UNIVERSITY OF INSUBRIA

DEPARTMENT OF BIOTECHNOLOGY AND LIFE SCIENCES

Ph.D. program in

Biotechnologies, Biosciences and Surgical Technologies

XXXI Cycle

Effects of some stable derivatives of butyrate used as feed additives on fish intestinal microbiota, histone modifications and the expression of genes related to epigenetic regulatory mechanisms and immune response

Ph.D. candidate:

Dr. Emi Gliozheni

N° 710871

Tutor:

Prof. Genciana Terova

Academic year 2017-2018

CONTENTS

ABSTRACT

Bacteria that inhabit the epithelium of the animals' digestive tract provide the essential biochemical pathways for fermenting otherwise indigestible dietary fibers, leading to the production of short-chain fatty acids (SCFAs). Of the major SCFAs, butyrate has received particular attention due to its numerous positive effects on the health of the intestinal tract and peripheral tissues.

Butyrate plays a major role in enhancing epithelial cell proliferation and differentiation and in improving the intestinal absorptive function. It has also potential immunomodulatory and anti-inflammatory properties in the intestine and may prevent colorectal cancer in humans.

The mechanisms of action of butyrate are different; this four-carbon chain organic acid is a histone deacetylase inhibitor that play a predominant role in the epigenetic regulation of gene expression and cell function, therefore many of its mechanisms are related to its potent regulatory effect on gene expression.

During the first year of PhD, my research activity was related to the study of the effects of dietary sodium butyrate on histone modifications and the expression of genes involved in epigenetic regulatory mechanisms and immune response in European sea bass (*Dicentrarchus Labrax*) fed a plant-based diet.

Accordingly, the effects of butyrate used as a feed additive on fish epigenetics as well as its regulatory role in mucosal protection and immune homeostasis through impact on gene expression, were investigated.

To meet the aims, seven target genes related to inflammatory response and reinforcement of the epithelial defense barrier [tnfα (tumor necrosis factor

alpha) il1β, (interleukin 1beta), il-6, il-8, il-10, and muc2 (mucin 2)] and five target genes related to epigenetic modifications [dicer1(double-stranded RNA-specific endoribonuclease), ehmt2 (euchromatic histone-lysine-N-methyltransferase 2), pcgf2 (polycomb group ring finger 2), hdac11 (histone deacetylase-11), and jarid2a (jumonji)] were analyzed in fish intestine and liver. We also investigated the effect of dietary butyrate supplementation on histone acetylation, by performing an immunoblotting analysis on liver core histone extracts. Results of the eight-week feeding trial showed no significant differences in weight gain or Specific Growth Rate (SGR) in sea bass that received 0.2% sodium butyrate supplementation in the diet in comparison to control fish that received a diet without Na-butyrate. Dietary butyrate led to a two-fold increase in the acetylation level of histone H4 at lysine 8, but showed no effect on the histone H3 at Lys9. Moreover, two different isoforms of histone H3 that might correspond to the H3.1 and H3.2 isoforms previously found in terrestrial animals were separated on the immunoblots. The expression of four (il1 β, il8, irf1, and tnfα) out of seven analyzed genes related to mucosal protection and inflammatory response was significantly different between the two analyzed tissues but only il10 showed differences in expression due to the interaction between tissue and butyrate treatment. In addition, butyrate caused significant changes *in vivo* in the expression of genes related to epigenetic regulatory mechanisms such as hdac11, ehmt2, and dicer1. Statistical analysis by two-way ANOVA for these genes showed not only significant differences due to the butyrate treatment, but also due to the interaction between tissue and treatment.

In the second year of my studies, I focused on a different fish species - gilthead sea bream (*Sparus aurata*), to investigate the effects of a specific

combination of short- and medium-chain 1-monoglycerides on intestinal microbiome, gene expression, and fish growth performance.

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

Currently, there is an increasing research interest towards the use of organic acids in commercial aqua-feeds, due to several beneficial effects they have on growth performance and intestinal tract's health of farmed fish. Among organic acids, monoglycerides of short-chain fatty acids (SCFAs) and medium-chain fatty acids (MCFAs) have attracted particular research attention also for their bacteriostatic and bactericidal properties. Accordingly, the present study aimed to evaluate the potential beneficial effects of SCFA and MCFA monoglycerides, used as a feed additive, on fish growth performance, and intestinal microbiota composition. For this purpose, a specific combination of short- and medium-chain 1-monoglycerides (SILOhealth 108Z) was tested in 600 juvenile gilthead sea bream (*Sparus aurata*) of 60 g mean initial weight that were fed for 90 days with plant-based diets. Two isoproteic and isolipidic diets were formulated. The control fish group received a plant-based diet, whereas the other group received the same control feed, but supplemented with 0.5% of SILOhealth 108Z. The Illumina MiSeq platform for high-throughput amplicon sequencing of 16S rRNA gene and QIIME pipeline were used to analyse and characterize the whole microbiome associated with both thefeeds and *S. aurata* intestine. The number of reads taxonomically classified according to the Greengenes database was 394,611. We identified 259 OTUs at 97% identity in sea bream fecal samples; 90 OTUs constituted the core gut microbiota. *Firmicutes*, *Proteobacteria* and

Actinobacteria represented the dominant phyla in both experimental groups. Among them, relative abundances of *Firmicutes* and *Proteobacteria* were positively and negatively affected by dietary SCFA monoglycerides supplementation, respectively. In summary, our findings clearly indicated that SILOhealth 108Z positively modulated the fish intestinal microbiota by increasing the number of beneficial lactic acid bacteria, namely, *Lactobacillus*, and reducing *Gammaproteobacteria*, which include several potential pathogenic bacteria. The specific composition of 1-monoglycerides of short- and medium-chain fatty acids contained in SILOhealth 108Z could thus have a great potential as a feed additive in aquaculture.

1. INTRODUCTION

1.1. AQUACULTURE

Human societies face the enormous challenge of having to provide food and livelihoods to a population well in excess of 9 billion people by the middle of the twenty-first century, while addressing the disproportionate impacts of climate change and environmental degradation on the resource base.

Food and agriculture are key to achieving the entire set of Sustainable Development Goals (SDGs), and many SDGs are directly relevant to fisheries and aquaculture.

The State of World Fisheries and Aquaculture 2018 highlights the critical importance of fisheries and aquaculture for the food, nutrition and employment of millions of people, many of whom struggle to maintain reasonable livelihoods.

The total fish production in 2016 reached an all-time high of 171 million tonnes, of which 88 percent was utilised for direct human consumption due to relatively stable capture fisheries production, reduced wastage and continued aquaculture growth. This production resulted in a record-high per capita consumption of 20.3 kg in 2016. Since 1961, the annual global growth in fish consumption has been twice as high as population growth.

While annual growth of aquaculture has declined in recent years, significant double-digit growth is still recorded in some countries, particularly in Africa and Asia (FAO, 2017). The sector's contribution to economic growth and the fight against poverty is growing. Strengthened demand and higher prices increased the value of global fish exports in 2017 to USD 152 billion, 54 percent originating from developing countries.

The fisheries and aquaculture sector is not without challenges, however, including the need to reduce the percentage of fish stocks captured beyond biological sustainability, currently 33.1 percent; to ensure that biosecurity and animal disease challenges are tackled successfully; and to maintain complete and accurate national statistics in support of policy development and implementation.

Aquaculture represent 47 percent of the total global fish production and 53 percent if non-food uses (including the reduction to fishmeal and fish oil) are excluded (FAO- Rome, 2018). Since the late 1980s, the overall capture of fishery production has been relatively static. Hence, aquaculture has been responsible for the continuing and important growth in the supply of fish for human consumption (Figure 1).

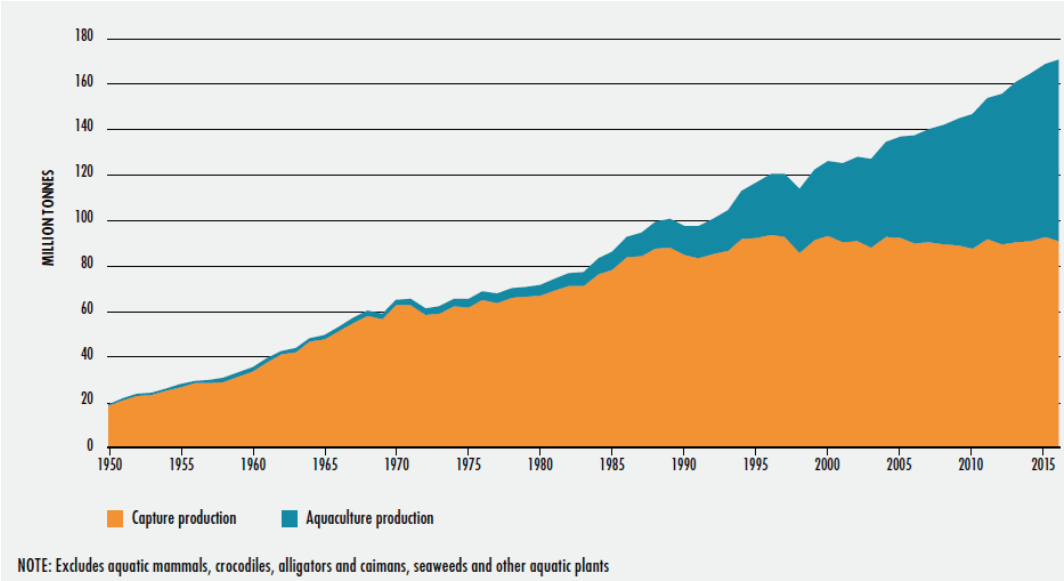

Figure 1. *World capture fisheries and aquaculture production (FAO- Rome, 2018)*

Between 1961 and 2016, regrettably the average annual increase in global food fish consumption (3.2 percent) outpaced the global population growth (1.6 percent) (Figure 2). This increase of fish demand exceeded even that of meat from all terrestrial animals combined (2.8 percent). (FAO - Rome, 2018)

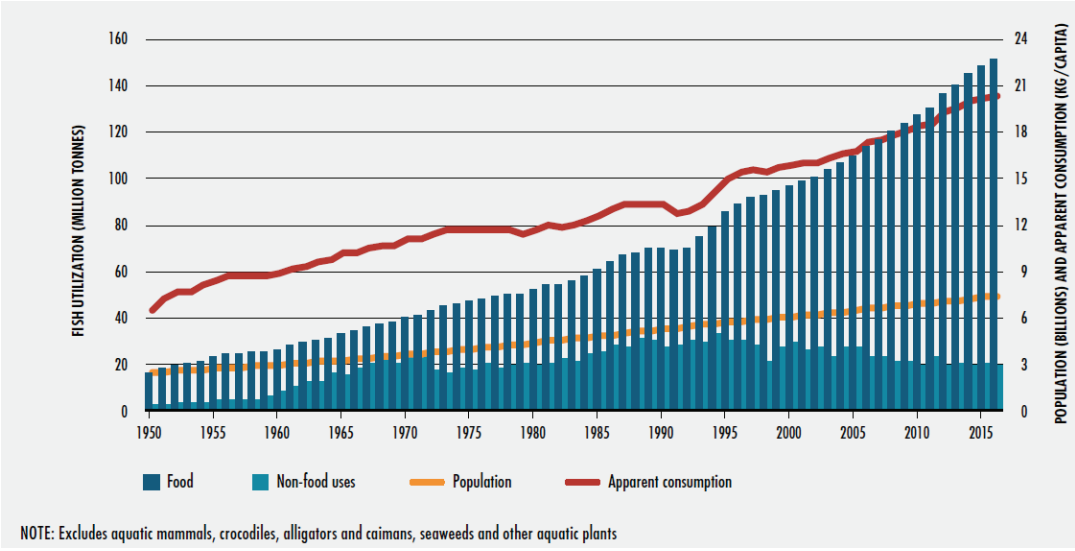

Figure 2. *World fish utilization and apparent consumption (FAO- Rome, 2018)*

In per capita terms, food fish consumption duplicated from 9.0 kg in 1961 to 20.2 kg in 2015, at an average rate of about 1.5 percent per year. Preliminary estimates for the next two years 2016 and 2017, suggest a further growth of about 20.3 and 20.5 kg, respectively. The expansion in consumption has been directed not only by increased production, but also by other several factors, including reduced wastage. About 3.2 billion people have been provided by fish consumption with almost 20 percent of their average per capita intake of animal protein. People in developing countries have a higher share of fish protein in their diets compared to those in developed countries, despite their relatively low levels of fish consumption.

The global capture fisheries production was 90.9 million tonnes in 2016 which is a small decrease in comparison to the two previous years (Table 1).

Category	2011	2012	2013	2014	2015	2016
Production						
Capture						
Inland	10.7	11.2	11.2	11.3	11.4	11.6
Marine	81.5	78.4	79.4	79.9	81.2	79.3
Total capture	**92.2**	**89.5**	**90.6**	**91.2**	**92.7**	**90.9**
Aquaculture						
Inland	38.6	42.0	44.8	46.9	48.6	51.4
Marine	23.2	24.4	25.4	26.8	27.5	28.7
Total aquaculture	**61.8**	**66.4**	**70.2**	**73.7**	**76.1**	**80.0**
Total world fisheries and aquaculture	**154.0**	**156.0**	**160.7**	**164.9**	**168.7**	**170.9**
Utilization[b]						
Human consumption	130.0	136.4	140.1	144.8	148.4	151.2
Non-food uses	24.0	19.6	20.6	20.0	20.3	19.7
Population (*billions*)[c]	7.0	7.1	7.2	7.3	7.3	7.4
Per capita apparent consumption (*kg*)	18.5	19.2	19.5	19.9	20.2	20.3

[a] Excludes aquatic mammals, crocodiles, alligators and caimans, seaweeds and other aquatic plants.
[b] Utilization data for 2014–2016 are provisional estimates.
[c] Source of population figures: UN, 2015e.

Table 1. *World fisheries and aquaculture production and utilization (million tonnes)*[a] *(FAO- Rome, 2018)*

Fisheries in marine and inland waters provided 87.2 and 12.8 percent of the global total, respectively (FAO- Rome, 2018).

About 88 percent of the 171 million tonnes of total fish production in 2016 (over 151 million tonnes) was utilised for direct human consumption and this data has been increasing significantly in recent decades.

The greatest part of the 12 percent used for non-food purposes (about 20 million tonnes) was reduced to fishmeal and fish oil. Live, fresh or chilled is often the most preferred and highly priced form of fish and represents the largest share of fish for direct human consumption (45 percent in 2016), followed by frozen (31 percent).

Fishmeal production reached the peak point in 1994 at 30 million tonnes (live weight equivalent) and since then the trend has been fluctuating but overall declining. A growing share of fishmeal is being produced from fish by-products, which previously were often wasted. It is estimated that by-products account for about 25 to 35 percent of the total volume of fishmeal and fish oil produced. Fishmeal and fish oil are still nowadays considered the most nutritious and most digestible ingredients for farmed fish feeds. Nevertheless, as they are used more selectively, their inclusion rates in compound feeds for aquaculture have shown a clear downward trend (FAO- Rome, 2018).

A significant, but declining, proportion of world fisheries production is processed into fishmeal and fish oil. This fraction contributes indirectly to human food production and consumption when these ingredients are used as feed in aquaculture and livestock raising. These products can be produced from whole fish, fish trimmings or other fish by-products resulting from processing. Several species, mostly of them small pelagic species, are used for the production of fishmeal and fish oil. Many of the species used, such as anchoveta (*Engraulis ringens*), have comparatively

high oil yields but are rarely used for direct human consumption (FAO-Rome, 2018).

Owing to the growing demand for fishmeal and fish oil, in particular from the aquaculture industry, and coupled with high prices, a growing share of fishmeal is being produced from fish by-products, which previously were often wasted. It is estimated that by-products account for about 25 to 35 percent of the total volume of fishmeal and fish oil produced, but there are also regional differences. For example, by-product use in Europe is comparatively high at 54 percent (Jackson and Newton, 2016). With no additional raw material expected to come from whole fish caught by reduction-dedicated fisheries (in particular, small pelagic fish), any increase in fishmeal production will need to come from use of by-products, which can, however, have a negative impact on the overall nutritional value as feed.

Fish oil represents the richest available source of long-chain polyunsaturated fatty acids (PUFAs) which are important in human diets for a wide range of functions. However, the Marine Ingredients Organisation (IFFO) estimates that approximately 75 percent of annual fish oil production still goes into aquaculture feeds (Auchterlonie, 2018). Because of the variable supply of fishmeal and fish oil production and associated price variation, commercial feed producers, nutritionists, and many researchers are seeking alternative sources of PUFAs, including large marine zooplankton stocks such as Antarctic krill (*Euphausia superba*) and the copepod *Calanus finmarchicus*, although concerns remain over the impacts for marine food webs (FAO- Rome, 2018).

However, in order to be included as a general oil or protein ingredient in fish feed, the cost of zooplankton products remains too high.

Fish silage (Kim and Mendis, 2006), a rich source of protein hydrolysate, is a less expensive alternative to fishmeal and fish oil and is increasingly important as a feed additive, for example in aquaculture and in the pet food industry. Silage, obtained by preserving whole fish or fish by-products with an acid and letting enzymes from the fish hydrolyse the proteins, has potential to increase growth and reduce mortality of animals that receive it in their feed.

Increasingly intensive aquaculture production methods, with greater use of crop-based feedstuffs and lower fishmeal and fish oil inclusion rates, are likely to influence the nutrient content of farmed aquatic products, particularly fat content and fatty acid profiles. A focus on the nutrient content of farmed aquatic foods is especially important where they have a key role in food-based approaches (FAO- Rome, 2018).

1.2. BUTYRATE

The availability of marine ingredients, fishmeal and fish oil, traditionally utilized in the preparation of feed for cultured fish, is finite. Consequently, the rapid rise of global aquaculture have forced the aquafeed industry to identify and utilize alternative and more sustainable ingredients that can guarantee fish growth and health (Tacon and Metian, 2008). In the last few years, significant advances have been made in this direction and the most commonly used alternatives to the limited and expensive fishery-derived raw materials have been of terrestrial plant origin (Gatlin et al., 2007). However, the main drawbacks of using vegetable feedstuff in aquafeeds are related to their suboptimal amino acid profile, poor in essential amino acids such as methionine and lysine, and to the presence of a wide variety of anti-nutritional factors (Francis et al., 2001). Those antinutritional components found in terrestrial plants, include phytic acid, saponins, and protease

inhibitors, which can damage the intestinal lining, compromise nutrient digestibility and absorption, thus leading to reduced fish growth, increased stressed and impaired resistance to diseases (Zhang et al., 2013; Penn et al., 2011; Santigosa et al., 2011; Francis et al., 2001). Therefore, there is an increasing interest to find feed additives that could prevent the adverse effects of plant-based ingredients normally used in fish diet formulations. One of the most promising feed additive has been sodium butyrate (Na-butyrate), a salt of butyric acid. The advantage of salts over free organic acids is that they are generally odourless and not volatile. Conversely, butyric acid isa short chain fatty acid (SCFA) with four carbon atoms and offensive odour, whose acidity is associated with the carboxyl group (−COOH) (Mallo et al., 2012; Lim et al., 2015).

SCFAs, also known as volatile fatty acids, are carboxylic acids with aliphatic tails of 1 to 6 carbon atoms that exist in straight- and branched-chain conformations. Common SCFAs include acetic (C2), propionic (C3), butyric (C4), valeric (C5), and caproic (C6) acid (Canani et al., 2012). Being weak acids with modest pKas of approximately 3.6 to 4.7, SCFAs do not completely dissociate or dissolve in water. Furthermore due to the pH of part of the gastrointestinal tract in which the fermentation occurs at nearly neutral level (the colonic pH is approximately 6.0-7.5), more than 90% of SCFAs are present as anions rather than as free acids (Bergman, 1990). The predominant anions in either the rumen or large intestine are the short, straight-chain FAs such as acetate, propionate, and butyrate, whereas the short branched-chain FAs, isobutyrate and isovalerate, which are produced by fermentation of the amino acids valine and leucine, respectively, are found in much smaller amounts (Bergman, 1990; Macfarlane and Macfarlane, 2011).

Butyric acid is one of the main end-products of anaerobic bacterial fermentation of otherwise undigested complex carbohydrates (cellulose, hemicellulose, pectin) in the animals' intestinal tract. This acid represents the major energy source for enterocytes and is involved in the maintenance of gut mucosal health, playing a central role in enhancing epithelial cell proliferation and differentiation and in improving the gut absorptive function (Gálfi and Neogrády 2002; Wong et al. 2006; Canani et al., 2011). Hence, in the last years, butyrate has received particular attention for its numerous and well documented beneficial effects on the health of intestinal tract and peripheral tissues in humans, and animals, including fish (Guilloteau et al., 2010; Mátis et al. 2013; Robles et al., 2013; Liu et al., 2014). Furthermore, there are several lines of evidence to suggest that butyrate has potential immunomodulatory and anti-inflammatory properties in the intestine and may prevent colorectal cancer in humans (Vinolo et al., 2009; Toden at al., 2007; Hammer et al., 2008;Terova et al., 2016; Rimoldi et al., 2016; Tian et al., 2017). In sea bream (*Sparus aurata*) Robles and colleagues (2013) firstly reported a positive effect of dietary butyrate on the availability of several essential amino acids and nucleotide derivatives associated with a significant improvement of fish growth rates. Similarly, Na-butyrate supplementation positively affected the growth performances of Pacific white shrimp (*Litopenaeus vannamei*) (Silva et al., 2016) and juvenile common carp (*Cyprinus carpio*) (Liu et al., 2014). However, the information on the effect of butyric acid and its salts on fish growth performances remains elusive. For example, in Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorynchus mykiss*) a supplemention of a diet with a mixture of SCFA, containing Na- butyrate, did not significantly improve growth rate or feed utilization (Bjerkeng et al., 1999; Gao et al., 2011).

1.2.1. EPIGENETIC EFFECTS OF BUTYRATE

In the mid-1970s several research groups reported that sodium butyrate alters DNA synthesis, arrests cell proliferation, alters cell morphology and increases or decreases gene expression. Some of these changes are similar to those produced by agents, which increase the intracellular level of adenosine 3', 5'-cyclic monophosphate (cAMP), or by analogs of cAMP. Sodium butyrate increases the intracellular level of cAMP by about two fold in neuroblastoma cells; therefore, some of the effects of sodium butyrate on these cells may in part be mediated by cAMP. Sodium butyrate appears to have properties of a good chemotherapeutic agent for neuroblastoma tumors because the treatment of neuroblastoma cells in culture causes cell death and "differentiation" (Prasad et al., 1976).

Although the exact underlying mechanisms of action have not yet been elucidated, the influence of butyrate on cell proliferation may be explained, at least in part, by its potent regulatory effect on the gene expression. This effect is often attributed to the ability of butyrate to inhibit the activity of many histone deacetylases, leading to hyper acetylation of histones (Hamer et al., 2008). Histone acetylation disrupts chromatin structure, allowing the binding of transcription factors and polymerases and hence, the beginning of transcription. The modulation of genome expression through core histone acetylation is one of the most relevant means by which cell function and DNA methylation are epigenetically regulated (Hamer et al., 2008; Canani et al., 2011; Biancotto et al., 2010).

