	7.685893	4°			
C5BS	7.989378	4°			

Supplementary Table 2: Water and sediment samples quartile distribution according to the Shannon alpha-diversity values. Color legend indicate samples corresponding sector for both water and sediment area.

Supplementary Table 3: Concentrations of the different biochemical compounds of organic matter in the sediment samples of the investigated area. Data are reported as mean ± standard deviation. **PRT**: Total Protein; **CHO**: Total Carbohydrates; **LIP**: Total Lipids; **Chl-a**: Chlorophyll-A; **Pheo**: Pheopigments; **BPC**: Biopolymeric C.

Station	PRT mg/g		CHO mg/g		LIP mg/g		Chl-a µg/g		Pheo µg/g		BPC mgC/g	
	Mean	Ds	Mean	Ds	Mean	Ds	Mean	Ds	Mean	Ds	Mean	Ds
C1S	1.99	0.47	0.31	0.07	0.30	0.09	0.70	0.07	8.96	0.15	1.32	0.17
C2S	2.39	0.22	0.39	0.02	0.33	0.04	0.94	0.04	10.20	0.30	1.57	0.14
C3S	1.24	0.23	0.31	0.04	0.25	0.11	0.59	0.12	8.27	1.05	0.92	0.15
C4S	4.15	1.46	0.45	0.03	0.36	0.08	0.69	0.01	7.08	0.65	2.49	1.04
C5.1S	1.78	0.06	0.26	0.01	0.28	0.02	0.63	0.11	9.65	0.31	1.19	0.03
C5.2S	4.26	0.62	0.56	0.02	0.73	0.02	2.14	0.04	24.08	1.49	2.85	0.32
C5.3S	1.63	0.12	0.24	0.01	0.25	0.06	0.57	0.05	6.49	0.58	1.08	0.08
C6.1S	3.06	1.55	0.46	0.30	0.51	0.11	1.40	0.19	14.90	2.40	2.07	0.80
C6.2S	1.43	0.09	0.27	0.01	0.39	0.04	1.23	0.20	14.49	1.46	1.08	0.08
C6.3S	1.61	0.15	0.43	0.11	0.58	0.20	1.59	0.61	18.29	4.48	1.29	0.02
C6B.1S	3.82	0.56	0.79	0.04	0.86	0.11	5.37	0.19	26.91	1.82	2.83	0.23
C6B.2S	4.54	0.16	0.39	0.10	0.69	0.11	0.57	0.08	10.97	0.97	2.89	0.13
C6B.3S	3.46	0.47	0.79	0.22	0.59	0.05	3.58	0.02	19.76	0.23	2.45	0.16
C7S	3.65	0.98	0.56	0.21	0.61	0.14	0.94	0.08	12.28	1.10	2.47	0.52
C8S	4.46	1.05	0.73	0.11	1.26	0.46	0.96	0.16	16.37	0.24	3.43	0.81
C9S	4.72	0.80	0.71	0.05	1.05	0.10	1.39	0.03	17.23	1.57	3.39	0.33
C10S	5.92	0.69	0.84	0.30	0.86	0.09	1.09	0.07	16.71	0.93	3.88	0.38
C11S	7.96	0.37	1.03	0.23	1.10	0.12	0.86	0.17	20.36	1.60	5.14	0.36
C12S	5.53	0.10	0.51	0.07	0.78	0.09	0.79	0.05	16.78	2.84	3.50	0.09
C13S	6.70	0.25	1.22	0.25	1.62	1.07	0.84	0.11	13.78	0.68	4.98	0.81
C14S	6.19	0.40	1.14	0.31	0.90	0.03	1.30	0.06	19.55	1.53	4.17	0.10
C15S	6.27	0.22	1.13	0.16	1.12	0.15	0.91	0.11	17.39	0.29	4.36	0.13
C16S	5.33	0.56	1.24	0.06	1.01	0.22	0.63	0.04	12.64	0.50	3.87	0.14
C17S	6.44	1.22	0.84	0.12	1.00	0.31	0.35	0.01	11.75	0.48	4.24	0.53
C18S	6.04	1.90	0.83	0.11	1.07	0.28	0.42	0.03	11.50	0.13	4.09	0.81