Several studies reveal that among the SCFAs, butyrate is the most effective in stimulating or repressing the expression of specific genes related to tumorigenic cells, in inducing differentiation and haemoglobin synthesis in erythroleukemic cells, inhibiting histone deacetylase (HDAC) activity, and arresting cell proliferation (Kruh et al., 1982, Davie et al., 2003).

Butyrate theoretically reactivates epigenetically-silenced genes by increasing global histone acetylation. However, the global gene expression profiles of bovine kidney epithelial cells indicate that there are more genes down-regulated than up-regulated by butyrate (Li et al., 2010). Similar results were also reported from human hepatocarcinoma (Li et al., 2006, Rada-Iglesias et al., 2007). It was suggested (Hinnebusch et al., 2002, Emenaker et al., 2001) that butyrate may also inhibit the development of colon cancer.

It has been shown that the SLC5A8 protein, which sits within the apical membrane of the enterocytes, is involved in the absorption of SCFAs such as butyrate into the colon (Park et al., 2008, Park et al., 2007). As such, SLC5A8 gene product that is involved in its transport through the colon mucosa has been labeled as a tumour suppressor gene (Park et al., 2007, Thangaraju et al. 2008).

Butyrate impacts cell proliferation through its effect on histones acetylation status. Histone modification is very instrumental in the expression level of genes within the cell. Butyrate transport by SLC5A8 gene product impacts the expression level of many genes that are likely involved in the anti-proliferative control of the cell cycle within the colon mucosa (Thangaraju et al. 2008). Of note, SLC5A8 transported compound, butyrate, affects chromatin structure through its effect on histones (Brim et al. 2011).

SLC genes are known to be involved in the transport of many solutes that differ from one gene to another and for the same gene, from one organ to the other (Li et al. 2008). SLC5A8 gene is involved in the transport of butyrate, propionate, and pyruvate that are all inhibitors of histone deacetylases (Ganapathy et al. 2008).

Accordingly, the first aim of the present study was to evaluate the potential effects of butyrate used as a feed additive on fish growth, as well as

butyrate's regulatory role on the gut mucosal protection and immune homeostasis through its effects on gene expression in the European sea bass (*Dicentrarchus labrax*). The target genes related to mucosal inflammatory response and reinforcement of the mucous defense barrier included: tnfα (tumor necrosis factor alpha), which is a cell-signaling protein (cytokine) that makes up the inflammatory acute phase reaction and possesses a wide range of pro-inflammatory actions (Locksley et al., 2001); interleukins such as il1β, il-6, il-8, and il-10, which are well-known cytokines that regulate immune responses, inflammatory reactions, and hematopoiesis; irf1 (interferon regulatory factor 1), which is a transcription factor that stimulates both, innate and acquired immune responses, by activating specific target genes expressed during inflammation, immune responses, and hematopoiesis (Brien et al. 2011); and muc2 (mucin 2), a major component of intestinal mucus gel secretions that serve as a barrier to protect the intestinal epithelium (Allen et al. 1998).

1.3. INTESTINAL MICROBIOTA

Bacteria associated with the epithelium of an animal's digestive tract play a critical role in establishing and maintaining their host's health. The intestinal microbiota is involved in the anaerobic fermentation of complex dietary carbohydrates (cellulose, hemicellulose, pectin), and oligosaccharides that are otherwise indigestible, as well as of digestible simple carbohydrates such as starch and glucose, that are well digested and absorbed in the small intestine (Bergman, 1990). Intestinal mucus, sloughed cells, and endogenous secretions provide other sources of fermentable substrates, especially proteins and polysaccharides (Bergman, 1990). Nearly 75% of the energy content of the carbohydrates is used for the production of metabolic end products such as volatile or SCFAs, which

are readily absorbed by the host; the remaining 25% is used for microbial growth and maintenance or is lost as hydrogen, carbon dioxide, and methane (Cummings and Macfarlane, 1991). Microbial fermentation mainly takes place in the forestomach (a fermentation chamber cranial to the acid-secreting part of the stomach) of foregut fermenters such as ruminants (cattle, sheep, goats, etc.) and in the cecum and large intestine of hindgut fermenters (the food is fermented after it has been digested by the stomach), such as rodents, elephants, and most carnivores and omnivores, including humans (Bergman, 1990; Guilloteau et al., 2010). The produced SCFAs are waste products to the microbes but represent the main source of metabolic energy for colonocytes in hindgut fermenters or serve as a principal source of energy for the entire animal in the case of foregut fermenters. Indeed, ruminants depend on SCFAs for 80% of their maintenance energy (Bergman, 1990; Canani et al., 2012; Louis and Flint, 2009).

Fish gut microbiota shares common intestinal microbiota features with other vertebrates. It plays important role in hosts' metabolism, absorption of nutrients, immunity, and resistance to diseases (Figure 3) (Rawls et al., 2004, 2006; Gómez and Balcázar, 2008; Llewellyn et al., 2014). The intestinal microbiota is also responsible for the synthesis of some vitamins, digestive enzymes and SCFAs, which are the main energy source of the gut epithelial cells (Maslowski e Mackay, 2010; Llewellyn et al., 2014; Nayak, 2010; Ghanbari et al., 2015; Ingerslev et al., 2014a).

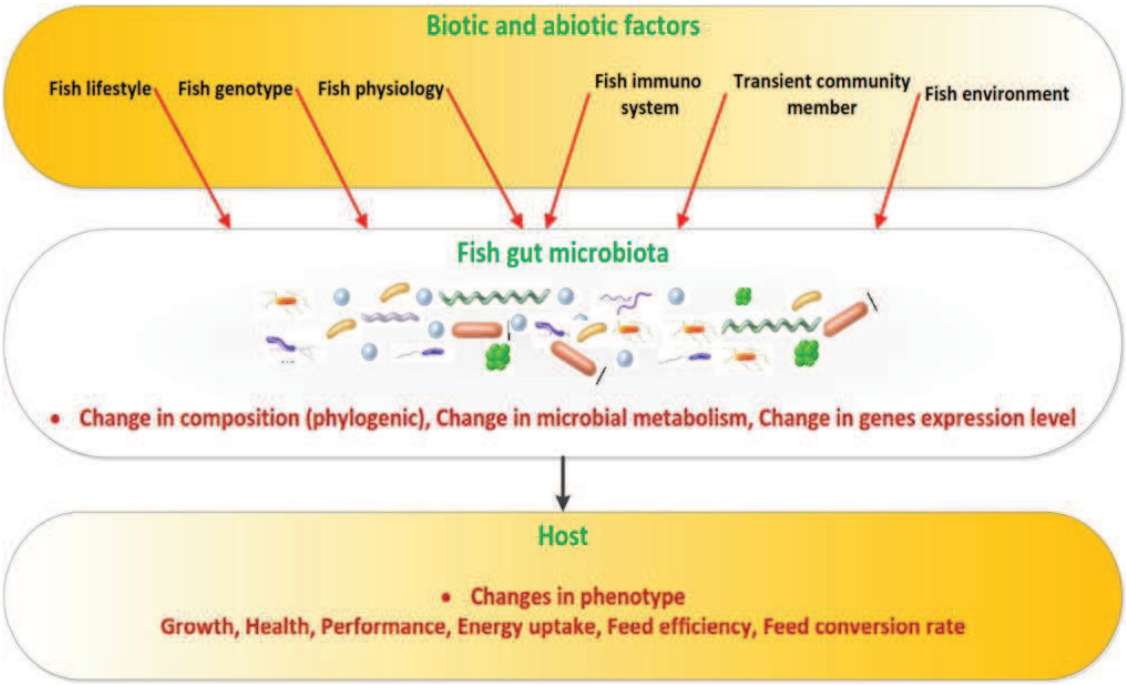

Figure 3. *A combination of biotic and abiotic factors (red arrows) such as genotype, fish physiological status (including properties of the innate and adaptive immune systems), fish pathobiology (disease status), fish lifestyle (including diet), fish environment and the presence of transient populations of microorganisms affect the composition, function and metabolic activity of the fish gut microbiota. These changes affect processes involved in growth, performance, energy storage and health in fish. (Ghanbari et al., 2015)*

Furthermore, it has recently been reported that the dietary supplementation of butyrate modulates the intestinal bacterial community of fish species such as European sea bass (*Dicentrarchus labrax*) (De Schryver et al., 2010), and common carp (Liu et al., 2014), as well as crustaceans such as Pacific white shrimp (da Silva et al. 2013, 2016; Anuta et al., 2011).

Changes in the gut microbiota following dietary butyrate typically consist of a shift in the dominant bacterial hierarchies. This is due to the lysis of Gram-negative bacteria, which is typically accompanied by an enrichment in "good" (beneficial) bacteria strains (Owen et al., 2006; Encarnacao, 2008; Hoseinifar et al., 2017). Most of the SCFAs and their salts are commonly known as acidifiers and are used as antimicrobial compounds in the livestock feed industry as well as in aquafeed production (Ng and Koh, 2017). Due to their capacity in reducing pH of the feed, they act as

preserving agents, inhibiting microbial growth and diminishing a possible intake of pathogenic organisms (Lückstädt, 2008). Similarly, in the intestinal tract, SCFAs cause a reduction of pH leading to growth inhibition of pathogenic bacteria, mainly belonging to Gram-negative species (Hoseinifar et al., 2017). However, the organic acids mechanism of action is very different from antibiotics. The antimicrobial activity of SCFAs is due to the ability of undissociated form of the acid to penetrate bacterial cell wall and, once inside, dissociate releasing its protons, thereby lowering the cytoplasmic ph. Consequently, the bacterium has to readdress its energies towards the efflux of the excess protons, thus exhausting the cell metabolism and leading to lower cell growth and even to cell death (Salsali et al., 2008; Hismiogullari et al. 2008).

For butyrate to exert its physiologic, cellular, and molecular effects, circulating concentrations would need to be maintained at a consistently high level. This is difficult to attain because plasma clearance of butyrate is very quick, with a half-life of about 6 min when given intravenously in humans (Miller et al., 2004). A possible solution to circumvent problems associated with rapid metabolism of butyrate would be to administer it orally by giving multiple daily doses of stable derivatives of butyrate. Indeed, when stable derivatives of butyrate were given orally as opposed to intravenously in humans, its half-life was increased to 40 min, and circulating butyrate concentrations reached high enough values to be efficacious (Miller et al., 2004).

However, in animals, the butyric acid and sodium butyrate have the disadvantage to be immediately absorbed by the upper digestive tract, thus limiting the delivery of a sufficient amount of butyric acid to intestine, where butyrate performs its aforementioned beneficial actions (Yin et al., 2016).

Butyrate glycerides, instead, have no such drawback since butyrate release from them requires the action of intestinal lipases. This means that butyric acid is protected from the absorption in the upper tract and its effectiveness in the rest of gut is improved (Sampugna et al., 1967; Namkung et al., 2011).

Therefore, butyrins (including mono-, di-, and tri-butyrate glycerides) have been developed to overcome this limit. Like butyrate salts, butyrins have no offensive odour. In broiler chickens, it has been reported that dietary addition of butyric acid glycerides improved the body weight gain, and breast weight gain (Leeson et al., 2005; Yin et al., 2016). Among short-chain fatty acid glycerides, monoglycerides have shown a more effective antimicrobial activity than di- and triglycerides of the same fatty acids (Namkung et al., 2011). To date, information regarding the effect of butyric acid glycerides on intestinal health and growth performances in fish is scarce.

Accordingly, the second aim of this study was to evaluate the potential beneficial effects of a mixure of SCFA and MCFA monoglycerides, used as a feed additive, on fish growth performance, and gut microbiota composition. For this purpose, a specific combination of short- and medium-chain 1-monoglycerides, namely SILOhealth 108Z, was tested in juvenile gilthead sea bream (*Sparus aurata*). Since sea bass and sea bream are the two main fish species on the Mediterranean Sea and economically very important for the local aquaculture, they have been both on purpose selected for this study.

2. MATERIAL, METHODS AND RESULTS

2.1 EFFECTS OF SODIUM BUTYRATE TREATMENT ON HISTONE MODIFICATIONS AND THE GENE EXPRESSION IN EUROPEAN SEA BASS

PLOS ONE

RESEARCH ARTICLE

Effects of Sodium Butyrate Treatment on Histone Modifications and the Expression of Genes Related to Epigenetic Regulatory Mechanisms and Immune Response in European Sea Bass (*Dicentrarchus Labrax*) Fed a Plant-Based Diet

Genciana Terova[1,3]*, Noelia Díaz[2¤], Simona Rimoldi[1], Chiara Ceccotti[1], Emi Gliozheni[1], Francesc Piferrer[2]

1 Department of Biotechnology and Life Sciences, University of Insubria, Via J.H.Dunant, 3, 21100, Varese, Italy, **2** Institut de Ciències del Mar, Consejo Superior de Investigaciones Científicas (CSIC), Passeig Marítim, 37–49, 08003, Barcelona, Spain, **3** Inter-University Centre for Research in Protein Biotechnologies "The Protein Factory"- Polytechnic University of Milan and University of Insubria, Varese, Italy

¤ Current address: Max Planck Institute for Molecular Biomedicine, Röntgenstraße 20, 48149 Münster, Germany
* genciana.terova@uninsubria.it

CrossMark
click for updates

OPEN ACCESS

Citation: Terova G, Díaz N, Rimoldi S, Ceccotti C, Gliozheni E, Piferrer F (2016) Effects of Sodium Butyrate Treatment on Histone Modifications and the Expression of Genes Related to Epigenetic Regulatory Mechanisms and Immune Response in European Sea Bass (*Dicentrarchus Labrax*) Fed a Plant-Based Diet. PLoS ONE 11(7): e0160332. doi:10.1371/journal.pone.0160332

Editor: Jorge M.O. Fernandes, Nord University, NORWAY

Received: March 15, 2016

Accepted: July 18, 2016

Published: July 29, 2016

Copyright: © 2016 Terova et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work has been funded under the seventh EU Framework Programme by the ARRAINA project N°288925: Advanced Research Initiatives for Nutrition & Aquaculture. Collaboration between the GT and FP labs was fostered by COST Action FA1201: Epigenetics and Periconception Environment ("Epiconcept"). The funders had no role in study design, data collection and analysis, decision

Abstract

Bacteria that inhabit the epithelium of the animals' digestive tract provide the essential biochemical pathways for fermenting otherwise indigestible dietary fibers, leading to the production of short-chain fatty acids (SCFAs). Of the major SCFAs, butyrate has received particular attention due to its numerous positive effects on the health of the intestinal tract and peripheral tissues. The mechanisms of action of this four-carbon chain organic acid are different; many of these are related to its potent regulatory effect on gene expression since butyrate is a histone deacetylase inhibitor that play a predominant role in the epigenetic regulation of gene expression and cell function. In the present work, we investigated in the European sea bass (*Dicentrarchus labrax*) the effects of butyrate used as a feed additive on fish epigenetics as well as its regulatory role in mucosal protection and immune homeostasis through impact on gene expression. Seven target genes related to inflammatory response and reinforcement of the epithelial defense barrier [*tnf*α (tumor necrosis factor alpha) *il1β*, (interleukin 1beta), *il-6*, *il-8*, *il-10*, and *muc2* (mucin 2)] and five target genes related to epigenetic modifications [*dicer1* (double-stranded RNA-specific endoribonuclease), *ehmt2* (euchromatic histone-lysine-N-methyltransferase 2), *pcgf2* (polycomb group ring finger 2), *hdac11* (histone deacetylase-11), and *jarid2a* (jumonji)] were analyzed in fish intestine and liver. We also investigated the effect of dietary butyrate supplementation on histone acetylation, by performing an immunoblotting analysis on liver core histone extracts. Results of the eight-week-long feeding trial showed no significant differences in weight gain or SGR (specific growth rate) of sea bass that received 0.2% sodium butyrate

to publish, or preparation of the manuscript. The views expressed in this work are the sole responsibility of the authors and do not necessary reflect the views of the European Commission.

Competing Interests: The authors have declared that no competing interests exist.

supplementation in the diet in comparison to control fish that received a diet without Na-butyrate. Dietary butyrate led to a twofold increase in the acetylation level of histone H4 at lysine 8, but showed no effect on the histone H3 at Lys9. Moreover, two different isoforms of histone H3 that might correspond to the H3.1 and H3.2 isoforms previously found in terrestrial animals were separated on the immunoblots. The expression of four (*il1 β*, *il8*, *irf1*, and *tnfα*) out of seven analyzed genes related to mucosal protection and inflammatory response was significantly different between the two analyzed tissues but only *il10* showed differences in expression due to the interaction between tissue and butyrate treatment. In addition, butyrate caused significant changes *in vivo* in the expression of genes related to epigenetic regulatory mechanisms such as *hdac11*, *ehmt2*, and *dicer1*. Statistical analysis by two-way ANOVA for these genes showed not only significant differences due to the butyrate treatment, but also due to the interaction between tissue and treatment.

Introduction

Bacteria associated with the epithelium of an animal's digestive tract play a critical role in establishing and maintaining their host's health. The intestinal microbiota is involved in the anaerobic fermentation of complex dietary carbohydrates (cellulose, hemicellulose, pectin), and oligosaccharides that are otherwise indigestible as well as of digestible simple carbohydrates such as starch, and glucose that escape digestion and absorption in the small intestine [1]. Intestinal mucus, sloughed cells from the epithelia, lysed microbial cells, and endogenous secretions provide other sources of fermentable substrates, especially proteins and polysaccharides [1]. Nearly 75% of the energy content of the fermented carbohydrates is used to produce metabolic end products such as short chain fatty acids (SCFAs), which are then readily absorbed by the host, whereas the remaining 25% is used for microbial growth and maintenance or lost as hydrogen, carbon dioxide, and methane [2,3,4,5].

SCFAs, also known as volatile fatty acids, are carboxylic acids with aliphatic tails of 1 to 6 carbon atoms that exist in straight- and branched-chain conformations. Common SCFAs include acetic (C2), propionic (C3), butyric (C4), valeric (C5), and caproic (C6) acid [4]. The predominant anions in either the rumen or large intestine are the short, straight-chain FAs such as acetate, propionate, and butyrate, whereas the short branched-chain FAs, isobutyrate and isovalerate, which are produced by fermentation of the amino acids valine and leucine, respectively, are found in much smaller amounts [1,6].

Among the SCFAs, butyrate has received particular attention due to its numerous positive effects on the health of intestinal tract and peripheral tissues [7]. In addition to being the main respiratory fuel source of the colonic bacteria, and preferred to glucose or glutamine, butyrate plays a major role in enhancing epithelial cell proliferation and differentiation and in improving the intestinal absorptive function [8,9,4]. Furthermore, there are several lines of evidence suggesting that butyrate has potential immunomodulatory and anti-inflammatory properties in the intestine and may prevent colorectal cancer in humans [10, 11, 12].

Although the exact underlying mechanisms of action have not yet been elucidated, the influence of butyrate on cell proliferation may be explained, at least in part, by its potent regulatory effect on gene expression. This effect is often attributed to the ability of butyrate to inhibit the activity of many histone deacetylases, leading to hyperacetylation of histones [12]. Histone acetylation modifies chromatin structure, allowing the binding of transcription factors and polymerases and hence, the beginning of transcription. The modulation of gene expression

through core histone acetylation is one of the most relevant means by which cell function and DNA methylation are epigenetically regulated [12,13,14]. A positive effect of butyrate on transcriptomic activity of some pivotal genes at the intestinal level has also been suggested in fish in two recent studies carried out on European sea bass (*Dicentrarchus labrax*) [15] and gilthead sea bream (*Sparus aurata*) [16].

Much of the research on butyrate has focused on its role in the gut, while less is known about whole-body metabolism of butyrate and, in particular, on how it might influence the metabolic potential of the liver *in vivo* [17, 18]. Although butyrate is largely taken up by the intestinal epithelium, a small fraction can also reach the liver through the blood stream via the portal vein [18, 19]. In liver, butyrate is readily converted in mitochondria to butyryl CoA to produce ketone bodies (rather unlikely in fed animals) and acetyl CoA, which then enters into the Krebs cycle [19, 20, 21]. Hepatic metabolism and clearance of butyrate are substantial since evidence shows that close to 100% was removed in the liver of rodents fed with a high-fiber diet [22], whereas butyrate released from the human gut *in vivo* into the circulatory system was counterbalanced by hepatic butyrate uptake [18], indicating that the liver is highly involved in butyrate metabolism [23,24].

For butyrate to exert its physiologic, cellular, and molecular effects, circulating concentrations would need to be maintained at a consistently high level. This is difficult to attain because plasma clearance of butyrate is very quick, with a half-life of about 6 min when given intravenously in humans [25]. A possible solution to circumvent problems associated with rapid metabolism of butyrate would be to administer it orally by giving multiple daily doses of stable derivatives of butyrate. Indeed, when stable derivatives of butyrate were given orally as opposed to intravenously in humans, its half-life was increased to 40 min, and circulating butyrate concentrations reached high enough values to be efficacious [25]. In farmed animals such as pigs and chickens, butyrate included in the diet has had a positive influence on body weight gain, feed utilization, and composition of intestinal microflora, as well as trophic effects on the intestinal epithelium through an increase in the villi length and crypt depth [26,27,28]. In poultry, butyrate applied as a nutritional supplement caused *in vivo* hyperacetylation of the hepatic core histones and modified the epigenetic regulation of hepatocyte's function [7]. In addition, some authors have suggested significant improvements in fish growth and feed conversion rates when butyrate is included in diets of some species such as catfish [29], tilapia, carp [30], and sea bream [16], but not in others such as salmon [31,32]. However, except for these studies, literature concerning the use of butyrate or its derivatives as an additive in fish feed is very scarce.

Accordingly, the present study aimed to evaluate in the European sea bass (*Dicentrarchus labrax*) the potential effects of butyrate as a feed additive on fish growth, as well as butyrate's regulatory role on the mucosal protection and immune homeostasis through its effects on gene expression. The target genes related to mucosal inflammatory response and reinforcement of the mucous defense barrier included: *tnfα*tumor necrosis factor alpha), which is a cell-signaling protein (cytokine) that makes up the inflammatory acute phase reaction and possesses a wide range of proinflammatory actions [33]; interleukins such as *il1β*, *il-6*, *il-8*, and *il-10*, which are well-known cytokines that regulate immune responses, inflammatory reactions, and hematopoiesis; *irf1* (interferon regulatory factor 1), which is a transcription factor that stimulates both innate and acquired immune responses by activating specific target genes expressed during inflammation, immune responses, and hematopoiesis [34]; and *muc2* (mucin 2), which is a major component of intestinal mucus gel secretions that serve as a barrier to protect the intestinal epithelium [35].

The second goal of the present study was to evaluate the epigenetic effects of dietary butyrate in sea bass by monitoring both the acetylation state of hepatic core histones and the

hepatic and intestinal expression of a suite of genes related to epigenetic modifications [36]. These genes included: *dicer 1*, which encodes an active, small RNA component that represses the expression of other genes [37]; *ehmt2* (euchromatic histone-lysine-N-methyltransferase 2), which demethylates Lys9 in histone 3 in euchromatin, creating a tag for epigenetic transcription repression [38,39]; *pcgf2* (polycomb group ring finger 2), which acts via chromatin remodeling and histone modification [40]; *hdac11* (histone deacetylase-11), which can modify core histone octamer packing chromatin in dense structures or controls various histone methyltransferase complexes [41]; and *jarid2a* (jumonji), which is a nuclear factor that functions as a powerful transcriptional repressor [42].

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the University of Insubria, Varese, Italy. The Committee on the Ethics of Animal Experiments of the same University approved all of the protocols performed. Fish handling was performed under tricaine methanesulfonate (MS222) anesthesia, and all efforts were made to minimize discomfort, and stress and to avoid pain to the animals.

Fish and Experimental Set Up

Juvenile European sea bass (*Dicentrarchus labrax*) were purchased from a commercial hatchery (Civitavechia, Italy). Upon arrival to the laboratory, fish were stocked for 40 days into two rectangular indoor tanks of 2.5 cubic meters to acclimate.

At the beginning of the trial, after removing fish deviating from the average weight of approximately 15 g, we distributed fish into six circular experimental tanks (3 replicates) of 600 L each, at a density of 35 fish per tank and let them to acclimate over a period of one week. There were no significant differences in fish weight between the experimental tanks at the onset of the experiment ($P>0.05$; data not shown).

Rearing Facility and Maintenance

All rearing tanks were located in an indoor facility. The tanks were equipped with re-circulating systems and photoperiod, temperature, and salinity could be strictly controlled with this equipment. The experimental layout consisted of six cylindrical 600 L fiberglass tanks, connected to a central main biofilter of 350 liters. The light source was the natural photoperiod enhanced with florescent light, providing a light intensity of 1200 lx during the day. The water was heated and maintained at 21 ± 1°C by using submersible aquarium heaters. The salinity was 22 ± 0.5 g/l throughout the experiment.

Twice a week the following parameters were measured: dissolved oxygen, pH, ammonia, and nitrite levels. The levels of all parameters remained within the range considered optimal for European sea bass growth throughout the experiment.

Diet Formulation, and Feeding

As a control diet we used a formulation of 40% crude protein and 16% fat, which was based on plant protein and fishmeal. The control diet was similar to feed commercially available for growing European seabass. Control diet was supplemented with 2g/kg (0.2%) of sodium butyrate to produce the experimental butyrate diet. A detailed diet composition is presented in Table 1. Diets were prepared using small-scale machinery for mixing ingredients and preparing

Table 1. Composition of the diets in g/100 g on a dry weight basis.

Ingredients (g/100g)	Control	Butyrate
Fish meal	10.00	10.00
Soybean meal	30.00	30.00
Pea concentrate	16.00	16.00
Corn gluten	14.20	14.20
Wheat gluten	5.00	5.00
Fish oil	14.00	14.00
Stay-C 35d	0.03	0.03
Vitamin Mix	0.40	0.40
Mineral Mix	1.00	1.00
DL-Methionine	0.25	0.25
Lysine (98%)	0.05	0.05
Fish Hydrolysate	2.00	2.00
Dextrin	1.56	1.56
Sodium alginate	0.79	0.79
Dicalcium phosphate	0.72	0.72
Filler (gelatin)	4.00	3.80
Na-butyrate	-	0.20
Total	100.00	100.00

doi:10.1371/journal.pone.0160332.t001

pellets of 3.5 mm in diameter. Na-butyrate substituted an equivalent amount of filler in the butyrate diet.

Each diet was provided to fish in triplicate (3 tanks/diet). Fish were fed twice a day and feeding rates were restricted to 3.0% of biomass. The feeding experiment was based on four-weekly fish weight measurements to adjust the feed ration to a similar percentage of fish biomass in both treatments. Feed consumption (g) in each tank was estimated from the difference between feed delivered into the tank and uneaten feed, which was collected from the bottom of the tank. The feeding trial lasted 8 weeks. Fish SGR was calculated using the following formula: (ln Wf—ln Wi)/t x 100, where Wf is the final weight (g), Wi is the initial weight (g), and t is growth time (days).

Fish Sampling

At the end of the eight-week-long feeding trial, fish of each tank were individually weighed after overnight food deprivation. Six fish from each treatment (three fish/tank) were then randomly fished, and sacrificed. Intestine and liver were excised from each sampled fish using sterile instruments, snap-frozen in dry ice, and then kept at minus 80°C until nucleic acid extraction and histone protein acetylation analysis.

Growth data statistical analysis. Growth data were analyzed by two-way analysis of variance (two-way ANOVA) considering diet, time and their interaction as sources of variation, followed by Tukey's HSD post hoc test. Significance level was set at $P< 0.05$.

Preparation of Liver Nuclear Protein Fraction

Liver nuclear protein extracts were prepared from six fish per group using 3 ml/g of tissue of extraction buffer containing: 10 mM Tris/HCl, pH 7.8, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM Pefabloc® (SIGMA-ALDRICH®), 0.5 mM DTT, 1 mM Na3VO4, and 1X protease inhibitor cocktail (SIGMA-ALDRICH®). Tissue lysis and homogenization were carried out in a closed

system using the gentleMACS™ Dissociator and single-use gentleMACS™ M tubes (Miltenyi Biotec). Liver lysates were then centrifuged at 1500 *g* for 20 min at 4°C. The supernatants containing the cytosolic protein fraction were discarded while the nuclear pellets were stored at minus 80°C until further histone isolation procedure.

Histone Isolation

Purified histone extracts were isolated from nuclear fractions using the Histone Purification Mini Kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. Active Motif's Histone Purification Kit preserves phosphoryl, acetyl, and methyl post-translational modifications on histones. Briefly, an equal volume of ice-cold extraction buffer was added to the nuclear suspension. After homogenization, samples were left overnight in the extraction buffer on a rotating platform at 4°C. Next day, tubes were centrifuged at maximum speed for 5 min in a microfuge at 4°C and the supernatants, which contained the crude histone extracts, were neutralized with one-fourth volume of 5x neutralization buffer (pH 8.0). Neutralized extracts were loaded to previously equilibrated histone isolation spin columns. After three washes with histone wash buffer, we eluted histones in 100 μl of histone elution buffer and precipitated overnight by adding 4% perchloric acid. On the following day, samples were centrifuged at maximum speed for 1 hour; histone pellets were washed first with 4% perchloric acid, later with acetone containing 0.2% HCl, and finally with pure acetone, after which they were air dried. Histones were suspended in sterile distilled water and the yield of total core histone proteins was quantified by measuring the absorbance at 230 nm.

Histone Acetylation Western Blots

Western blotting analyses were performed on four samples of purified histones, given that the quantity of histones isolated from the other two nuclear protein extracts resulted not sufficient. For the analysis, we followed the instructions of the Acetyl Histone Antibody Sampler Kit (Cell Signaling) and the protocol applied by Mátis *et al.* [7]. Before using "Acetyl-Histone H4 (Lys8) Antibody #2594"and Histone H4 (L64C1) #2935 provided with the kit, we used ClustalW to perform a multiple sequence alignment between the human histone H4 peptide sequence that was used for the production of antibodies and the ortholog sequences in European seabass (*Dicentrarchus labrax*), and other teleosts such as zebrafish (*Danio rerio*), Nile tilapia (*Oreochromis niloticus*), and Atlantic salmon (*Salmo salar*). As shown in S1 Fig, the histone H4 peptide sequence in European sea bass presents 100% similarity with the human sequence, and it is the same for the other teleosts' histone H4 sequences. This suggest that antibodies were suitable for the detection of the antigen in our target species.

Histone proteins were diluted by 2x SDS and β -mercaptoethanol containing loading buffer (supplemented with 50 mM DTT), sonicated for 15 s, and heat denatured at 95°C for 5 min. Histones were separated by SDS-PAGE on polyacrylamide (4–20%) precast gradient gels (Bio-Rad); 3 μg histone protein per lane were loaded for the detection of histones H2A, H2B, and H3, whereas 6 μg per lane were loaded for histone H4. After electrophoresis, proteins were blotted onto PVDF membranes (0.22-μm pore size, Bio-Rad). Before proceeding to the immunodetection process, a reversible Ponceau staining was applied to membranes to test equal loading of gels and protein transfer. Histones were identified using antibodies furnished by the Acetyl Histone Antibody Sampler Kit. After blocking with 5% fat-free milk containing PBST for 3 h, the immunoblots were incubated overnight at 4°C with primary antibodies against histone H2A (1:1000), H2B (1:500), H3 (1:1000), H4 (1:500), and their acetylated forms. Each acetyl histone antibody was specific for the target histone modified at the lysine residue of the most frequent acetylation site (AcH2A and AcH2B: Lys 5, AcH3: Lys 9, AcH4: Lys 8). The

primary antibody was detected using an anti-rabbit secondary antibody (1:2000) or an anti-mouse secondary antibody (1:900) for the non-acetylated H4 histone. Both secondary antibodies were coupled with horseradish peroxidase. Primary antibodies were diluted in PBST containing 5% BSA, with the exception of anti H4, which was diluted in PBST containing 5% of defatted milk. Secondary antibodies were diluted in PBST containing 5% fat-free milk. Signals were detected using an enhanced chemiluminescence system (SuperSignal®west Dura Extended Duration Substrate, Thermo Scientific) and exposing to clear-blue X-ray film. After film exposure, densitometry was used to quantify protein levels on the western blots by means of Quantity One 1-D software (Bio-Rad). The protein levels were expressed as adjusted volume, Adj. Vol. [OD*mm^2] = [{Sum of the intensities of the pixels inside the volume boundary} x {area of a single pixel in mm^2}]–{the background volume}).

RNA Extraction and cDNA Synthesis for Gene Expression Analysis

RNA from 12 sea bass livers and 12 intestines was extracted using a semi-automatic system (Maxwell® 16 Instrument, Promega) and a total RNA purification kit (Maxwell® 16 Tissue LEV). RNA purity and concentration were assessed by a ND-2000 spectrophotometer (NanoDrop product, Thermo Scientific).

One hundred nanograms of the total extracted RNA were reverse transcribed to cDNA using SuperScript III and random hexamers (Life Technologies, Italy) following the manufacturer's instructions. Two rounds of cDNA synthesis per sample were carried out and then merged.

Quantitative Real-Time PCR (qRT-PCR)

For the already cloned genes in European sea bass, FASTA sequences were taken from the NCBI repository (http://www.ncbi.nlm.nih.gov/) and primers were designed by using Primer3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus.cgi). For the genes not cloned yet, exon sequences from other fish species (stickleback or tilapia) were taken from the Ensembl Genome Browser (http://www.ensembl.org/) and blasted against the European sea bass genome database (http://seabass.mpipz.de/) [43]. Only when the match was annotated in the sea bass genome the exon was considered for primer design (S1 Table and S2 Fig). Primer efficiency was evaluated by analyzing the slope of a linear regression from six different dilutions using a pool with all the samples involved in the analysis: six fish per treatment in the two different tissues. Efficiencies ranged from 1.8 to 2.4. In addition, the correct binding of the primers and hence the presence of a single amplicon generation was assessed by adding a melting-curve analysis (95°C for 15 s, 60°C for 15 s and 95°C for 15 s) after the amplification phase.

qRT-PCR was performed on an ABI 7900HT (Life Technologies) under a standard cycling program (UDG decontamination cycle: 50°C for 2 min; initial activation step: 95°C for 10 min; 40 cycles of 15 s denaturation at 95°C and 1 min annealing/extension at 60°C). A final dissociation step was also added (95°C for 15 s and 60°C for 15 s).

For qRT-PCR gene analysis, cDNA was diluted 1:10 for all the target genes except for the reference gene, *r18S*, which was diluted 1:500. All samples were run in triplicate in a 384-well plate in a final volume of 10 μl. Each well contained a mix of 5 μl SYBR Green Supermix (Life Technologies), 2 μl distilled water, 2 μl primer mix (forward and reverse at 10 μM concentration), and 1 μl cDNA. Negative controls were added in duplicate. The software SDS 2.3 and RQ Manager (Life Technologies) were used to collect data and calculate gene expression levels (cycle thresholds, Cts), respectively. The expression of housekeeping gene *r18S* (the endogenous control) was used to correct for intra- and inter-assay variations.

Data Analysis

qRT-PCR Raw Data Analysis. Ct values were adjusted, taking into account primer efficiencies per each gene when calculating 2^ddCt values. Expression data for each target gene were also normalized to the housekeeping gene (*r18S*) and fold-change calculations were made based on the Schmittgen and Livak's method [44].

qRT-PCR Statistical Analysis. qRT-PCR analyses were performed using 2^ddCt values in the IBM SPSS Statistics 19 software. Data were evaluated for normality and homoscedasticity of variance; outliers (no more than one per condition) were eliminated when needed. Treated versus control groups, in liver and intestine, were analyzed in two steps: 1) by analyzing fold-change differences with respect to the controls [44] and 2) by a Student *t*-test analysis. In addition, a two-way analysis of variance (ANOVA) was carried out, taking into consideration both treatment and tissue for analyzing not only the contributions of each variable but also their interactions.

Results

Effect of Butyrate on Growth Performance

The initial weight of 14.91±1.73 g of the control fish group (Fig 1) increased to 20.63±4.17 g after 4 weeks of feeding and to 30.22±5.61 g after 8 weeks of feeding. Fish receiving the butyrate-supplemented diet had an initial mean body weight of 15.80±1.60 g, which increased to 20.51±4.74 g after 4 weeks and to 28.97±8.09 g after 8 weeks of feeding. However, the results of

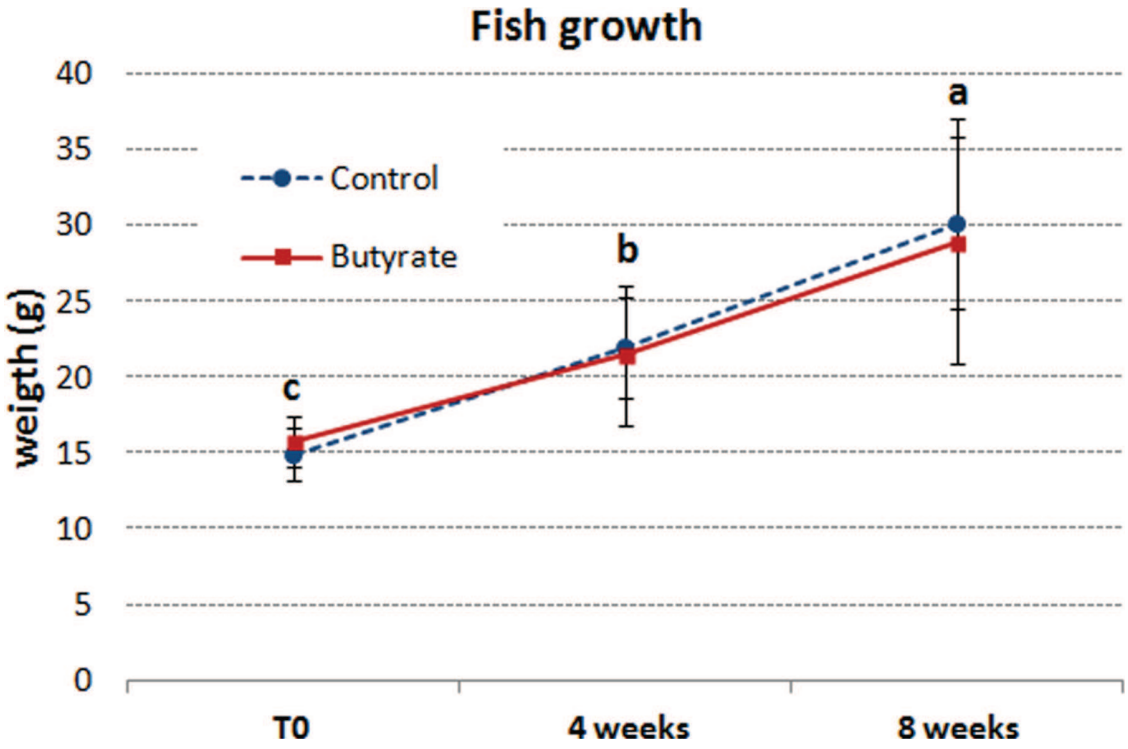

Fig 1. Effects of dietary butyrate on European sea bass growth. The data were tested by ANOVA followed by Tukey's HSD test to determine whether there were any significant differences between different groups. Fish were fed for 8 weeks two different diets, a control diet, and an experimental diet, which was the control diet supplemented with 2g/kg (0.2%) of Na-butyrate. Each histogram shows the mean ± SEM of 105 animals. Different letters indicate significant differences ($P < 0.05$).

doi:10.1371/journal.pone.0160332.g001

two-way ANOVA showed that, starting from the 4th week of the feeding trial, there was only a time effect on the fish growth, whereas the interaction effect between diet and time was not significant. By considering the main effect of time (S2 Table), the average weight of fish fed butyrate was not significantly different from that of the control fish from the 4th week until the end of the feeding trial.

Survival was high (around 95%) with no significant differences between the groups of fish fed different diets. The SGR of fish fed the butyrate-supplemented diet was 1.06±0.02 after 4 weeks of feeding and 1.19±0.03 at the end of the experiment, whereas that of the control group was 1.34±0.04 and 1.33±0.07 after 4 and 8 weeks of feeding, respectively. There were no significant differences in SGR between the fish fed control and butyrate diet (data not shown).

Effect of Butyrate on Core Histone Acetylation

To investigate the effect of dietary supplementation of sodium butyrate on histone acetylation in European sea bass, we performed an immunoblotting analysis on liver core histone extracts of four fish from each group. The results of this analysis are presented in Fig 2, whereas the intensity values (Adj. Vol [OD*mm2]) of each band are reported in Table 2. Among the primary antibodies furnished by the Acetyl-Histone Antibody Sampler Kit (Cell Signaling Technology) anti-H2A (non-acetylated form), anti-H3 (acetylated and non-acetylated forms), and anti-H4 (acetylated and non-acetylated forms) recognized respective sea bass's epitopes. In contrast, none of the anti-H2B, anti-AcH2B, and anti Ac-H2A antibodies recognized any of the sea bass epitopes.

Immunoblotting on hepatocyte core histone extracts (Table 2) revealed that dietary butyrate intake decreased the relative protein expression level of the H2A histone ($P<0.05$), which was poorly expressed in butyrate-treated fish but was detected at high amounts (fivefold more) in control fish. Screening of the principal acetylation sites of core histones revealed that butyrate treatment caused hyperacetylation of histone H4. Indeed, the addition of sodium butyrate to the diet significantly increased the ratio of AcH4/H4 at lysine 8 ($P<0.05$), leading to an approximately twofold increase in comparison to the control group (no butyrate) (Table 2). In contrast, the acetylation state of histone H3 at Lysine 9 was not significantly influenced by butyrate dietary intake. Interestingly, two different isoforms of histone H3 were separated on in the immunoblots, which could correspond to the H3.1 and H3.2 isoforms previously found in chicken [7].

Genes Related to Epigenetic Regulatory Mechanisms

Regardless of treatment, a two-way ANOVA showed that the differences between hepatic and intestinal levels of expression of five target genes related to epigenetic regulatory mechanisms were statistically significant ($P<0.05$) or highly significant ($P<0.01$; $P<0.001$) (Table 3 and S3 Table), being in general higher in the intestine. However, pairwise individual comparisons between control and treated fish for each tissue and gene analyzed by a Student's t-test showed no differences in any case, despite fold-change ranges of 0.49 to 2.66 in the intestine and of 1.67 to 14.74 in the liver. This could be due to the high variability observed between fish. Furthermore, regardless of tissue, *ehmt2* showed significant differences due to butyrate treatment ($P = 0.002$), with significant differences ($P = 0.010$) for the interaction between tissue and treatment, too. Similarly, *dicer1* and *hdac11* showed statistically significant differences due to the interaction between tissue and treatment ($P = 0.050$ and $P = 0.038$, respectively). Fold-change differences in the expression of genes that reached significance due to tissue, treatment, or both are shown in Fig 3A–3C.

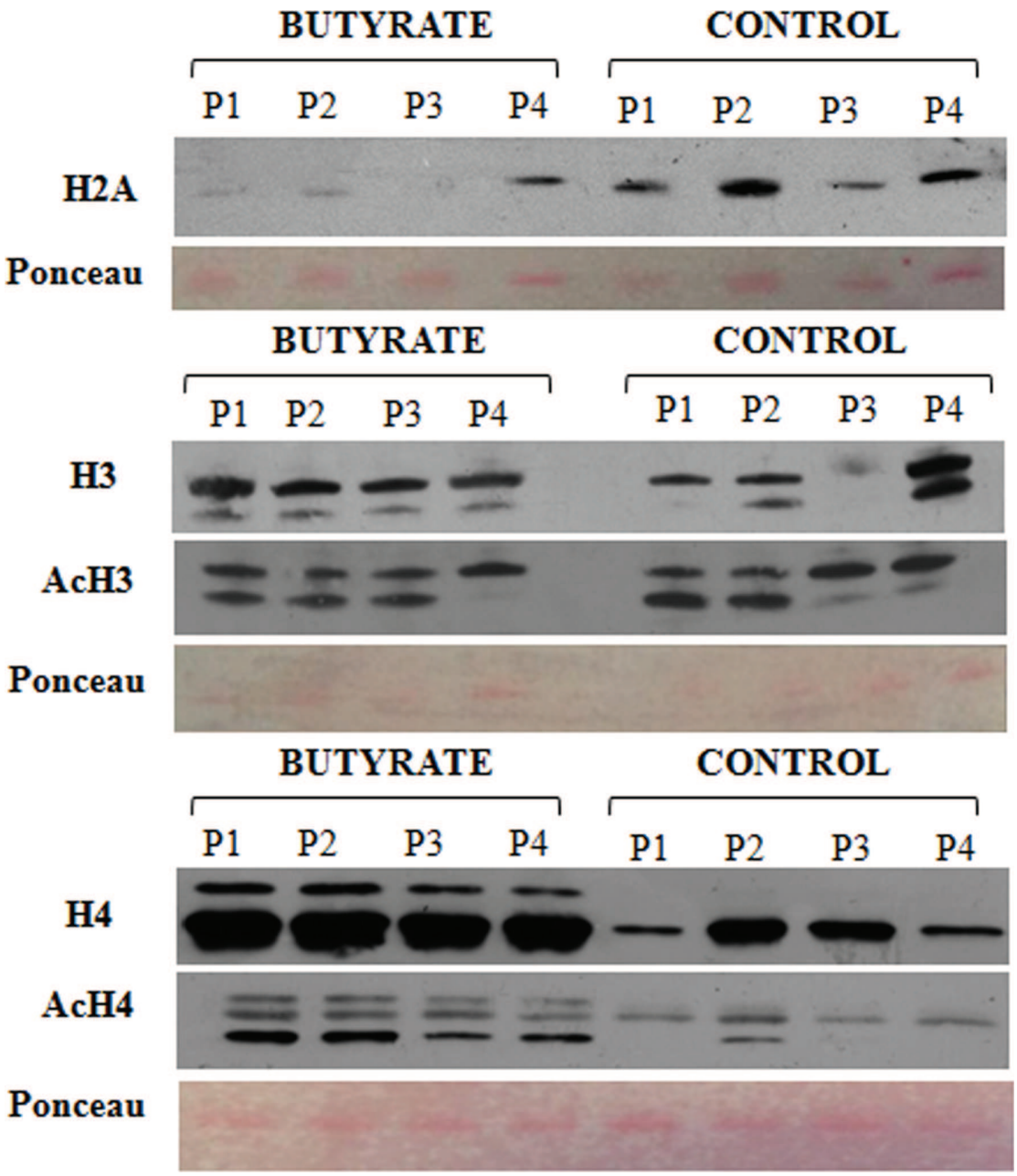

Fig 2. Effects of butyrate on the acetylation state of histones from isolated hepatocytes in European sea bass. One–dimensional immune-blotting analysis of histones H2A and H3 as well as H3, H4 acetylated histones is shown. Each column represents individual fish. 3 μg histone protein per lane were loaded for the detection of histones H2A, and H3, and 6 μg per lane for histone H4. Before immunodetection, a reversible Ponceau staining was applied to membranes to test equal loading of gels and protein transfer. After X-ray film exposure, densitometry was used to quantify protein levels on the western blots by means of Quantity One 1-D software (Bio-Rad). Putative isoforms for histone H3 [H3.1 (upper band) and H3.2 (lower band)] were accounted for the densitometry analysis.

doi:10.1371/journal.pone.0160332.g002

Genes Related to Mucosal Protection and Inflammatory Response

Statistical analysis by two-way ANOVA revealed that the expression of four (*il1 β*, *il8*, *irf1*, and *tnfα*) out of seven target genes related to inflammatory response and immune system was

Table 2. Quantification of core histone protein expression (Adj. Vol [OD*mm2]) and H4 acetylation ratio by densitometry. (*) (**) indicate statistical significant differences between experimental groups with $P < 0.05$ and $P < 0.01$, respectively.

	BUTYRATE	CONTROL	
Histone	**Adj. Vol [OD*mm^2]**		***t*-test**
H2A	1.12 ± 1.10*	5.46 ± 2.62	$P < 0.05$
H3	5,43 ± 1.36	5.78 ± 4.64	
AcH3	6.42 ± 1.33	8.36 ± 1.20	
H4	38.23 ± 6.48**	10.18 ± 7.81	$P < 0.01$
AcH4	6.55 ± 3.30**	0.53 ± 0.44	$P < 0.01$
	Acetylation ratio		
AcH4/H4	0.16 ± 0.05*	0.07 ± 0.04	$P < 0.05$

doi:10.1371/journal.pone.0160332.t002

significantly different ($P<0.05$) between the two analyzed tissues (liver and intestine) but only the *il10* gene showed differences in expression ($P = 0.003$) due to the butyrate treatment (Table 4 and S4 Table). This effect was also demonstrated with pairwise comparisons using Student's *t*-test ($P = 0.002$). In contrast to what was observed with the epigenetic regulatory mechanism-related genes and with the exception of *il10* in the liver (fold change 25.09±17.18; Fig 3D), the magnitude of fold change in the other two genes (*il6*, *muc2*) was lower (range 0.01–4.74). Furthermore, in contrast to the epigenetic regulatory mechanism-related genes, the interaction effect between tissue and treatment did not reach statistical significance for any of the seven target genes related to the inflammatory response and mucosal protection.

Discussion

Due to the paucity of oceanic resources utilized in the preparation of diets for cultured fish, the amount of fishmeal (FM) included in compound aquafeeds is steadily decreasing and commercial feed producers have been trying to replace FM by using alternative protein sources such as

Table 3. Two-way ANOVA statistical analysis of the expression of genes involved in epigenetic regulatory mechanisms.

Gene	2- way ANOVA	
dicer 1	F (Ts)	14.661 ($P = 0.001$)***
	F (Tr)	0.025 ($P = 0.875$)
	F (Ts x Tr)	2.219 ($P = 0.050$)*
ehmt2	F (Ts)	61.878 ($P = 0.000$)***
	F (Tr)	13.426 ($P = 0.002$)**
	F (Ts x Tr)	8.093 ($P = 0.010$)**
pcgf2	F (Ts)	7.211 ($P = 0.014$)*
	F (Tr)	0.003 ($P = 0.096$)
	F (Ts x Tr)	0.024 ($P = 0.878$)
jarid2a	F (Ts)	6.159 ($P = 0.022$)*
	F (Tr)	0.825 ($P = 0.374$)
	F (Ts x Tr)	0.385 ($P = 0.542$)
hdac11	F (Ts)	45.051 ($P = 0.000$)***
	F (Tr)	0.002 ($P = 0.969$)
	F (Ts x Tr)	4.843 ($P = 0.038$)*

Note: Asterisks mark statistical differences (*$P<0.05$; ** $P<0.01$; *** $P<0.001$). Ts = Tissue, Tr = Treatment, Ts x Tr denotes de interaction between Tissue and Treatment.

doi:10.1371/journal.pone.0160332.t003

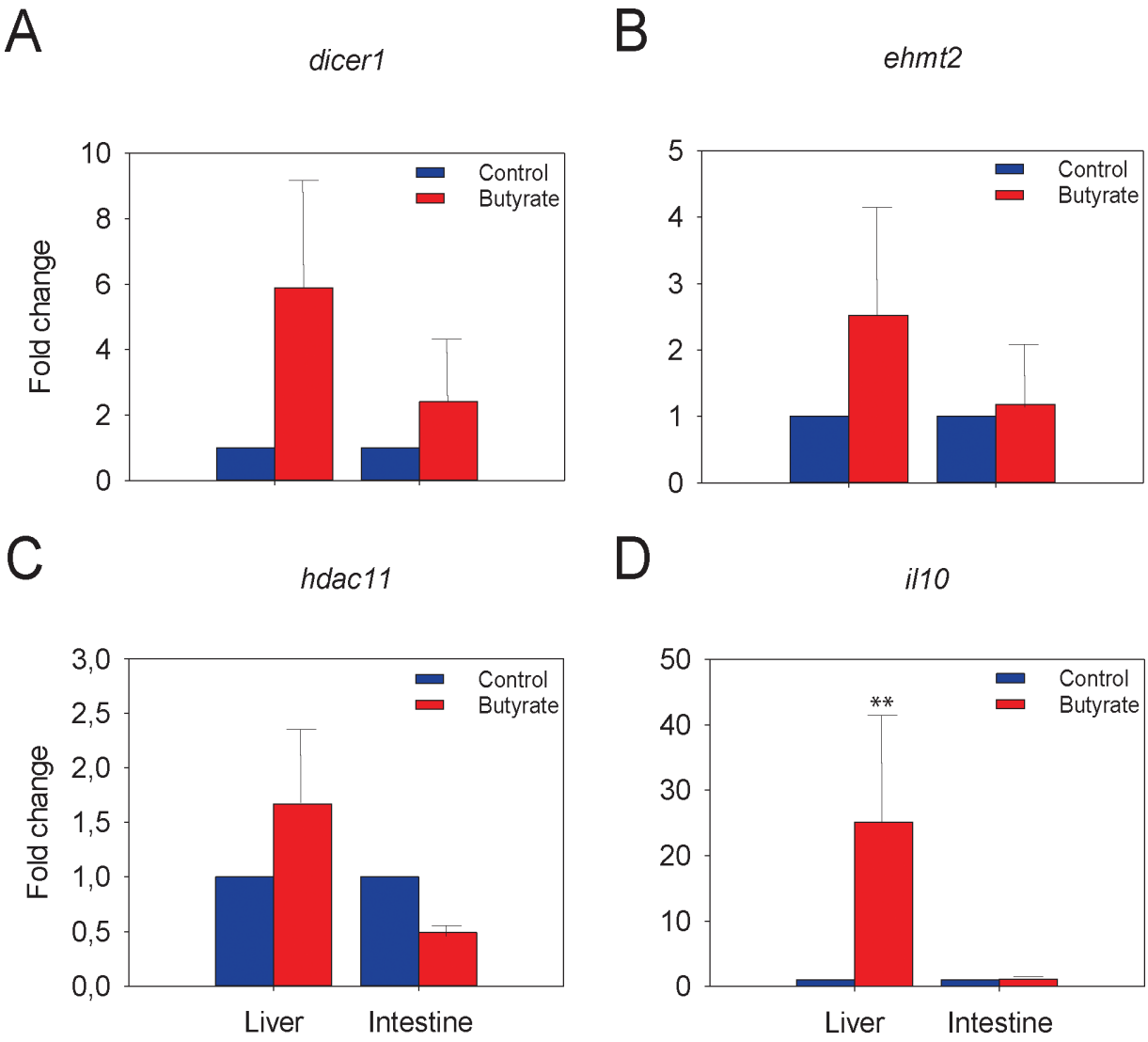

Fig 3. Effects of dietary butyrate on gene expression in two tissues of the European sea bass: liver and intestine, as determined by qRT-PCR analysis. Only those genes that showed statistical differences for the interaction between tissue and treatment (A: *dicer1*, B: *ehmt2* and C: *hdac11*), or differences in expression solely due to the treatment (D: *il10*) are depicted. Fish were fed for 8 weeks two different diets, a control diet, similar to feed commercially available for growing European seabass, and the experimental diet, which was the control diet supplemented with 2 g/kg (0.2%) of Na-butyrate. The means of six animals in each group are shown.

doi:10.1371/journal.pone.0160332.g003

vegetable proteins meals (VMs) [45]. VMs are able to replace a substantial part of the FM. However, these products have limitations due to unbalanced amino acid profiles, high fiber content, antinutritional factors and competition with use for human consumption [46]. Therefore, to further proceed with low FM inclusion levels, fish feeds should be adequately supplemented with natural feed additives such as butyrate [47] or other organic acids, which have generated increasing interest in the industry. Currently, there is strong interest in the use of organic acids and their salts as natural feed additives since such products seem to have growth-promoting effects in livestock. Their positive effects are well documented in terrestrial livestock production [28,48,49,50], but some questions remain regarding their efficacy in fish farming,

Table 4. Two-way ANOVA statistical analysis of the expression of genes involved in inflammatory response, mucosal protection, and immune homeostasis.

Gene	2- way ANOVA	
il1 β	F (Ts)	11.368 ($P = 0.003$)**
	F (Tr)	0.000 ($P = 1.000$)
	F (Ts x Tr)	0.000 ($P = 1.000$)
il6	F (Ts)	2.068 ($P = 0.165$)
	F (Tr)	1.126 ($P = 0.301$)
	F (Ts x Tr)	0.949 ($P = 0.341$)
il8	F (Ts)	8.129 ($P = 0.009$)**
	F (Tr)	0.632 ($P = 0.435$)
	F (Ts x Tr)	0.660 ($P = 0.425$)
il10	F (Ts)	0.036 ($P = 0.851$)
	F (Tr)	10.881 ($P = 0.003$)**
	F (Ts x Tr)	1.007 ($P = 0.326$)
irf1	F (Ts)	48.930 ($P = 0.000$)***
	F (Tr)	2.401 ($P = 0.136$)
	F (Ts x Tr)	1.505 ($P = 0.233$)
tnfα	F (Ts)	55.649 ($P = 0.000$)***
	F (Tr)	0.000 ($P = 1.000$)
	F (Ts x Tr)	0.000 ($P = 1.000$)
muc2	F (Ts)	4.241 ($P = 0.059$)
	F (Tr)	0.148 ($P = 0.706$)
	F (Ts x Tr)	0.070 ($P = 0.795$)

Note: Asterisks mark statistical differences (** $P<0.01$; *** $P<0.001$). Ts = Tissue, Tr = Treatment, Ts x Tr denotes interaction between Tissue and Treatment.

doi:10.1371/journal.pone.0160332.t004

and conflicting reports exist on the subject. Indeed, growth was significantly enhanced in some fish species, such as rainbow trout (*Oncorhynchus mykiss*), when fed an organic acid blend supplement mainly consisting of formate and sorbate [51], but not in trout fed other commercial supplements such as lactic acid [52] or citric acid [52,53]. On the other hand, neither hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) fed potassium diformate [54] nor Atlantic salmon (*S. salar*) fed sodium salts of acetic, propionic, and butyric acid (5:5:2 w/w/w) showed any growth enhancement [55, 31]. Species differences may thus occur. The results of our work are in accordance with the last two studies, Gislason et al., [55], and Bjerkeng et al., [31] as we did not find differences in the growth of European sea bass fed a diet supplemented with Na-butyrate. To date, literature related to the use of butyric acid or its salts in fish feed is still scarce and mainly focused on the effects of butyrate on fish growth performance, intestinal morphology, and metabolism [55,32,16,56]. Only few reports have described butyrate-induced epigenetic and transcriptional changes in intestinal and hepatic genes of farmed fish [15,56]. In view of this, the present study aims to contribute to the current understanding of the epigenetic regulatory effects of butyrate in European sea bass, which is one of the most important species in Mediterranean aquaculture.

Butyrate belongs to a well-known class of epigenetic factors known as histone deacetylase inhibitors (HDACi) [4]. Histone deacetylases (HDACs) are critical enzymes involved in epigenetic transcriptional regulation, i.e., histone acetylation associated with chromatin structure and function [57,58].There are very compelling data showing that sodium butyrate increases the quantities of acetylated H3 and H4 core histone proteins in certain cells and tissues [59–

62]. However, very limited evidence can be found in the literature regarding butyrate-induced histone acetylation *in vivo*. The only data available were obtained in chicken, mice, and pigs [7,63,64,65]; hence, the present study represents the first in fish. Our results on sea bass clearly confirmed the capability of butyrate to induce histone hyperacetylation even *in vivo*. In agreement with what Mátis and colleagues [7] observed in liver of chickens fed a low dose of butyrate (0.25 g/kg body weight, BW), no significant differences were found in the acetylation state of total histone H3 at lysine 9 after the dietary administration of 2 g/kg feed of Na-butyrate in sea bass. Interestingly, a higher dose of butyrate (1.25 g/kg BW) caused, instead, a relevant increase in H3 acetylation ratio in chicken [7]. This indicated that the level of histone H3 acetylation was dose-dependent and therefore the failed hyperacetylation observed in sea bass fed butyrate could be explained by the amount of Na-butyrate in the diet (2 g/kg feed), which was perhaps not sufficient to induce histone H3 hyperacetylation. Moreover, likewise in chicken [66], two isoforms of histone H3 were separated on the immunoblots in sea bass; in mammals, in contrast, three H3 variants have been characterized (H3.1, H3.2, H3.3) [67].

Butyrate treatment undoubtedly induced an increase of histone H4 acetylation in sea bass liver. In chicken, hyperacetylation of histone H4 occurred independently of the dietary intake levels of butyrate [7]. Similarly, acetylation of histone H4 in mammals [64] seemed to be independent of the butyrate dose, since both low and high diet content of Na-butyrate increased acetylated H4 levels in mouse hippocampus. Furthermore, in functional studies such as transcription factor-binding assays or gene expression analysis, acetylation of histone H4 was often found to be inversely correlated with acetylation of H3 [68–70]. Therefore, it would not be surprising if histone H3 and H4 differ from each other in response to dietary butyrate and this could be tested in a future research.

Among all core histones, H2A has the largest number of variants. In mammalian Jurkat cells, at least thirteen H2A variants were identified [71]. According to Brower-Toland et al., [72], and Ishibashi et al., [73] acetylation of H2A is involved in conformational changes of nucleosomes, which influence some strong, specific, and key histone-DNA interactions. In contrast, Gansen et al. [70] suggested that acetylation of H2A and H2B histones did not influence nucleosome stability, but could instead affect the nucleosome entry-exit region. However, multiple studies revealed that butyrate caused hyperacetylation of H2A both *in vivo* [7] and in cell culture [60,73,74]. We could not verify in sea bass whether butyrate induced H2A hyperacetylation since the antibody we used did not recognized our species epitope. However, we found that dietary butyrate caused a significant decrease in the total amount of H2A histone in European sea bass hepatocytes.

Concerning gene transcript abundance analysis, this study clearly showed tissue-dependent differences in the expression of five target genes involved in epigenetic regulatory mechanisms [75]; the expression was in general, higher in the liver than in the intestine. As previously found in European sea bass reared in different temperatures [36], three of target genes (*dicer1*, *ehmt2*, and *hdac11*) exhibited increased expression in the liver as a consequence of butyrate treatment, suggesting that these genes are involved in physiological processes in charge of coping with external insults.

The Dicer1 family is known to participate in the innate immune response to pathogens, mainly in RNA silencing-based antiviral immunity [76,77]. Indeed, studies in the past twenty years have established a completely new RNA-based immune system against viruses that is mechanistically related to RNA silencing or RNA interference. This viral immunity begins with recognition of viral double-stranded or structured RNA by the Dicer nuclease family of host immune receptors. Moreover, *dicer1* knockdown experiments showed an increase in the interferon response against pathogens [77]. Although our results showed a slightly increase in the

expression of *irf1*, a higher expression of *dicer1* was also observed in the liver, suggesting that in butyrate-treated fish dicer 1 was inhibiting an interferon response against the external insult.

The higher expression of *ehmt2* found in both tissues due to butyrate treatment could probably be related to the histone H3 dimethylation of lysine residue 10, as this is the expected effect of this enzyme. As demonstrated previously, this creates an epigenetic mark on nucleosomes associated to the *il6* promoter that may repress its expression and alter the *il6* signaling pathway [78]. A similar effect is possible in our experiment with butyrate treatment since *il6* expression was downregulated (although not significantly) in both the intestine and liver.

Finally, *hdac11* has also been related to the immune system by downregulating the expression of *il10* in antigen-presenting cells [79]. Overexpression of *hdac11* is thought to inhibit *il10* expression and activate T-cell responses. Our results in intestine showed a decrease in *hdac11* expression and a slight increase in *il10* levels. This suggests that, in butyrate-treated fish, antigen-specific T-cell responses could be impaired, which probably activates immune tolerance. This situation is known to prevent self-tissue damage [80] and the scenario fits nicely with the known anti-inflammatory effect of butyrate in the fish that received the supplemented diet.

Conclusions

Results of the 8-week-long feeding trial showed no significant differences in weight gain and SGR of sea bass that received 0.2% sodium butyrate supplementation in the diet in comparison to control fish that received a diet without Na-butyrate.

Butyrate in the feed significantly increased the acetylation state of histone H4 at lysine 8, leading to a twofold increase in comparison to the control group, but no changes were found in the acetylation of histone H3 at Lys9. Interestingly, for histone H3 two different isoforms were separated on the immunoblots, which could correspond to H3.1 and H3.2 isoforms previously found in terrestrial animals.

Concerning gene expression, butyrate applied as a nutritional supplement caused significant changes *in vivo* in the expression of genes related to epigenetic regulatory mechanisms such as *hdac11*, *ehmt2*, and *dicer1*. Statistical analysis by two-way ANOVA for these genes showed significant differences due to the butyrate treatment ($P = 0.002$) and to the interaction between tissue and treatment ($P = 0.010$). The expression of four (*il1 β*, *il8*, *irf1*, and *tnfα*) out of seven target genes related to mucosal protection and inflammatory response was significantly different between the two analyzed tissues but only for the *il10* gene were differences observed in the expression ($P = 0.003$) due to the butyrate treatment. Thus, in this study we reveal some of the effects of butyrate supplementation. This information is essential for the development of substitution diets in the efforts to improve the sustainability of the aquaculture of carnivorous species.

Supporting Information

S1 Fig. Multiple sequence alignment between the human peptide sequence used for the production of "Acetyl-Histone H4 (Lys8) Antibody #2594", and the ortholog sequences in European sea bass (*Dicentrarchus labrax*) and other teleost fish.
(PDF)

S2 Fig. Genome position of all the primers used in the study.
(PDF)

S1 Table. Quantitative real time PCR primer characteristics.
(PDF)

S2 Table. Statistical analysis by two-way ANOVA of fish growth data.
(PDF)

S3 Table. Quantitative real time PCR: fold changes (FC) in the expression of genes related to epigenetic regulatory mechanisms and statistical analysis.
(PDF)

S4 Table. Quantitative real time PCR: fold changes (FC) in the expression of genes related to inflammatory response, mucosal protection, and immune homeostasis plus statistical analysis.
(PDF)

Acknowledgments

EG is a PhD student of the 'Dottorato in Biotecnologie, Bioscienze e Tecnologie Chirurgiche' at the "Università degli Studi dell'Insubria".

Author Contributions

Conceptualization: GT FP. Funding acquisition: GT FP. Methodology: SR ND CC EG. Software: SR ND. Validation: CC EG. Writing - original draft: GT ND SR. Writing - review & editing: GT ND.

References

1. Bergman FN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev. 1990; 70:567–590. PMID: 2181501
2. Cummings JH, Macfarlane GT. The Control and Consequences of Bacterial Fermentation in the Human Colon. Journal of Applied Bacteriology. 1991; 70(6):443–459. PMID: 1938669
3. Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel F. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr Res Rev. 2010; 23:366–384. doi: 10.1017/S0954422410000247 PMID: 20937167
4. Canani RB, Di Costanzo M, Leone L. The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. Clinical Epigenetics. 2012; 4(1):4. doi: 10.1186/1868-7083-4-4 PMID: 22414433
5. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett. 2009; 294:1–8. doi: 10.1111/j.1574-6968.2009.01514.x PMID: 19222573
6. Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. The Proceedings of the Nutrition Society. 2003; 62(1):67–72. PMID: 12740060
7. Mátis G, Neogrády Z, Csikó G, Kulcsár A, Kenéz Á, Huber K. Effects of orally applied butyrate bolus on histone acetylation and cytochrome P450 enzyme activity in the liver of chicken–a randomized controlled trial. Nutrition & Metabolism. 2013; 10:12.
8. Gálfi P, Neogrády S. The pH-dependent inhibitory action of n-butyrate on gastrointestinal epithelial cell division. Food Res Int. 2002; 34:581–586.
9. Wong JMWRD, de Souza RRD, Kendall CWC, Emam A, Jenkins DJA. Colonic Health: Fermentation and Short Chain Fatty Acids. Journal of Clinical Gastroenterology. 2006; 40(3):235–243. PMID: 16633129
10. Vinolo MA, Hatanaka E, Lambertucci RH, Curi R. Effects of short chain fatty acids on effector mechanisms of neutrophils. Cell Biochem Funct. 2009; 27(1):48–55. doi: 10.1002/cbf.1533 PMID: 19107872
11. Toden S, Bird AR, Topping DL, Conlon MA. Dose-dependent reduction of dietary protein-induced colonocyte DNA damage by resistant starch in rats correlates more highly with caecal butyrate than with other short chain fatty acids. Cancer Biol Ther. 2007; 6(2):e1–e6.
12. Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. Alimentary Pharmacology & Therapeutics (Aliment Pharmacol Ther). 2008; 27:104–119.

13. Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World J Gastroenterol. 2011; 17:1519–28. doi: 10.3748/wjg.v17.i12 PMID: 21472114
14. Biancotto C, Frigè G, Minucci S. Histone modification therapy of cancer. Adv Genet. 2010; 70:341–386. doi: 10.1016/B978-0-12-380866-0.60013-7 PMID: 20920755
15. Scollo G, Terova G, Rimoldi S, Bernardini G, Antonini M, Saroglia M. Does butyrate have a role in the protection of fish intestine? Results from a preliminary study on European sea bass (*D. labrax*) Aquaculture Europe. Book of abstracts. Prague. 2012.
16. Robles R, Lozano AB, Sevilla A, Márquez L, Nuez-Ortín W, Moyano FJ. Effect of partially protected butyrate used as feed additive on growth and intestinal metabolism in sea bream (*Sparus aurata*). Fish Physiol. Biochem. 2013; 39: 1567–1580. doi: 10.1007/s10695-013-9809-3 PMID: 23737146
17. Björkholm B, Bok CM, Lundin A, Rafter J, Hibberd ML, Pettersson S. Intestinal microbiota regulate xenobiotic metabolism in the liver. PLoS ONE. 2009; 4:e6958. doi: 10.1371/journal.pone.0006958 PMID: 19742318
18. Bloemen JG, Venema K, van de Poll MC, Olde Damink SW, Buurman WA, Dejong CH. Short chain fatty acids exchange across the gut and liver in humans measured at surgery. Clin Nutr. 2009; 28:657–661. doi: 10.1016/j.clnu.2009.05.011 PMID: 19523724
19. Demigné C, Yacoub C, Rémésy C. Effects of absorption of large amounts of volatile fatty acids on rat liver metabolism. J Nutr. 1986; 116:77–86. PMID: 3003291
20. Beauvieux MC, Tissier P, Gin H, Canioni P, Gallis JL. Butyrate impairs energy metabolism in isolated perfused liver of fed rats. J Nutr. 2001; 131:1986–1992. PMID: 11435518
21. Gallis JL, Tissier P, Gin H, Beauvieux MC. Decrease in oxidative phosphorylation yield in presence of butyrate in perfused liver isolated from fed rats. BMC Physiol. 2007; 7:8. PMID: 17725817
22. Gallis JL, Gin H, Roumes H, Beauvieux MC. A metabolic link between mitochondrial ATP synthesis and liver glycogen metabolism: NMR study in rats re-fed with butyrate and/or glucose. Nutr Metab. 2011; 8:38.
23. Evans JA. The effects of diet and feeding frequency on peripheral nutrient supply and growth traits of the lamb. PhD Thesis. University of Nottingham. 1998. Available: http://eprints.nottingham.ac.uk/14420/.
24. Topping DL, Clifton PM. Short-chain fatty acids and human colonic function: roles of resistant starch and non-starch polysaccharides. Physiol Rev. 2001; 81: 1031–64. PMID: 11427691
25. Miller SJ. Cellular and physiological effects of short-chain fatty acids. Mini Rev Med Chem. 2004; 4 (8):839–845. PMID: 15544545
26. Gálfi P, Bokori J. Feeding trial in pigs with a diet containing sodium n-butyrate. Acta Vet Hung. 1990; 38(1–2):3–17. PMID: 2100936
27. Kotunia A, Wolinski J, Laubitz D, Jurkowska M, Rome V, Guilloteau P, Zabielski R. Effect of sodium butyrate on the small intestine development in neonatal piglets fed correction of feed by artificial sow. J Physiol Pharmacol. 2004; 55 Suppl 2:59–68. PMID: 15608361
28. Hu Z, Guo Y. Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens. Anim Feed Sci Tech. 2007; 132:240–249.
29. Owen MAG, Waines P, Bradley G, Davies S. The effect of dietary supplementation of sodium butyrate on the growth and microflora of Clarias gariepinus (Burchell, 1822). XII international symposium fish nutrition and feeding. 2006 May 28–June 1; Book of abstracts: pp 149.
30. Zheng, RG. The effect of sodium butyrate on the growth performance and intestinal mucous structure of fresh water fish. PhD thesis (2009). Available: http://www.globethesis.com/?t=2143360272979206.
31. Bjerkeng B, Storebakken T, Wathne E. 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. Aquaculture Nutrition. 1999; 5:181–191.
32. Gao Y, Storebakken T, Shearer KD, Penn M, Øverland M. Supplementation of fishmeal and plant protein-based diets for rainbow trout with a mixture of sodium formate and butyrate. Aquaculture. 2011; 311: 233–240.
33. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. 2001; 104(4):487–501. PMID: 11239407
34. Brien JD, Daffis S, Lazear HM, Cho H, Suthar MS, Gale M Jr, et al. Interferon Regulatory Factor-1 (IRF-1) Shapes Both Innate and CD8+ T Cell Immune Responses against West Nile Virus Infection. PLoS Pathog. 2011; 7(9): e1002230. doi: 10.1371/journal.ppat.1002230 PMID: 21909274
35. Allen A, Hutton DA, Pearson JP. The MUC2 gene product: a human intestinal mucin. Int J Biochem Cell Biol. July 1998; 30(7):797–801. PMID: 9722984

36. Díaz N, Piferrer F. Lasting effects of early exposure to temperature on the gonadal transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed genetic and environmental sex determination. BMC Genomics. 2015 Sep 4; 16(1):679.
37. Jaskiewicz L, Filipowicz W: Role of Dicer in posttranscriptional RNA silencing. Current Topics in Microbiology and Immunology. 2008; 320:77–97. PMID: 18268840
38. Nishio H, Walsh MJ. CCAAT displacement protein/cut homolog recruits G9a histone lysine methyltransferase to repress transcription. PNAS. 2004; 101:11257–11262. PMID: 15269344
39. Duan Z, Zarebski A, Montoya-Durango D, Grimes HL, Horwitz M. Gfi1 coordinates epigenetic repression of p21Cip/WAF1 by recruitment of histone lysine methyltransferase G9a and histone deacetylase 1. Molecular and Cellular Biology. 2005; 25: 10338–10351. PMID: 16287849
40. Guo WJ, Datta S, Band V, Dimri GP. Mel-18, a polycomb group protein, regulates cell proliferation and senescence via transcriptional repression of Bmi-1 and c-Myc oncoproteins. Molecular Biology of the Cell. 2007; 18:536–546. PMID: 17151361
41. Gao L, Cueto MA, Asselbergs F, Atadja P. Cloning and functional characterization of HDAC11, a novel member of the human histone deacetylase family. The Journal of Biological Chemistry. 2002; 277:25748–25755. PMID: 11948178
42. Kim TG, Kraus JC, Chen J, Lee Y. JUMONJI, a critical factor for cardiac development, functions as a transcriptional repressor. The Journal of Biological Chemistry. 2003; 278: 42247–42255. PMID: 12890668
43. Tine M, Kuhl H, Gagnaire P-A, Louro B, Desmarais E, Martins RS, et al. European sea bass genome and its variation provide insights into adaptation to euryhalinity and speciation. Nature Commun. 2014; 5:5770. doi: 10.1038/ncomms6770
44. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature Protocols. 2008; 3(6):1101–1108. PMID: 18546601
45. Tacon AGJ, Metian M. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future perspectives. Aquaculture. 2008; 285: 146–158.
46. Hardy RW. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquacult. Res. 2010; 41: 770–776.
47. Benedito-Palos L, Ballester-Lozano GF, Simó P, Karalazos V, Ortiz Á, Calduch-Giner J, Pérez-Sánchez J. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (Sparus aurata). Aquaculture. 2016; 454: 8–18.
48. Øverland M, Granli T, Kjos NP, Fjetland O, Steien SH, Stokstad M. Effect of dietary formates on growth performance, carcass traits, sensory quality, intestinal microflora, and stomach alterations in growing–finishing pigs. Journal of Animal Science. 2000; 78:1875–1884. PMID: 10907830
49. Øverland M, Kjos NP, Borg M, Skjerve E, Sørum H. Organic acids in diets for entire male pigs: effect on skatole level, microbiota in digesta, and growth performance. Livestock Science. 2008; 115:169–178.
50. Lückstädt C. The use of acidifiers in fish nutrition. CAB Reviews: Perspectives in Agriculture Veterinary Science. Nutr and Nat Resour. 2008; 3(044):1–8.
51. De Wet L. Organic acids as performance enhancers. Aqua Feeds: Formulation and Beyond. 2005; 2:12–14.
52. Pandey A, Satoh S. Effects of organic acids on growth and phosphorus utilization in rainbow trout *Oncorhynchus mykiss*. Fisheries Science. 2008; 74:867–874.
53. Vielma J, Ruohonen K, Lall SP. Supplemental citric acid and particle size of fish bone-meal influence the availability of minerals in rainbow trout *Onchorynchus mykiss* (Walbaum). Aquaculture Nutrition. 1999; 5:65–71.
54. Zhou Z, Liu Y, He S, Shi P, Gao X, Yao B, Ringø E. Effects of dietary potassium diformate (KDF) on growth performance, feed conversion and intestinal bacterial community of hybrid tilapia (*Oreochromis niloticus*×*O. aureus*). Aquaculture. 2009; 291:89–94.
55. Gislason G, Olsen RE, RingØ E. Lack of growth-stimulating effect of lactate on Atlantic salmon, *Salmo salar* L. Aquaculture and Fisheries Management. 1994; 25:861–862.
56. Liu W, Yang Y, Zhang J, Gatlin DM, Ringø E, Zhou Z. Effects of dietary microencapsulated sodium butyrate on growth, intestinal mucosal morphology, immune response and adhesive bacteria in juvenile common carp (*Cyprinus carpio*) pre-fed with or without oxidized oil. Br J Nutr. 2014; 112:15–29. doi: 10.1017/S0007114514000610 PMID: 24774835
57. Dokmanovic M, Marks PA. Prospects: histone deacetylase inhibitors. J Cell Biochem. 2005; 96:293–304. PMID: 16088937

58. Waterborg JH. Dynamics of histone acetylation *in vivo*. A function for acetylation turnover? Biochim Cell Biol. 2002; 80:363–78.

59. Candido EPM, Reeves R, Davie JR. Sodium butyrate inhibits histone deacetylation in cultured cells. Cell. 1978; 14:105–113. PMID: 667927

60. Mohana KB, Song HJ, Cho SK, Balasubramanian S, Choe SY, Rho GJ. Effect of histone acetylation modification with sodium butyrate, a histone deacetylase inhibitor, on cell cycle, apoptosis, ploidy and gene expression in porcine fetal fibroblasts. Journal of Reproduction and Development. 2007; 53:903–913. PMID: 17558190

61. Koprinarova M, Markovska P, Iliev I, Anachkova B, Russev G. Sodium butyrate enhances the cytotoxic effect of cisplatin by abrogating the cisplatin imposed cell cycle arrest. BMC Molecular Biology. 2010; 11:49. doi: 10.1186/1471-2199-11-49 PMID: 20576112

62. Paskova L, Trtkova KS, Fialova B, Benedikova A, Langova K, Kolar Z. Different effect of sodium butyrate on cancer and normal prostate cells. Toxicology in Vitro. 2013; 27:1489–1495. doi: 10.1016/j.tiv.2013.03.002 PMID: 23524101

63. Shimazu T, Hirschey MD, Newman J, He W, Shirakawa K, Le Moan N, Grueter CA, Lim H, Saunders LR, Stevens RD, Newgard CB, Farese RV Jr, de Cabo R, Ulrich S, Akassoglou K, Verdin E. Suppression of oxidative stress by *b*-hydroxybutyrate, an endogenous histone deacetylase inhibitor. Science. 2013; 339: 211. doi: 10.1126/science.1227166 PMID: 23223453

64. Gundersen BB, Blendy JA. Effects of the histone deacetylase inhibitor sodium butyrate in models of depression and anxiety. Neuropharmacology. 2009; 57:67–74. doi: 10.1016/j.neuropharm.2009.04.008 PMID: 19393671

65. Kien CL, Peltier CP, Mandal S, Davie JR, Blauwiekel R. Effects of the *in vivo* supply of butyrate on histone acetylation of cecum in piglets. JPEN-Parenter Enter Nutr. 2008; 32:366–384.

66. Zhang K, Tang H, Huang L, Blankenship JW, Jones PR, Xiang F, Yau PM, Burlingame AL. Identification of acetylation and methylation sites of histone H3 from chicken erythrocytes by high-accuracy matrix-assisted laser desorption ionization-time-of-flight, matrix-assisted laser desorption ionization-postsource decay, and nanoelectrospray ionization tandem mass spectrometry. Analytical Biochemistry. 2002; 306:259–269. PMID: 12123664

67. Hake SB, Garcia BA, Duncan EM, Kauer M, Dellaire G, Shabanowitz J, Bazett-Jones DP, Allis CD, Hunt DF. Expression patterns and post-translational modifications associated with mammalian histone H3 variants. J Biol Chem. 2006; 281:559–568. PMID: 16267050

68. Kurdistani SK, Tavazoie S, Grunstein M. Mapping global histone acetylation patterns to gene expression. Cell. 2004; 117:721–733. PMID: 15186774

69. Agricola E, Verdone L, Di Mauro E, Caserta M. H4 acetylation does not replace H3 acetylation in chromatin remodeling and transcription activation of Adr1-dependent genes. Mol Microbiol. 2006; 62:1433–1446. PMID: 17121596

70. Gansen A, Tóth K, Schwarz N, Langowski J. Opposing roles of H3- and H4-acetylation in the regulation of nucleasome structure-a FRET study. Nucleic Acid Research. 2015; 43(3):1433–1443.

71. Bonenfant D, Coulot M, Towbin H, Schindler P, van Oostrum J. Characterization of histone H2A and H2B variants and their post-translational modifications by mass spectrometry. Mol Cell Proteomics. 2006; 5:541–552. PMID: 16319397

72. Brower-Toland B, Wacker DA, Fulbright RM, Lis JT, Kraus WL, Wang MD. Specific contributions of histone tails and their acetylation to the mechanical stability of nucleosomes. J Mol Biol. 2005; 346:135–146. PMID: 15663933

73. Ishibashi T, Dryhurst D, Rose KL, Shabanowitz J, Hunt DF, Ausió J. Acetylation of vertebrate H2A.Z and its effects on the structure of nucleosome. Biochemistry. 2009; 48: 5007–5017. doi: 10.1021/bi900196c PMID: 19385636

74. Tobisawa Y, Imai Y, Fukuda M, Kawashima H. Sulfation of colonic mucins by N-acetylglucosamine 6-O-sulfotransferase-2 and its protective function in experimental colitis in mice. J Biol Chem. 2010; 285:6750–6760. doi: 10.1074/jbc.M109.067082 PMID: 20018871

75. Piferrer F. Epigenetics of sex determination and gonadogenesis. Developmental Dynamics. 2013; 242:360–370. doi: 10.1002/dvdy.23924 PMID: 23335256

76. Aliyari R, Ding SW. RNA-based viral immunity initiated by the Dicer family of host immune receptors. Immunology Review. 2009; 227(1):176–188.

77. Chiappinelli KB, Haynes BC, Brent MR, Goodfellow PJ. Reduced DICER1 elicits an interferon response in endometrial cancer cells. Molecular Cancer Research. 2012; 10: 316–325. doi: 10.1158/1541-7786.MCR-11-0520 PMID: 22252463

78. Takahashi H, Katayama K, Sohya K, Miyamoto H, Prasad T, Matsumoto Y, Ota M, Yasuda H, Tsumoto T, Aruga J, Craig AM. Selective control of inhibitory synapse development by Slitrk3-PTPd trans-synaptic interaction. Nature Neurosci. 2012; 15: 389–400. doi: 10.1038/nn.3040 PMID: 22286174

79. Villagra A, Cheng F, Wang HW, Suarez I, Glozak M, Maurin M, Nguyen D, Wright KL, Atadja PW, Bhalla K, Pinilla-Ibarz J Seto E, Sotomayor EM. The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. Nature Immunology. 2009; 10(1): 92–100. doi: 10.1038/ni.1673 PMID: 19011628

80. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR Jr, Muller W, Rudensky AY. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity. 2008; 28:546–558. doi: 10.1016/j.immuni.2008.02.017 PMID: 18387831

2.2. EFFECT OF A SPECIFIC COMPOSITION OF FATTY ACIDS ON GROWTH PERFORMANCES AND GUT MICROBIOTA IN GILTHEAD SEA BREAM

Effect of a specific composition of short- and medium-chain fatty acid 1-Monoglycerides on growth performances and gut microbiota of gilthead sea bream (*Sparus aurata*)

Simona Rimoldi[1], Emi Gliozheni[1], Chiara Ascione[1], Elisabetta Gini[1] and Genciana Terova[1,2]

[1] Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy
[2] Inter-University Centre for Research in Protein Biotechnologies, "The Protein Factory", Polytechnic University of Milan and University of Insubria, Varese, Italy

ABSTRACT

In aquaculture research, one important aim of gut microbiota studies is to provide the scientific basis for developing effective strategies to manipulate gut microbial communities through the diet, promoting fish health and improving productivity. Currently, there is an increasing commercial and research interest towards the use of organic acids in aquafeeds, due to several beneficial effects they have on growth performance and intestinal tract's health of farmed fish. Among organic acids, monoglycerides of short-chain fatty acids (SCFAs) and medium-chain fatty acids (MCFAs) have attracted particular research attention also for their bacteriostatic and bactericidal properties. Accordingly, the present study aimed to evaluate the potential beneficial effects of SCFA and MCFA monoglycerides, used as a feed additive, on fish growth performance, and intestinal microbiota composition. For this purpose, a specific combination of short- and medium-chain 1-monoglycerides (SILOhealth 108Z) was tested in 600 juvenile gilthead sea bream (Sparus aurata) of about 60 g mean initial weight that were fed for 90 days with plant-based diets. Two isoproteic and isolipidic diets were formulated. The control fish group received a plant-based diet, whereas the other group received the same control feed, but supplemented with 0.5% of SILOhealth 108Z. The Illumina MiSeq platform for high-throughput amplicon sequencing of 16S rRNA gene and QIIME pipeline were used to analyse and characterize the whole microbiome associated both to feeds and S. aurata intestine. The number of reads taxonomically classified according to the Greengenes database was 394,611. We identified 259 OTUs at 97% identity in sea bream fecal samples; 90 OTUs constituted the core gut microbiota. Firmicutes, Proteobacteria and Actinobacteria represented the dominant phyla in both experimental groups. Among them, relative abundance of Firmicutes and Proteobacteria were positively and negatively affected by dietary SCFA monoglycerides supplementation, respectively. In summary, our findings clearly indicated that SILOhealth 108Z positively modulated the fish intestinal microbiota by increasing the number of beneficial lactic acid bacteria, namely, Lactobacillus, and reducing Gammaproteobacteria, which include several potential pathogenic bacteria. The specific composition of 1-monoglycerides of short- and medium-chain fatty acids contained in SILOhealth 108Z could thus have a great potential as a feed additive in aquaculture.

Submitted 7 February 2018
Accepted 7 July 2018
Published 31 July 2018

Corresponding author
Genciana Terova,
genciana.terova@uninsubria.it

Academic editor
Luis Conceição

Additional Information and Declarations can be found on page 20

DOI **10.7717/peerj.5355**

Copyright
2018 Rimoldi et al.

Distributed under
Creative Commons CC-BY 4.0

OPEN ACCESS

How to cite this article Rimoldi et al. (2018), Effect of a specific composition of short- and medium-chain fatty acid 1-Monoglycerides on growth performances and gut microbiota of gilthead sea bream (*Sparus aurata*) . **PeerJ 6:e5355; DOI 10.7717/peerj.5355**

Subjects Aquaculture, Fisheries and Fish Science, Marine Biology
Keywords Next-generation sequencing, Metagenomics, Gut microbiome, Feed additive, Aquaculture, 1-Monoglycerides, SILOhealth 108Z, 16S rRNA gene

INTRODUCTION

Aquaculture, with an average annual rate of 8 percent, is probably the fastest-growing food-producing sector in the world. It provides nearly 50 percent of the seafood consumed by humans (*FAO, 2014*) and the World Bank projects that aquaculture will increase to provide 2/3 of the world's fish in 2030. Currently, about 68% and 88% of the demand for fishmeal (FM) and fish oil (FO), respectively, comes from aquaculture (*Naylor et al., 2009*). However, with most wild fish capture fisheries at or above maximum sustainable yield, aquaculture can no longer rely on oceanic resources for the manufacturing of aquafeeds and such feed options are simply not sustainable (*Naylor et al., 2000*). To defray rising costs and avert ecological harm, commercial feed producers and fish farmers have made substantial efforts to reduce the proportion of FM and FO in aquaculture feed, by replacing ground-up forage fish with terrestrial plants (*Tacon & Metian, 2008*; *Gatlin et al., 2007*). However, the use of vegetable feedstuff in aquafeed production has several drawbacks that are related to the low level of indispensable amino acids (in particular lysine and methionine) and to the presence of a wide variety of anti-nutritional factors that could damage the intestine, thus reducing nutrient absorption, and consequently, affecting fish growth and resistance to stress and diseases (*Zhang et al., 2013*; *Penn et al., 2011*; *Santigosa et al., 2011*; *Francis, Makkar & Becker, 2001*).

For this reason, nutritionists and feed manufacturers are investing great effort to find feed additives that could prevent or alleviate the adverse effects at the gut level of plant-based ingredients that are actually used in fish diet formulations.

Here, the most promising feed additives seem to be organic acids that are compounds with acidic properties associated with their carboxyl group (−COOH) (*Lim et al., 2015*).

Among them, short- and medium-chain fatty acids (SCFAs and MCFAs) are known to play a central role as energy-source for enterocytes. SCFAs are fatty acids with aliphatic tails of one to six carbon atoms, the most common being acetic (C2), propionic (C3), and butyric (C4) acid, whereas MCFA comprise fatty acids with seven to 12 carbon atoms. SCFAs are produced within the intestinal lumen by bacterial fermentation of undigested dietary carbohydrates and fibers (cellulose, hemicellulose, pectin). Contrariwise, MCFAs mainly arise from dietary triglycerides and natural sources of MCFAs are generally coconut oil, palm kernel oil, and milk. The use of SCFAs as additive in aquafeeds and their impact on fish growth, nutrient utilization, and disease resistance were recently reviewed (*Ng & Koh, 2017*). Among SCFAs, butyric acid has received particular attention for its various well-documented beneficial effects on the health of intestinal tract and peripheral tissues in human and farmed animals, including fish (*Guilloteau et al., 2010*; *Mátis et al., 2013*; *Robles et al., 2013*; *Liu et al., 2014*). Butyrate represents a major energy source for enterocytes and is involved in maintaining gut mucosal health, playing a central role in enhancing epithelial cell proliferation and differentiation and in improving the intestinal

absorption (*Gálfi & Neogrády, 2001*; *Wong et al., 2006*; *Canani et al., 2011*). Butyrate has anti-inflammatory properties and the potential to stimulate the immune system, too (*Vinolo et al., 2011*; *Hamer et al., 2008*; *Terova et al., 2016*; *Rimoldi et al., 2016*; *Tian et al., 2017*). However, the data on the effect of butyric acid and its salts (sodium butyrate) on the growth performance of cultured fish and crustaceans are still controversial. In juvenile common carp (*Cyprinus carpio*) (*Liu et al., 2014*), and Pacific white shrimp (*Litopenaeus vannamei*) (*Da Silva et al., 2016*), butyrate supplementation positively affected the growth performance. On the other hand, a dietary supplementation of a mixture of SCFAs, containing butyrate, did not significantly improve growth rate or feed utilization in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), and European sea bass (*Bjerkeng, Storebakken & Wathne, 1999*; *Gao et al., 2011*; *Terova et al., 2016*). Recently, *Simó-Mirabet et al. (2017)* reported that sodium salt of coconut fatty acid distillate, particularly rich in lauric acid (C12), increased feed intake, improved gut development and nutrient absorption, thus enhancing growth rate of gilthead sea bream (*Sparus aurata*). Moreover, MCFAs have been suggested to have a role in immunological response regulation (*Wang et al., 2006*). Organic acids, their salts or combinations thereof, are commonly known as acidifiers and are used as storage preservatives in terrestrial livestock feeds as well as in aquafeeds (*Ng & Koh, 2017*). Due to their capacity to reduce pH, they inhibit microbial growth and diminish a possible contamination of feed by pathogenic organisms such as *Salmonella* and *Escherichia coli* (*Lückstädt, 2008*; *Van Immerseel et al., 2003*; *Van Immerseel et al., 2004*; *Skřivanová et al., 2009*). The mechanism of action of SCFAs and MCFAs differs from that of antibiotics. *Salsali, Parker & Sattar (2008)* firstly proposed that SCFAs and MCFAs bacteriostatic and bactericidal activities could be due to the ability of the undissociated form of the acid to penetrate the bacterial cell wall and, once inside, to dissociate releasing protons, thereby lowering the cytoplasmic pH. Consequently, the bacterium must redirect its energy towards the efflux of the excess protons, thus exhausting cell metabolism and leading to lower bacterial cell growth and even to cell death (*Salsali, Parker & Sattar, 2008*; *Hismiogullari et al., 2008*; *Ng & Koh, 2017*). In the digestive tract, organic acids cause a pH reduction in the intestine via the delivery of H^+ ions (*Lim et al., 2015*). Actually, in fish, dietary administration of acidifiers inhibits overgrowth of pH-sensitive pathogenic bacteria favouring the growth of beneficial intestinal flora (*Zhou et al., 2009*; *Hoseinifar, Sun & Caipang, 2017*; *Abu Elala & Ragaa, 2015*; *Ringøet al., 2016*; *Da Silva et al., 2013*; *Da Silva et al., 2016*; *Anuta et al., 2011*; *De Schryver et al., 2010*; *Liu et al., 2014*; *Piazzon et al., 2017*). Indeed, although the bacteriostatic activity of organic acids is preserved at the intestinal level, their bactericidal efficacy is limited because of the intestinal pH. Being weak acids with modest pKas of approximately 3.6 to 4.7, the majority of organic acids at neutral or slightly alkaline pH, are present as anions rather than as undissociated forms (free acids) that are assumed to penetrate the lipid membrane, destroying the bacterial cell (*Yoon et al., 2018*).

Dietary free organic acids and their salts have also the disadvantage to be easily absorbed by the upper digestive tract, thus limiting their delivery to the desired target, i.e., lower intestinal tract, where they exert the aforementioned beneficial actions.

On the contrary, monoglycerides, which are esters formed by glycerol and one molecule of fatty acid, have no such drawbacks. The great advantage of monoglycerides is that organic acid is released from the glycerol backbone only under the action of intestinal lipases. This means that SCFA or MCFA remains protected from absorption in the upper gastrointestinal tract and could reach the final portion of intestine, where it would exert its major functions (*Sampugna et al., 1967*; *Namkung et al., 2011*). Moreover, monoglycerides possess a more effective antimicrobial activity than the corresponding free fatty acids, since their efficacy is independent from environmental pH (*Bergsson et al., 2001*; *Sun, O'Connor & Roberton, 2003*; *Thormar, Hilmarsson & Bergsson, 2006*). Due to their amphipathic properties, monoglycerides show a membrane-lytic action, which leads to bacterial membrane destabilization and pore formation. Membrane-destabilizing activity causes increased cell permeability and cell lysis, leading to inhibition of growth and cell death (*Yoon et al., 2018*). MCFA monoglycerides are able to penetrate also the peptidoglycan layer of Gram-positive bacteria's cell wall (*Bergsson et al., 2001*).

Up to date, antimicrobial and growth-promoting action of monoglycerides have been widely investigated in poultry (*Bedford & Gong, 2018*; *Yang et al., 2018*; *Jahanian & Golshadi, 2015*; *Leeson et al., 2005*), whereas in fish their effects have been poorly explored. Accordingly, the present study aimed to evaluate the potential beneficial effects of dietary SCFA and MCFA monoglycerides on fish growth performances and intestinal microbiota composition. For this purpose, a specific synergic combination of 1-monoglycerides of short- and medium-chain fatty acids (SILOhealth 108Z), commercially available from SILO SpA, Florence, Italy (http://www.silohealth.com/), was tested in juvenile gilthead sea bream (*Sparus aurata*) fed a plant-based diet. The Illumina MiSeq platform for high-throughput sequencing of 16S rRNA gene was utilized to analyse and characterize the whole gut microbiome of gilthead sea bream.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the indoor experimental facility of Civita Ittica (Civitavecchia, Italy), and in accordance with EU Directive 2010/63/E U for animal experiments. The Committee on the Ethics of Animal Experiments of the same experimental facility approved all of the study protocols (approval n. 120/2008-A of 03/09/2008 (Art.12 of D.Lgs.116/92)). Fish handling was performed under tricaine methanesulfonate (MS222) anesthesia, and all effort was made to minimize discomfort, stress, and pain to the fish.

Experimental diets

The two experimental diets were formulated and manufactured by VRM S.r.l. Naturalleva (Verona, Italy). Feeds were prepared using small-scale machinery for mixing ingredients and preparing pellets of 3.0 mm in diameter. The formulation and proximate composition of diets are shown in Tables 1 and 2. The diets were isoenergetic (17.5 MJ kg^{-1}), *isoproteic* (50%), and *isolipidi* c (16%), fully satisfying the gilthead sea bream nutritional demands (Table 2). The control group (CTRL) received a commercial plant-based diet;

Table 1 Formulation (g kg^{-1} diet) of experimental diets.

Ingredient	CTRL	Sh108
Fish meal	280.0	280.0
Corn gluten	220.0	220.0
Guar germ meal	132.0	132.0
Soybean seed meal	120.0	120.0
Wheat middlings	120.0	120.0
Fish oil (94%)	64.5	62.4
Rapeseed oil	44.3	41.4
DL-methionine	4.5	4.5
Lysine hydrochloride	2.7	2.7
Taurine	4.5	4.5
Vitamin C (stay-C 35)	0.6	0.6
Vitamin and mineral premix[a]	7.0	7.0
SILOhealth108	–	5.0

Notes.

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

Table 2 Proximate composition (g kg^{-1} diet) of the experimental diets.

	DIET	
	CTRL	Sh108
Moisture	42.1	42.1
Crude protein	500.0	500.0
Crude lipids	160.0	160.0
Crude fibre	19.6	19.6
NFE	213.3	213.3
Ash	65.0	65.0
DP	403.9	403.9
DE (MJ kg^{-1})	17.5	17.5
DP/DE (g MJ^{-1})	22.9	23.0
EPA	12.3	11.8
DHA	8.2	7.8
$n-3/n-6$	1.3	1.3
DHA/EPA	0.6	0.6

Notes.

NFE, Nitrogen-free extract; DP, digestible protein; DE, digestible energy; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid; $n-3$, omega-3 fatty acids; $n-6$, omega-6 fatty acids.

the treated group (Sh108) received the same control feed but it was supplemented with 0.5% of SILOhealth 108Z commercially available from SILO SpA, Florence, Italy (http://www.silohealth.com/). SILOhealth 108Z is composed of a specific combination of 1-monoglycerides of short- and medium-chain fatty acids (from C3 to C12), in which 1-monobutyrin represents 65% of total blend (Table 3).

Table 3 **Fatty acid composition (%) of SILOhealth 108Z.**

	Fatty acid	Quantity (%)
C3:0	Propionic acid	20
C4:0	Butyric acid	65
C6:0, C7:0, C8:0, C9:0, C12	Blend of caproic, heptanoic, caprylic, lauric acid	15

Table 4 **Growth and feed efficiency indices.** Final mean body weight, specific growth rate (SGR), relative growth rate (RGR), biological feed conversion ratio (bFCR), and economic feed conversion ratio (eFCR) values of sea bream fed with two experimental diets (CTRL and Sh108). The weight data represent the mean value ± SD ($n = 300$ fish/per diet). SGR, RGR, bFCR, and eFCR were tank-based determined ($n = 3$) and reported as mean ± SD. Different letters indicate statistically significant differences between groups (Student's t-test, $P < 0.05$).

Diet	Initial weight	Final weight	SGR (% day^{-1})	RGR (%)	bFCR	eFCR
CTRL	60.56 ± 1.44	126.84 ± 1.90	0.75 ± 0.01	109.49 ± 2.49	1.53 ± 0.05	1.55 ± 0.05^{a}
Sh108	60.50 ± 0.70	129.39 ± 1.12	0.77 ± 0.01	113.88 ± 3.27	1.47 ± 0.01	1.48 ± 0.01^{b}

Fish and feeding trial

Six hundred juvenile gilthead sea bream of about 60 g mean initial body weight (Table 4) were randomly distributed into six fiberglass tanks of 2 m^3 each (100 fish/tank) at the indoor experimental facility of Civita Ittica (Civitavecchia, Italy). The tanks were supplied with filtered sea water (salinity of 37 mg/l) at a temperature and average dissolved oxygen level of 21.2 ± 1.4 °C and 11.7 ± 0.6 mg/l, respectively. Fish were kept under a 12:12 h light:dark photoperiod regimen. Feeding rate was restricted to 2.0% of biomass during the feeding experiment based on four-weekly fish weight measurements. During the experiment that lasted 90 days, fish in triplicate groups (three tanks/diet) were fed with their respective diet twice a day (7:00 am and 4:00 pm) for 6 days per week, except Sunday. Feed consumption (g) in each tank was estimated from the difference between feed delivered into the tank and uneaten feed. Uneaten feed was collected from the bottom of the tank one hour after each meal by siphoning, dried at 70 °C and then weighed. Fish mortality was checked and recorded every day. At the end of the feeding trial, all fish in the tank were individually weighed and measured for their length. Specific growth rate (SGR), relative growth rate (RGR), and biological and economic feed conversion ratio (bFCR and eFCR, respectively) values were calculated. The bFCR is the net amount of feed used to produce one kg of fish, whereas the eFCR considers all the feed used, meaning that the effects of feed losses and mortalities are included (*Robb & Crampton, 2013*).

The each ratio values were calculated using the following formulas:

$$\mathrm{bFCR} = \text{Total feed} / (\text{Final weight}(W_t) + \text{mass mortality}) - \text{Initial weight } (W_0)$$

$$\mathrm{eFCR} = \text{Total feed} / (\text{Final weight}(W_t) - \text{Initial weight } (W_0))$$

$$\mathrm{SGR} = 100 \times (\ln W_t / \ln W_0) / \text{Days}$$

$$\mathrm{RGR} = 100 \times (W_t - W_0) / W_0.$$

The day of fecal sampling, fish were fed at 6:00 am and after 6 h from the last meal, six fish/diet (2 fish/tank) were randomly collected and euthanized with an overdose (320 mg/L at 22 °C) of anesthetic (tricaine-methasulfonate MS-222). To avoid gut content

contamination by the body surface microflora during dissection, external abdominal surface of each fish was wiped thoroughly with a sterile 70° alcohol moistened cotton with an area of 10 cm^2. Then, with the aid of sterile scissors and forceps, the entire intestine (excluding pyloric ceca) was exposed from the ventral side and aseptically removed. The fecal content was obtained by squeezing out and scrapping the intestinal mucosa with a sterile spatula, in order to collect both, the digesta- and the mucosa-associated microbiota. The fecal samples were immediately frozen in dry ice and stored at minus 80 °C until the metagenomics analysis.

Microbial DNA extraction

Two hundred and fifty mg of intestinal content from each fish (12 × 250 mg samples in total) and 200 mg of each dietary pellet (2 × 200 mg samples in total) were processed for DNA extraction using DNeasy PowerSoil Kit (Qiagen, Milan, Italy). The bacterial cells were disrupted via high-speed shaking in plastic tubes with stainless steel beads (TissueLyser II, Qiagen, Milan, Italy) for 2 min at 25 Hz. Total DNA was then extracted according to the manufacturer's instructions. A sample with only lysis buffer was processed in parallel to the biological samples as a negative control to check if external DNA contamination was introduced during the extraction procedure. Bacterial DNA concentration was measured spectrophotometrically by using NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Monza, Italy) and then stored at −20 °C until further processing.

16S rRNA gene library preparation and sequencing

The 16S ribosomal RNA gene library was prepared according to the Illumina protocol "16S Metagenomic Sequencing Library Preparation" (#15044223 rev.B). PCR amplifications of the V3-V4 region of the 16S rRNA gene were carried out in 25-µl reactions containing bacterial DNA (500 ng), buffer (10X), dNTPs (0.2 mM), $MgSO_4$ (1.5 mM), Platinum® Taq DNA Polymerase High Fidelity (1U) (Thermo Fisher Scientific, Monza, Italy), forward primer (5′-CCTACGGGNBGCASCAG-3′), and reverse primer (5′-GACTACNVGGGTATCTAATCC-3′) (400 nM each). The universal primers used were selected by *Takahashi et al. (2014)* and were designed with Illumina adapters at their 5′ end. All the procedure for 16S rRNA gene library preparation and sequencing is described in detail in *Rimoldi et al. (2018)*. However, briefly, PCR cycling conditions for 16S rRNA gene amplification were 94 °C for 1 min, 30 cycles of 94 °C for 30 s, 55 °C for 1 min, and 68 °C for 1.30 min, with a final extension step at 68 °C for 10 min. The resulting size of 16S rRNA gene amplicons was about 550 bp. Dual indices and Illumina sequencing adapters (P5 and P7) were then attached to the amplicons using Nextera XT Index Kit (Illumina, San Diego, CA, USA), according to manufacturer's instructions, to produce the final libraries. Final libraries were quantified by quantitative PCR (qPCR) using KAPA Library Quantification Kits for Illumina® platforms (Kapa Biosystems Ltd., Dorset, UK) and a set of six diluted DNA standards to generate a standard curve. Final libraries were pooled in equimolar amounts, denatured and diluted to 6 pM. Before loading onto the MiSeq flow cell, 15% of the PhiX control library was combined with the amplicon library. Sequencing was performed on an Illumina MiSeq platform using v3 reagent and a 2 × 300

bp paired end protocol, according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

Sequencing raw data analysis

Raw sequences were processed using the open-source bioinformatics pipeline QIIME v1.9.1 (*Caporaso et al., 2010*) by BMR Genomics NGS service (Padova, Italy). Sequences were trimmed using Trimmomatic v0.32. Only reads above 36 nucleotides in length were included in the downstream analysis. The remaining sequences were grouped by diet according to their barcodes. For original amplicon reconstruction, overlapping R1 and R2 paired reads were joined using FLASH v1.2.11 software (http://sourceforge.net/projects/flashpage) and filtered for base quality ($Q > 30$). Amplicons were dereplicated, sorted, and clustered at $\geq$ 97% identity. Amplicon clusters (Operational Taxonomic Units, OTUs) were then identified against reference QIIME-formatted Greengenes database v.13.8 (http://greengenes.lbl.gov) by using QIIME script 'pick_closed_reference_otus.py' and only the OTUs that represented at least 0.005% of total reads were kept. The taxonomical classification was performed down to species level. To determine the abundance of each bacterial taxon, OTUs obtained from each sample were binned according to their consensus sequences, and the final OTU-table output files, in txt and biom format, were created using 'summarize_taxa_through_plots.py' custom script. OTUs assigned to the phylum *Cyanobacteria* (class *Chloroplast*) were removed from the analysis as potential plant contaminants, as described in *Rimoldi et al. (2018)*. Reads of mitochondrial or eukaryotic origin were also excluded.

Alpha and beta diversity statistics were performed as described in *Rimoldi et al. (2018)*. Alpha diversity metrics were calculated based on a rarefied OTU table using 'observed species', 'Chao1 index' (species richness estimator), 'Shannon's diversity index', 'Good's coverage', and 'PD whole tree'. OTUs diversity among sample communities (beta diversity) was assessed by applying weighted (presence/absence/abundance matrix) and unweighted (presence/absence matrix) UniFrac distance matrices (*Lozupone & Knight, 2005*; *Lozupone et al., 2007*). The distance matrices were visualized by principal coordinate analysis (PCoA) three-dimensional plots.

The common core microbiome (OTUs shared, regardless of the diet, and found in at least five out of the six samples per dietary group) was identified using the 'compute_core_microbiome.py' script. The Venn diagrams representing the results of the core microbiota were drawn using the web tool http://bioinformatics.psb.ugent.be/webtools/Venn/.

Statistics

All data were presented as means $\pm$ standard deviation. The number of reads across samples was normalized by sample size and the relative abundance (%) of each taxon was calculated. Only those taxa with an overall abundance of more than 1% (up to order) and more than 0.5% at family and genus level were considered for statistical analysis. Before being statistically analysed, the resulting microbial profiles were calculated as the angular transformation (arcsine of the square root). All data were tested for normality and

homogeneity of variances by Shapiro–Wilk's and Levene's test, respectively. Differences between two groups were analysed by unpaired Student's *t*-test or non-parametric Mann–Whitney U test, depending if the data were or not normal distributed. Welch's *t*-test was used instead of Student's *t*-test when variances were unequal between groups. Statistical significance was set at $P < 0.05$. Correction of multiple testing was done using Benjamini–Hochberg False Discovery Rate (FDR) method with a false discovery rate (Q) set to 0.20. All analyses were performed using Past3 software (*Hammer, Harper & Ryan, 2001*). To verify the significance of differences in the beta diversity of bacterial communities, analysis of similarities (ANOSIM), and permutational multivariate analysis of variance (adonis function) were performed with 999 permutations. Both tests were accomplished using QIIME script 'compare_categories.py'.

RESULTS

Fish growth performance and feeding conversion

During the 90 days of the feeding trial, the mortality rate was lower than 1%. Specifically, two fish of CTRL and four fish of Sh108 group died during the first week of feeding trial, with no further mortalities recorded for the rest of the test. Fish growth performance indexes such as SGR, and RGR did not reveal any significant differences between control and SILOhealth 108Z-supplemented dietary groups, meaning that all fish grew efficiently, regardless of the fatty acid monoglycerides supplementation. At the end of the feeding trial, all fish doubled their body mass reaching a final mean body weight of 126.84 ± 1.90 g, and 129.39 ± 1.12 g in CTRL and Sh108 group, respectively. On the contrary, economic FCR differed between two groups, resulting lower in fish fed diet Sh108 (Table 4).

Characterization of microbial communities of the diets

Bacterial communities associated to feeds were analysed using the QIIME pipeline, which revealed that the two microbial profiles were qualitatively and quantitatively equivalent. After filtering for quality, trimming length, and generating consensus lineages, the number of reads taxonomically classified according to the Greengenes database was 47,791 and 44,483 for CTRL and Sh108 diet, respectively. The total number of OTUs at 97% identity found in CTRL and Sh108 feed samples amounted to 193 and 188, respectively. The overall amount of reads of eukaryotic origin was around 70%. The microbial profiles of feed samples at the phylum, family, and genus taxonomic level are reported in Figs. 1A–1C. The most abundant bacterial taxa (relative abundance >1%) were mainly comprised of 3 phyla, four classes, six orders, seven families, eight genera, and eight species (Figs. 1A–1C; Dataset S1).

QIIME data analysis and taxonomic characterization of gut microbiome

The twelve fecal samples were processed via Illumina MiSeq platform and analysed using the QIIME pipeline. During bioinformatics analysis process, two CTRL samples were discarded following OTU-picking step, due to their inadequate number of sequences. The total number of reads taxonomically classified according to the Greengenes database was

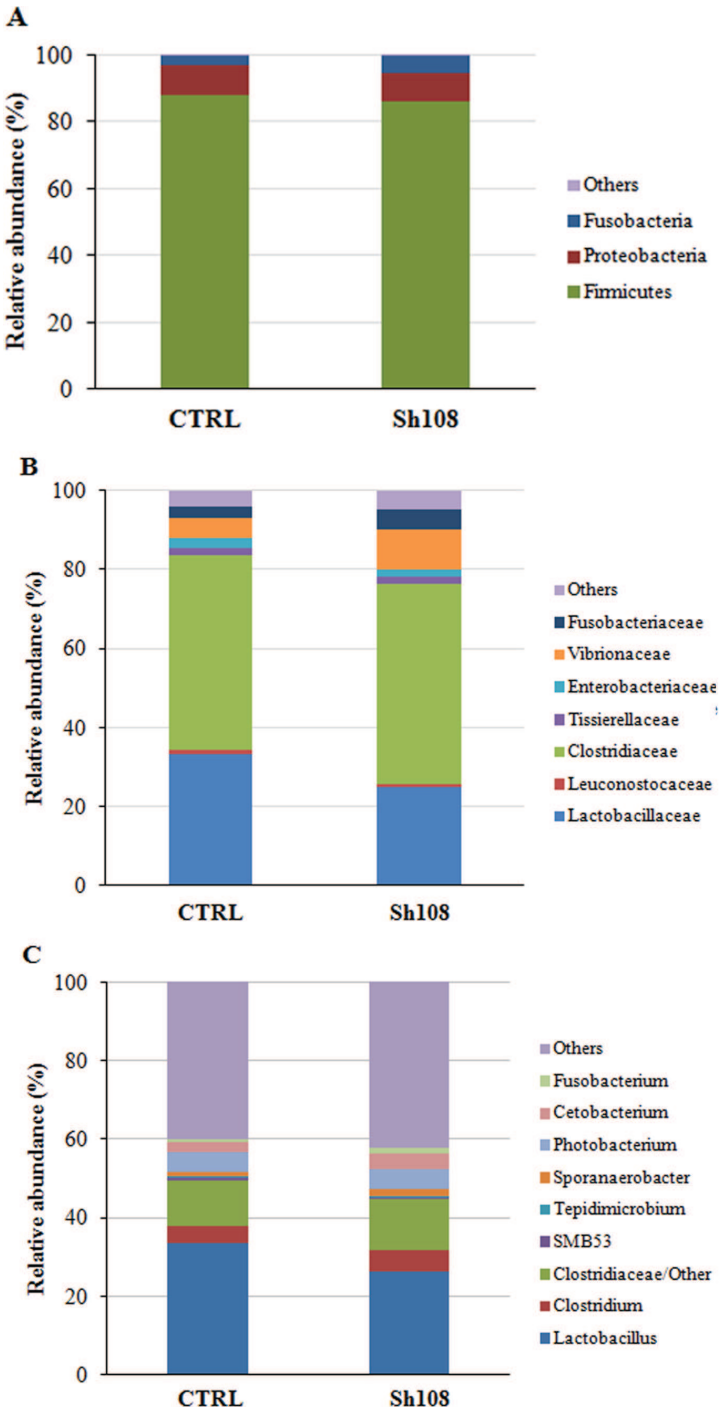

Figure 1 Bacterial relative abundance (%) in the feeds. The amount (%) of the most prevalent bacteria in CTRL and Sh108 feeds at (A) phylum; (B) family, and (C) genus level. Only bacteria with an overall abundance of $\geq$ 1% (at genus level) and $\geq$ 0.5% (at family and genus level), were reported. Bacteria with lower abundance were pooled and indicated as "Others".

Full-size DOI: 10.7717/peerj.5355/fig-1

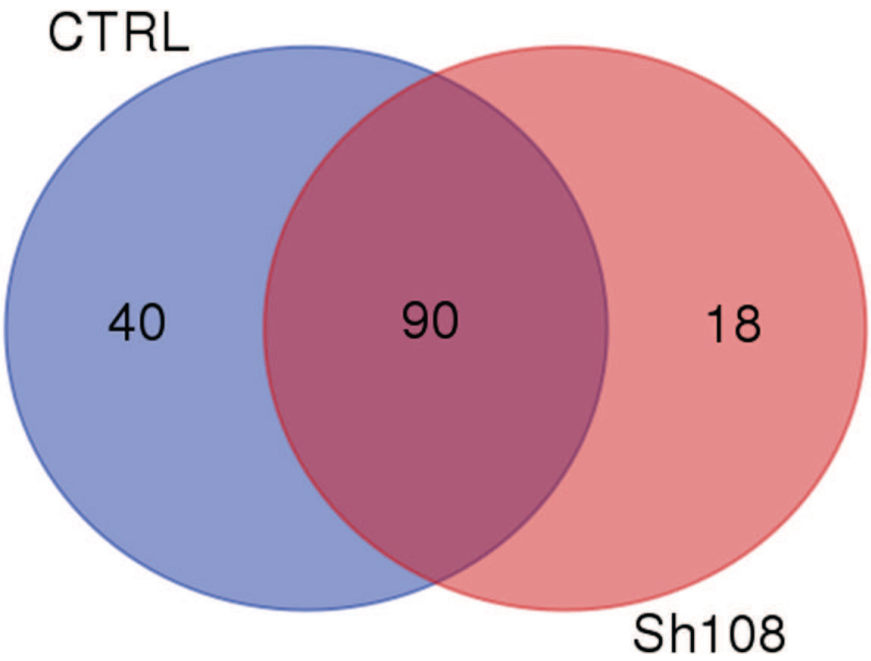

Figure 2 Intestinal core microbiota. Venn diagram representing unique and shared OTUs between fish of the CTRL and Sh108 dietary groups.

Full-size DOI: 10.7717/peerj.5355/fig-2

Table 5 Alpha diversity results of gut microbiota of seabream fed two tested diets. Number of reads per sample assigned to OTUs, and alpha diversity metrics values (normalized at the lowest sample size: 20,052 reads) of gut microbial community of gilthead sea bream fed CTRL ($n = 4$) or Sh108 ($n = 6$) diets for 90 days. Data are expressed as means ± SD. Different letters indicate statistically significant differences between groups (Student's t-test, $P < 0.05$).

Diet	Reads	Observed species	Good's coverage	PD Whole tree	Chao1	Shannon
CTRL	26,828 ± 7,248[b]	160 ± 19	0.99 ± 0.0	13.8 ± 1.0	172 ± 19	3.3 ± 0.7
Sh108	47,883 ± 9,482[a]	154 ± 24	0.99 ± 0.0	13.6 ± 1.9	172 ± 21	2.4 ± 0.7
Total number of reads taxonomically classified						394,611
Mean number of reads/sample						39,461 ± 13,626
Total number of OTUs						259

394,611, which corresponded to an average number of 39,461 ± 13,626 reads per sample (Table 5). Sequences of eukaryotic origin were 51% of total reads. Sequencing data were exported as individual fastq files and deposited in the European Nucleotide Archive (EBI ENA) under the accession code: PRJEB25441.

We identified 259 OTUs at 97% identity in sea bream fecal samples (Dataset S2). Ninety OTUs constituted the core gut microbiota, i.e., those OTUs found in at least three out of the four control samples and at least five out of the six Sh108 samples (or OTUs present in at least 75% of fecal samples) and shared, regardless of the diet (Fig. 2). Among these, 43 OTUs were common to 100% of samples, showing a dominance of *Firmicutes* (26 OTUs) (Dataset S3). Good's coverage values for both dietary groups were >0.99, indicating that sequencing coverage was attained and that the OTUs found in the samples were

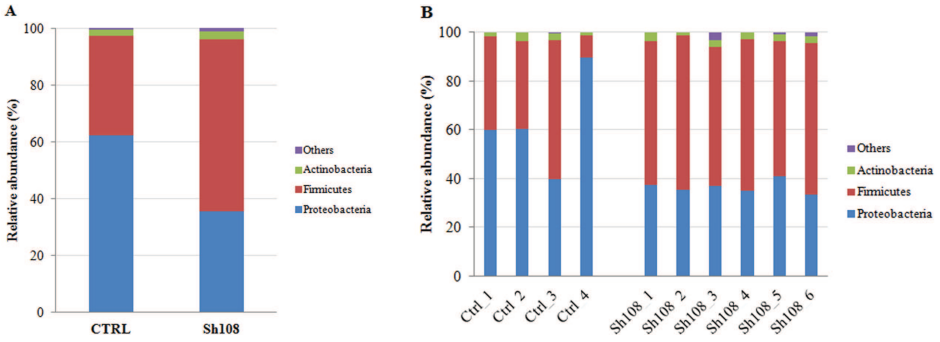

Figure 3 Relative abundance (%) of the overall most prevalent bacterial phyla in the gut of (A) all, and (B) individual fish fed with CTRL and Sh108 diets. All bacteria with an overall abundance of ≥ 1% were reported. Bacteria with lower abundance were pooled and indicated as "Others".

Full-size DOI: 10.7717/peerj.5355/fig-3

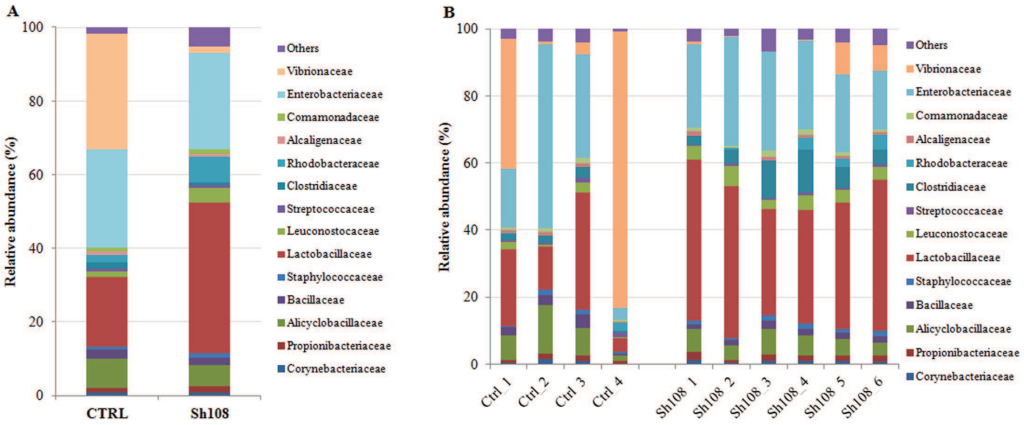

Figure 4 Relative abundance (%) of the overall most prevalent bacterial families in the gut of (A) all, and (B) individual fish fed with CTRL and Sh108 diets. All bacteria with an overall abundance of ≥ 0.5% were reported. Bacteria with lower abundance were pooled and indicated as "Others".

Full-size DOI: 10.7717/peerj.5355/fig-4

representative of the whole population (Table 5). The whole microbial community profile of samples, excluding reads from eukaryotic origin, was successfully outlined, resulting in nine phyla, 14 classes, 25 orders, 44 families, 75 genera, and 38 species (Dataset S2). However, only taxa with an overall abundance of more than 1% (at the phylum, class, and order level) and more than 0.5% (at family and genus level) were considered for statistical analysis. The mean relative abundance changes at species level between groups were not considered to be informative since the number of unassigned sequences was remarkable (74-92%) and they were consequently excluded from analysis. Therefore, considering only the most abundant taxa, the overall gut microbial community was comprised of three phyla, six classes, eight orders, 14 families, 12 genera, and 13 species. The profiles of intestinal microbial communities for each dietary group and individual fish are presented at the phylum (Figs. 3A, 3B), family (Figs. 4A, 4B), and genus (Figs. 5A, 5B) level.

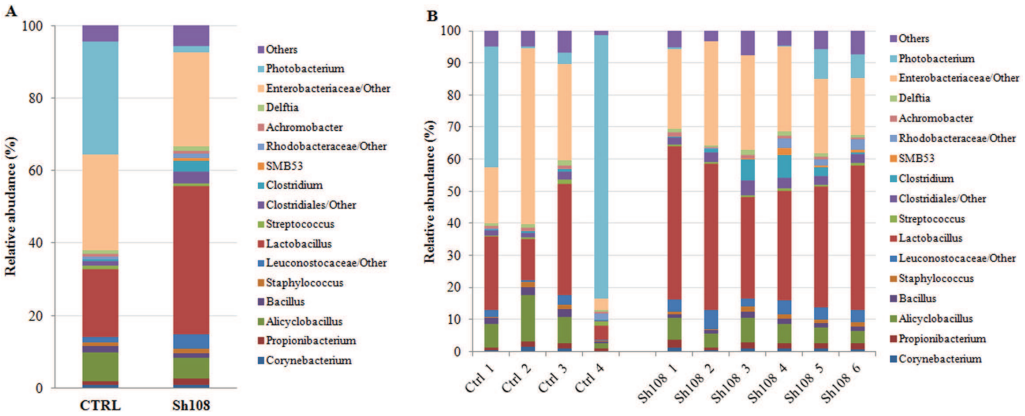

Figure 5 Relative abundance (%) of the overall most prevalent bacterial genera in the gut of (A) all, and (B) individual fish fed with CTRL and Sh108 diets. All bacteria with an overall abundance of $\geq 0.5\%$ were reported. Bacteria with lower abundance were pooled and indicated as "Others".

Full-size DOI: 10.7717/peerj.5355/fig-5

Different α-diversity metrics were applied, including observed species count, phylogenetic diversity (PD Whole tree), and Chao1 and Shannon indices. All the rarefaction curves, normalized to the sample with the lowest number of sequences (20,052 reads), tended to plateau (Figs. S1A–S1C). As reported in Table 5, neither of the indices of diversity and species richness was affected by adding of SILOhealth 108Z to the diet. In particular, Shannon diversity index reached a stable value in all samples, indicating that bacterial diversity in these communities was mostly covered and did not differ between the two experimental groups. Only the number of reads was significantly higher in Sh108 samples compared to control.

Analysis of intestinal microbiome changes in response to different diets

To understand the between-group differences, the mean relative abundances of individual taxa were compared and the results are reported in Table 6. *Firmicutes*, *Proteobacteria* and *Actinobacteria* represented the dominant phyla in both experimental groups (Fig. 3A). Among them, amount of *Firmicutes* and *Proteobacteria* were significantly influenced by dietary monoglycerides supplementation. Our data revealed that the relative abundance of *Firmicutes* was significantly higher (60.64 ± 1.63%) in fish fed with diet Sh108 than in fish fed the control diet (35.11 ± 19.63%) (Table 6). In contrast, fish fed the control diet were characterized by a higher percentage of bacteria assigned to *Proteobacteria* phylum (62.38 ± 20.50%) than fish receiving diet Sh108 (35.60 ± 1.63%) (Table 6). *Bacilli* and *Gammaproteobacteria* classes were dominant in both dietary groups. However, fewer *Gammaproteobacteria* were found in the group Sh108 (28.41 ± 3.01%) than in the control group (58.63 ± 20.88%) (Table 6). In the same fish, at order level, a higher percentage of *Lactobacillales* was found. The increased proportion of *Lactobacillales* was due to a significant enrichment in bacteria belonging to *Lactobacillaceae* (40.90 ±7.41%) and *Leuconostocaceae* (4.15 ± 1.21%) families in comparison to the control group (Fig. 4A,

Table 6 Mean relative abundance (%) ± SD of the most prevalent bacterial phyla, classes, orders, families, and genera found in fecal samples of gilthead sea bream fed with two tested diets.

	CTRL	Sh108	*P*-value	Benjamini Hochberg *P*-value
Phylum				
Actinobacteria	2.14 ± 1.09	2.68 ± 0.78	0.413	0.591
Firmicutes	35.11 ± 19.63	60.64 ± 1.63	0.021	0.135
Proteobacteria	62.38 ± 20.50	35.60 ± 1.63	0.022	0.135
Class				
Actinobacteria	2.16 ± 1.10	2.80 ± 0.89	0.367	0.591
Bacilli	33.01 ± 18.52	55.25 ± 6.51	0.039	0.209
Clostridia	2.47 ± 1.54	7.60 ± 4.67	0.069	0.211
Alphaproteobacteria	1.11 ± 0.92	2.21 ± 2.11	0.339	0.591
Betaproteobacteria	2.07 ± 0.85	2.53 ± 1.18	0.531	0.671
Gammaproteobacteria	58.63 ± 20.98	28.41 ± 3.01	0.014	0.135
Order				
Actinomycetales	2.16 ± 1.10	2.80 ± 0.89	0.367	0.591
Bacillales	11.80 ± 7.18	9.52 ± 2.00	0.513	0.668
Lactobacillales	21.21 ± 14.52	45.73 ± 8.07	0.014	0.135
Clostridiales	2.47 ± 1.54	7.60 ± 4.67	0.069	0.211
Rhodobacterales[a]	0.64 ± 1.18	1.73 ± 2.13	0.241	0.545
Burkholderiales	1.95 ± 0.77	2.12 ± 0.96	0.792	0.874
Enterobacteriales	26.72 ± 21.86	26.19 ± 5.56	0.959	0.959
Vibrionales[a]	31.53 ± 38.20	1.78 ± 3.22	0.066	0.211
Family				
Corynebacteriaceae	0.75 ± 0.58	0.83 ± 0.34	0.820	0.874
Propionibacteriaceae	1.15 ± 0.44	1.75 ± 0.59	0.138	0.364
Alicyclobacillaceae	7.98 ± 5.33	5.70 ± 1.66	0.389	0.591
Bacillaceae	2.5 ± 1.42	1.88 ± 0.33	0.363	0.591
Staphylococcaceae	0.88 ± 0.67	1.23 ± 0.48	0.402	0.591
Lactobacillaceae	18.75 ± 13.23	40.90 ± 7.41	0.015	0.135
Leuconostocaceae	1.55 ± 1.34	4.15 ± 1.21	0.018	0.135
Streptococcaceae	0.89 ± 0.55	0.65 ± 0.18	0.446	0.599
Clostridiaceae	1.94 ± 1.16	7.02 ± 4.63	0.068	0.211
Rhodobacteraceae[a]	0.64 ± 1.18	1.73 ± 2.13	0.241	0.545
Alcaligenaceae	0.90 ± 0.32	0.85 ± 0.43	0.834	0.874
Comamonadaceae	1.00 ± 0.43	1.14 ± 0.53	0.689	0.822
Enterobacteriaceae	26.72 ± 21.86	26.18 ± 5.55	0.958	0.959
Vibrionaceae[a]	31.29 ± 38.13	1.75 ± 3.20	0.066	0.211
Genus				
Corynebacterium	0.75 ± 0.58	0.83 ± 0.34	0.820	0.874
Propionibacterium	1.15 ± 0.44	1.75 ± 0.59	0.138	0.364
Alicyclobacillus	7.98 ± 5.33	5.70 ± 1.66	0.389	0.591

(*continued on next page*)

Table 6 (*continued*)

	CTRL	Sh108	*P*-value	Benjamini Hochberg *P*-value
Bacillus	1.78 ± 0.89	1.34 ± 0.31	0.333	0.591
Staphylococcus	0.86 ± 0.65	1.18 ± 0.51	0.439	0.599
Lactobacillus	18.73 ± 13.20	40.86 ± 7.36	0.014	0.135
Streptococcus	0.89 ± 0.55	0.62 ± 0.15	0.400	0.591
Clostridium	0.39 ± 0.24	3.09 ± 3.33	0.144	0.364
SMB53	0.11 ± 0.08	0.70 ± 0.93	0.258	0.554
Achromobacter	0.83 ± 0.31	0.77 ± 0.38	0.802	0.874
Delftia	0.95 ± 0.38	1.11 ± 0.52	0.629	0.822
Photobacterium[a]	31.04 ± 38.02	1.74 ± 3.20	0.066	0.211

Notes.

Significance of the differences ($P < 0.05$) was obtained by Student's *t*-test or non-parametric Mann-Whitney *U* test (a) depending on normal distribution of data. Benjamini-Hochberg FDR method was applied for multiple test correction with Q set to 0.20.

Table 6). Accordingly, the number of bacteria assigned to the *Lactobacillus* genus was significantly higher in Sh108 samples (Fig. 5A, Table 6). At the species level, the number of unassigned bacteria was sizeable, more than 90% for Sh108 group and around 70% for control, thus making a comparison between the two groups meaningless at this taxonomical level. However, although the percentage of unassigned sequences was remarkable at this taxonomical level, the only species of *Lactobacillus* identified, namely *L. agilis,* was found at a higher percentage in fish receiving Sh108 diet than in control group (0.15%).

Beta diversity metrics of gut bacterial communities

QIIME pipeline 'beta_diversity_trough_plots.py' was used to compute microbial beta diversity metrics; both weighted and unweighted UniFrac analyses were performed. Sample UniFrac distances were visualized using principal coordinate analysis (PCoA) onto a three-dimensional plot (Figs. 6A, 6B). Unweighted PCoA showed no sharp separation between samples, which clustered together regardless of the diet (Fig. 6A). Contrariwise, weighted PCoA revealed a clear clustering of samples by diet and principal coordinates PC1 and PC2 together explained 93% of the variation between individuals (Fig. 6B). The permutational multivariate analysis Adonis totally confirmed the PCoA plots results, revealing a significant difference in microbial communities of gut microbiota between the two groups (F Model $= 7.92$, $P = 0.02$; $R^2 = 0.49$). The R^2 value, from Adonis test, indicated that the sample grouping explained the 49% of the variation in distances. Similarly, ANOSIM test was significant only for weighted Unifrac distance matrix ($P = 0.01$; $R = 0.58$), indicating that the divergences between samples were due more to differences in bacterial abundance rather than to the presence or absence of specific taxa. Results of multivariate analysis are summarized in Fig. 6.

DISCUSSION

We tested a specific mix of 1-monoglycerides of short- and medium-chain organic acids (SILOhealth 108Z) in the diet of gilthead sea bream, to determine the effects on fish intestinal microbiota and growth performance. This product is a synergic combination

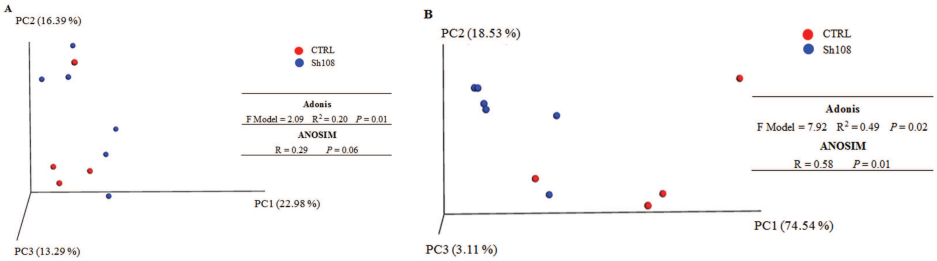

Figure 6 Beta diversity metrics. Principal Coordinate Analysis of (A) Unweighted, and (B) Weighted Unifrac distances of gut microbial communities associated to two experimental diets. Each dot represents an individual sample plots according to its microbial profile at genus level. Results of Permutational multivariate analysis of variance (adonis function) and Analysis of similarity (ANOSIM) are reported next to the PCoA plot to which they are referred. Significance was set at $P < 0.05$.

Full-size DOI: 10.7717/peerj.5355/fig-6

of short and medium chain 1-monoglycerides (from C3 to C12), particularly rich in monobutyrin. It has been widely demonstrated that butyrate, despite being the least abundant of the three-primary gastrointestinal SCFAs (acetate, propionate and butyrate), exerts important protective and anti-inflammatory functions in the gut of several fish species, ultimately enhancing gut health and improving fish performance (*Benedito-Palos et al., 2016*; *Liu et al., 2014*; *Terova et al., 2016*; *Rimoldi et al., 2016*). These previous, promising results prompted the idea that, as feed additive, butyric acid monoglycerides, could represent an effective strategy to improve fish growth performance, feed conversion, and disease resistance by promoting the establishment of a healthy intestinal microbiota. Indeed, esterification with glycerol protect butyric acid from being absorbed in the upper part of the digestive system targeting its release in the deeper tracts of intestine where butyrate would exert its major functions.

Use of monoglycerides as feed additive has been widely investigated in poultry (*Bedford & Gong, 2018*; *Yang et al., 2018*; *Jahanian & Golshadi, 2015*; *Leeson et al., 2005*). On the contrary, research dealing with their use in aquaculture is very scarce to date, despite the increasing commercial interest in the use of SCFAs and MCFAs in aquafeeds for farmed fish species. In this perspective, our findings represent a first contribution which could help to fill this knowledge gap.

We tested a dietary inclusion level of 0.5% for SILOhealth 108Z. This inclusion level was chosen based on studies conducted in Pacific white shrimp (*Penaeus vannamei*) and white sturgeon (*Acipenser transmontanus*) that were recently presented at some aquaculture conferences by *Parini & Paoli (2016)*, and *Parini (2016)*. The authors of these studies reported that the inclusion of 0.5% of SILOhealth 108Z in shrimp feed increased SGR and improved FCR, whereas in sturgeon infected with *Aeromonas hydrophila*, the addition of 0.8% of SILOhealth 108Z to the diet, improved fish growth performance, and increased the survival rate. However, considering that no bacterial challenge was planned in our study for gilthead sea bream, a nutritional dosage of 0.5% of SILOhealth 108Z was decided to be included in the diet of this species.

The dietary supplementation of 0.5% SILOhealth 108Z did not significantly improve fish growth performance. However, even if not significant, SGR mean value of fish receiving Sh108 diet showed an improvement of 3% in comparison to control fish. Interestingly, even if the biological FCR did not differ between two groups, the economic FCR value was lower (improved) in fish fed with Sh108 diet. The eFCR is a very strong tool for farmers and feed companies to monitor the performance of feeds as it takes into account not only the nutritional value of the feed, but also the health status of the fish (*Robb & Crampton, 2013*). Indeed, factors well outside the control of the feed quality, such as fish disease and mortalities, can strongly affect eFCR and in order to reduce (improve) the eFCR, farmers should follow a series of corrective actions as described in *Robb & Crampton (2013)*.

Similarly to the present study, no consistent effects in growth rates were observed in rainbow trout (*Gao et al., 2011*), European sea bass (*Terova et al., 2016*; *Rimoldi et al., 2016*) or gilthead sea bream fed dietary butyrate (*Benedito-Palos et al., 2016*). On the other hand, a diet supplemented with medium-chain fatty acids in the form of a sodium salt of coconut fatty acid distillate enhanced the overall feed intake and growth rates of sea bream (*Simó-Mirabet et al., 2017*). As suggested by *Ng & Koh (2011)*, in addition to the amount of organic acid included in the diet, various factors may influence fish growth, including organic acid type, fish species and age, diet composition, and farming condition, which could explain these apparently conflicting and inconsistent results reported in literature.

A precious contribution to our understanding of the controversial mechanism of action of organic acids could come from studies of fish gut microbiota. Recently, the advent of next-generation sequencing (NGS) technologies has substantially improved our knowledge of changes in the gut microbial ecosystem in fish, in response to a variety of factors, including diet. To the best of our knowledge, this study represents the first investigation on the effects of dietary 1-monoglycerides on gut bacterial community of gilthead sea bream. In agreement with previous metagenomics studies conducted on the same fish species, our results indicated that *Firmicutes* and *Proteobacteria* were the most dominant phyla of the gut microbiome regardless of the diet (*Parma et al., 2016*; *Estruch et al., 2015*). Similarly, *Piazzon et al. (2017)* found a dominance of *Proteobacteria* in intestine of juvenile sea bream unrelated to the diet; however, compared to our findings, the relative abundance of *Firmicutes* was much lower, from 0.5% to 27.9%. This divergence could be related to the fact that Piazzon and colleagues (*2017*) investigated only changes in the autochthonous bacterial community, whereas we considered both the luminal- (allochthonous) and mucosa-associated communities (autochthonous). Actually, *Firmicutes* are generally the dominant phylum of transient microbial community in the distal intestine with a relative abundance of around 70% (*Parma et al., 2016*; *Estruch et al., 2015*).

Although we did not observe an overall effect of 0.5% SILOhealth dietary supplementation on the bacterial richness and diversity, the composition of gut microbiota in terms of relative abundance of specific taxa, was significantly influenced by the dietary treatment. As revealed by weighted UniFrac PCoA of bacterial communities, there was a significant relationship between diet type and microbiota associated to fish intestine. Weighted UniFrac β-diversity measurement showed a clear clustering of samples by diet, statistically validated by ANOSIM and adonis test. Our data revealed that including

SILOhealth 108Z in the diet was associated with a higher *Firmicutes*:*Proteobacteria* ratio than in the control diet, which instead favoured, the presence of *Proteobacteria*. Specifically, adding 1-monoglycerides to the diet induced a twofold increase in intestinal *Firmicutes* relative abundance as compared to the control diet. A similar trend was described in sea bream following butyrate dietary administration (*Piazzon et al., 2017*), but in this case a 139-fold increase with respect to the control diet was registered. The *Firmicutes* phylum includes different genera of lactic acid bacteria such as *Streptococcus*, *Lactobacillus*, and *Leuconostoc*. They are generally thought to be beneficial microorganisms associated with a healthy intestinal epithelium and are often used as probiotics for fish and other vertebrates; therefore, an increase in their number is mostly considered desirable (*Kim, Bhatnagar & Kang, 2012*; *Askarian et al., 2011*; *Ringø& Gatesoupe, 1998*). Moreover, *Firmicutes* include several bacterial genera, which play an important role in degrading otherwise indigestible carbohydrates, such as resistant starch and dietary fiber, thus contributing to a more efficient food energy utilization. In particular, the relative abundance of lactic acid bacteria belonging to the *Leuconostocaceae* and *Lactobacillaceae* families, the latter mainly represented by *Lactobacillus* genus, were positively affected by our tested feed additive. In agreement with our findings, dietary Na-butyrate supplementation increased the abundance of *Lactobacillus* and decreased the number of harmful bacteria *Aeromonas* and *Escherichia coli* in the intestine of grass carp (*Ctenopharyngodon idella*) (*Tian et al., 2017*). Similarly, the lactic acid bacteria, but not the total intestinal bacterial count, significantly increased in common carp fry fed different levels of a blend of SCFAs (*Hoseinifar, Sun & Caipang, 2017*). Furthermore, it has been reported that the supplementation of potassium diformate to plant protein-based diets stimulated the colonization of some lactic acid bacteria in the gut of tilapia (*Oreochromis niloticus*) (*Abu Elala & Ragaa, 2015*) and hybrid tilapia (*Oreochromis niloticus* ♀ × *Oreochromis aureus* ♂) (*Zhou et al., 2009*), whereas butyrate supplementation at 0.4% in a plant-based diet, induced a partial reversion to gut microbial phenotype of fish fed control diet (based on fishmeal and fish oil), with a decrease in *Photobacterium* (*Piazzon et al., 2017*). A similar effect was found in our samples; indeed, two fish of the control group showed very high percentage of this bacterial genus, whereas the relative abundance of *Photobacterium* was definitely less in all samples of Sh108 group. Actually, besides *Firmicutes,* the number of *Proteobacteria*, in particular *Gammaproteobacteria,* was affected by adding SILOhealth 108Z to the diet. Indeed, sea bream fed with Sh108 diet showed a reduced percentage of this taxon in comparison to control group. The dominance of *Proteobacteria* phylum in gut microbiome has been described in several marine carnivorous fish (*Sullam et al., 2012*), including gilthead sea bream (*Kormas et al., 2014*; *Piazzon et al., 2017*; *Estruch et al., 2015*). However, the most abundant *Proteobacteria* harboured in the gut of sea bream from either a wild population or fed conventional fishmeal-based diets, are usually *Betaproteobacteria* (*Desai et al., 2012*) and not *Gammaproteobacteria*, as in the present study. Generally, a high amount of *Gammaproteobacteria* has been associated with vegetable ingredients in the diet (*Piazzon et al., 2017*; *Desai et al., 2012*; *Estruch et al., 2015*). Indeed, the *Gammaproteobacteria* class includes several species of bacteria, belonging, for example, to *Photobacterium* genus, capable to degrade cellulose. However, the *Proteobacteria* phylum includes also many

potential pathogenic genera, such as *Pseudomonas*, the same *Photobacterium*, and *Vibrio*. Therefore, when this phylum represents the dominant clade of intestinal microflora, it might indicate an alteration in the gut microbiota balance. An imbalanced microbiota, could negatively affect the intestinal immune mechanisms, thus contributing to easier development of diseases in fish (*Savas, Kubilay & Basmaz, 2005*). In the present study, 0.5% of organic acid monoglycerides in the diet was sufficient to significantly reduce the amount of *Proteobacteria* in the intestine of gilthead sea bream and, at the same time, to favour the proliferation of *Firmicutes*. Interestingly, Kollanoor and colleagues (*2007*) demonstrated *in vitro* antibacterial activity of caprylic acid (C9) and its monoglyceride that is a component of SILOhealth 108Z blend, against fish pathogens, including *Edwardsiella* species that belong to *Gammaproteobacteria* class. Additionally, low concentrations of SILOhealth 108Z (from 0.01% to 0.1%) inhibited growth of pathogenic bacteria *in vitro*, without inhibiting the beneficial *Lactobacillus plantarum* and *Lactobacillus acidophilus* (*Parini & Paoli, 2016*). This *in vitro* test proved that SILOhealth 108Z selectively exerts antibacterial action against *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Bacillus cereus*, and *Photobacterium damselae*. Accordingly, the inclusion of SILOhealth 108Z in white sturgeon, rohu (*Labeo rohita*) and shrimp diets reduced the mortality caused by pathogenic bacteria *A. hydrophila* and *V. parahaemolyticus* (*Parini, 2016*). The antimicrobial action of SILOhealth 108Z is strictly related to the amphipathic structure of monoglycerides that enables them to interact with cell membranes of several enteric pathogenic bacteria, thus altering membrane integrity and causing inhibition of bacterial growth up to cell death (*Yoon et al., 2018*; *Salsali, Parker & Sattar, 2008*).

In this regard, even *Lactobacilli* could have an active role in host defense against pathogenic bacterial invasion at the intestinal level. It is known that lactic acid bacteria inhibit the growth of pathogens by producing antibacterial compounds, such as lactic acid, hydrogen peroxide, and bacteriocins and by releasing biosurfactants. These are a structurally diverse group of surface-active compounds synthesized by microorganisms and characterized by amphipathic nature. Biosurfactants enhance the solubility of water-insoluble compounds, facilitating their uptake into the cell. They participate in processes such as biofilm formation and defense against other microorganisms by affecting microorganisms' adhesion to different surfaces and exhibiting antibacterial activity. In our study, *L. agilis* was the only species of *Lactobacillus* present in small amounts in fish fed Sh108 diet, but not in fish fed the control diet. Also of interest, it has been recently reported that this bacterial species has the ability to produce a biosurfactant compound, which is a glycoprotein with antimicrobial and anti-adhesive activities that are effective against pathogens such as *Staphylococcus aureus*, *Streptococcus agalactiae* and *Pseudomonas aeruginosa* (*Gudiña et al., 2015*).

CONCLUSIONS

In summary, the present study indicated that there were no differences in growth performance between gilthead sea bream fed the diet supplemented with 0.5% of SILOhealth 108Z and fish fed the control diet. Economic feed conversion ratio (eFCR) was,

instead, significantly improved by dietary administration of 1-monoglycerides. Our findings clearly indicated that SILOhealth 108Z positively modulated the fish intestinal microbiota by increasing the relative abundance of beneficial lactic acid bacteria, namely, *Lactobacillus*. Therefore, the specific composition of 1-monoglycerides of short- and medium-chain fatty acid contained in SILOhealth 108Z has great potential as a feed additive in aquaculture. The present study provides a further confirmation that it possible through diet manipulation to obtain positive effects on gut microbiota, which is known to have a very important role in growth performance, feed conversion, and disease resistance of farmed fish. However, further experiments are needed to elucidate which feed ingredients have the highest impact on changes in the gut microbiota and how these changes can interact with host metabolism.

ACKNOWLEDGEMENTS

Emi Gliozheni and Chiara Ascione are PhD students of the ''Dottorato in Biotecnologie, Bioscienze e Tecnologie chirurgiche'' at the ''Università degli Studi dell'Insubria'', Varese, Italy.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by the AGER project Fine Feed for Fish (4F), Rif. nr. 2016-01-01, and by Silo International S.r.l. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
AGER project Fine Feed for Fish (4F), Rif. nr. 2016-01-01.
Silo International S.r.l.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Simona Rimoldi analyzed the data, authored or reviewed drafts of the paper, approved the final draft.
- Emi Gliozheni performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Chiara Ascione performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Elisabetta Gini analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Genciana Terova conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the indoor experimental facility of Civita Ittica (Civitavecchia, Italy), and in accordance with EU Directive 2010/63/EU for animal experiments. The Committee on the Ethics of Animal Experiments of the same experimental facility approved all of the study protocols [approval n. 120/2008-A of 03/09/2008 (Art.12 of D.Lgs.116/92).

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

Sequencing data has been deposited in European Nucleotide Archive (EBI ENA) under the accession code: PRJEB25441.

Data Availability

The following information was supplied regarding data availability:

All the raw data are included in the Tables, Supplemental Materials and Figshare: Terova, Genciana, and 0000-0002-1995-263x. "Sea Bream Gut Bacterial DNA Sequencing". 25 July 2018. Web. 26 July 2018. DOI: 10.6084/m9.figshare.5858310.v1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.5355#supplemental-information.

REFERENCES

Abu Elala NM, Ragaa NM. 2015. Eubiotic effect of a dietary acidifier (potassium diformate) on the health status of cultured Oreochromis niloticus. *Journal of Advanced Research* **6**:621–629 DOI 10.1016/j.jare.2014.02.008.

Anuta JD, Buentello A, Patnaik S, Lawrence AL, Mustafa A, Hume ME, Gatlin DM, Kemp MC. 2011. Effect of dietary supplementation of acidic calcium sulfate (Vitoxal) on growth, survival, immune response and gut microbiota of the pacific white shrimp, Litopenaeus vannamei. *Journal of the World Aquaculture Society* **42(6)**:834–844 DOI 10.1111/j.1749-7345.2011.00519.x.

Askarian F, Kousha A, Salma W, Ringø E. 2011. The effect of lactic acid bacteria administration on growth, digestive enzyme activity and gut microbiota in Persian sturgeon (Acipenser persicus) and beluga (Huso huso) fry. *Aquaculture Nutrition* **17**:488–497 DOI 10.1111/j.1365-2095.2010.00826.x.

Bedford A, Gong J. 2018. Implications of butyrate and its derivatives for gut health and animal production. *Animal Nutrition* **4(2)**:151–159 DOI 10.1016/j.aninu.2017.08.010.

Benedito-Palos L, Ballester-Lozano GF, Simó P, Karalazos V, Ortiz Á, Calduch-Giner J, Pérez-Sánchez J. 2016. Lasting effects of butyrate and low FM/FO diets

on growth performance, blood haematology/biochemistry and molecular growth-relatedmarkers in gilthead sea bream (*Sparus aurata*). *Aquaculture* **454**:8–18 DOI 10.1016/j.aquaculture.2015.12.008.

Bergsson G, Arnfinnsson J, Steingrímsson Ó, Thormar H. 2001. Killing of gram-positive cocci by fatty acids and monoglycerides. *APMIS* **109**:670–678 DOI 10.1034/j.1600-0463.2001.d01-131.x.

Bjerkeng B, Storebakken T, Wathne E. 1999. 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. *Aquaculture Nutrition* **5(3)**:181–191 DOI 10.1046/j.1365-2095.1999.00103.x.

Canani RB, Costanzo MD, Leone L, Pedata M, Meli R, Calignano A. 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World Journal of Gastroenterology* **17**:1519–1528 DOI 10.3748/wjg.v17.i12.1519.

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7**:335–336 DOI 10.1038/nmeth.f.303.

Da Silva BC, Vieira FN, Mouriño JLP, Bolivar N, Seiffert WQ. 2016. Butyrate and propionate improve the growth performance of *Litopenaeus vannamei*. *Aquaculture Research* **47(2)**:612–623 DOI 10.1111/are.12520.

Da Silva BC, Vieira F do N, Mouriño JLP, Ferreira GS, Seiffert WQ. 2013. Salts of organic acids selection by multiple characteristics for marine shrimp nutrition. *Aquaculture* **384–387**:104–110.

De Schryver P, Sinha AK, Kunwar PS, Baruah K, Verstraete W, Boon N, De Boeck G, Bossier P. 2010. Poly- β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. *Applied Microbiology and Biotechnology* **86(5)**:1535–1541 DOI 10.1007/s00253-009-2414-9.

Desai AR, Links MG, Collins SA, Mansfield GS, Drew MD, Van Kessel AG, Hill JE. 2012. Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **350–353**:134–142.

Estruch G, Collado MC, Peñaranda DS, Tomás Vidal A, Jover Cerdá M, Pérez Martínez 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 DOI 10.1371/journal.pone.0136389.

Food and Agriculture Organization (FAO). 2014. The state of world fisheries and aquaculture. Rome, 223. In: FAO Fisheries and Aquaculture Department [online]. *Available at www.fao.org/3/a-i3720e.pdf* .

Francis G, Makkar HPS, Becker K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture* **199(3–4)**:197–227 DOI 10.1016/S0044-8486(01)00526-9.

Gálfi P, Neogrády S. 2001. The pH-dependent inhibitory action of n-butyrate on gastrointestinal epithelial cell division. *Food Research International* **34(7)**:581–586 DOI 10.1016/S0963-9969(01)00075-8.

Gao Y, Storebakken T, Shearer KD, Penn M, Øverland M. 2011. Supplementation of fishmeal and plant protein-based diets for rainbow trout with a mixture of sodium formate and butyrate. *Aquaculture* **311(1–4)**:233–240 DOI 10.1016/j.aquaculture.2010.11.048.

Gatlin DM, Barrows FT, Brown P, Dabrowski K, Gaylord TG, Hardy RW, Herman E, Hu G, Krogdahl Å, Nelson R, Overturf K, Rust M, Sealey W, Skonberg D, Souza EJ, Stone D, Wilson R, Wurtele E. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research* **38(6)**:551–579 DOI 10.1111/j.1365-2109.2007.01704.x.

Gudiña EJ, Fernandes EC, Teixeira JA, Rodrigues LR. 2015. Antimicrobial and anti-adhesive activities of cell-bound biosurfactant from Lactobacillus agilis CCUG31450. *RSC Advances* **5**:90960–90968 DOI 10.1039/C5RA11659G.

Guilloteau P, Martin L, Eeckhaut V, Ducatelle R, Zabielski R, Van Immerseel F. 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. *Nutrition Research Reviews* **23(2)**:366–384 DOI 10.1017/S0954422410000247.

Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer R-J. 2008. Review article: the role of butyrate on colonic function. *Alimentary Pharmacology & Therapeutics* **27(2)**:104–119.

Hammer Ø, Harper DAT, Ryan PD. 2001. PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4(1)**:Article 4.

Hismiogullari SE, Hismiogullari AA, Sahin F, Toksoy OE, Yenice S, Karasartova D. 2008. Investigation of antibacterial and cytotoxic effects of organic acids including ascorbic acid, lactic acid and acetic acids on mammalian cells. *Journal of Animal and Veterinary Advances* **7(6)**:681–684.

Hoseinifar SH, Sun YZ, Caipang CM. 2017. Short-chain fatty acids as feed supplements for sustainable aquaculture: an updated view. *Aquaculture Research* **48(4)**:1380–1391 DOI 10.1111/are.13239.

Jahanian R, Golshadi M. 2015. Effect of dietary supplementation of butyric acid glycerides on performance, immunological responses, ileal microflora, and nutrient digestibility in laying hens fed different basal diets. *Livestock Science* **178**:228–236 DOI 10.1016/j.livsci.2015.05.038.

Kim SK, Bhatnagar I, Kang KH. 2012. Development of marine probiotics: prospects and approach. *Advances in Food and Nutrition Research* **65**:353–362 DOI 10.1016/B978-0-12-416003-3.00023-8.

Kollanoor A, Vasudevan P, Nair MKM, Hoagland TH, Venkitanarayanan K. 2007. Inactivation of bacterial fish pathogens by mediumchain lipid molecules (caprylic acid, monocaprylin and sodium caprylate). *Aquaculture Research* **38**:1293–1300 DOI 10.1111/j.1365-2109.2007.01799.x.

Kormas KA, Meziti A, Mente E, Frentzos A. 2014. Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). *Microbiology Open* **3(5)**:718–728 DOI 10.1002/mbo3.202.

Leeson S, Namkung H, Antongiovanni M, Lee EH. 2005. Effect of butyric acid on the performance and carcass yield of broiler chickens. *Poultry Science* **84**:1418–1422 DOI 10.1093/ps/84.9.1418.

Lim CC, Lückstdt CD, Webster P, Kesius. 2015. Organic acids and their salts. In: Lee CS, Lim C, Gatlin DM, Webster CD, eds. *Dietary nutrients, additives and fish health*. Hoboken: Wiley-Blackwell, 305–315.

Liu W, Yang Y, Zhang J, Gatlin DM, Ringo E, Zhou Z. 2014. Effects of dietary microencapsulated sodium butyrate on growth, intestinal mucosal morphology, immune response and adhesive bacteria in juvenile common carp (*Cyprinus carpio*) pre-fed with or without oxidised oil. *British Journal of Nutrition* **112(1)**:15–29 DOI 10.1017/S0007114514000610.

Lozupone CA, Hamady M, Kelley ST, Knight R. 2007. Quantitative and qualitative β diversity measures lead to different insights into factors that structure microbial communities. *Applied and Environmental Microbiology* **73**:1576–1585 DOI 10.1128/AEM.01996-06.

Lozupone C, Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Applied and Environmental Microbiology* **71**:8228–8235 DOI 10.1128/AEM.71.12.8228-8235.2005.

Lückstädt C. 2008. The use of acidifiers in fish nutrition. CAB reviews. *Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* **3**:Article 44 DOI 10.1079/PAVSNNR20083044.

Mátis G, Neogrády Z, Csikó G, Kulcsár A, Kenéz Á, Huber K. 2013. Effects of orally applied butyrate bolus on histone acetylation and cytochrome P450 enzyme activity in the liver of chicken—a randomized controlled trial. *Nutrition & Metabolism* **10(1)**:Article 12 DOI 10.1186/1743-7075-10-12.

Namkung H, Yu H, Gong J, Leeson S. 2011. Antimicrobial activity of butyrate glycerides toward salmonella typhimurium and clostridium perfringens. *Poultry Science* **90(10)**:2217–2222 DOI 10.3382/ps.2011-01498.

Naylor LR, Goldburg J, Primavera HJ, Kautsky N, Beveridge MCM, Clay J, Folke C, Lubchenco J, Mooney H, Troell M. 2000. Effect of aquaculture on world fish supplies. *Nature* **405**:1017–1024 DOI 10.1038/35016500.

Naylor RL, Hardy RW, Bureau DP, Chiu A, Elliott M, Farrell AP, Forster L, Gatlin DM, Goldburg RJ, Hua K, Nichols PD. 2009. Feeding aquaculture in an era of finite resources. *Proceedings of the National Academy of Sciences* **106**:15103–15110 DOI 10.1073/pnas.0905235106.

Ng WK, Koh CB. 2011. Application of organic acids in aquafeeds: impacts on fish growth, nutrient utilization and disease resistance. In: Lückstädt C, ed. *Standards for acidifiers—principles for the use of organic acids in animal nutrition. Proceeding of the 1st international acidifier summit*. Nottingham: Nottingham University Press, 49–58.

Ng WK, Koh CB. 2017. The utilization and mode of action of organic acids in the feeds of cultured aquatic animals. *Reviews in Aquaculture* **9**:342–368 DOI 10.1111/raq.12141.

Parini M. 2016. Species independent efficacy of a specific composition of Monoglycerides of short and medium chain fatty acids in preventing EMS and controlling

Vibrio parahaemolyticus, *Aeromonas spp.*, *Streptococcus uberis*, *Flavobacterium spp.*, *Yersinia ruckery*, *Pseudomonas spp* and *Bacillus cereus* affecting shrimps and other aquatic species. In: *Book of abstracts: 2nd Global Summit on Aquaculture & Fisheries*. Kuala Lumpur: OMICS International.

Parini M, Paoli A. 2016. Efficacy of a specific composition of 1-monoglycerides of short- and medium-chain fatty acids in controlling *Aeromonas salmonicida*, *Yersinia ruckeri*, *Flavobacterium psychrophilum* in salmonids and *Vibrio parahaemolyticus* and *Vibrio harveyi* affecting shrimp. In: *Book of abstracts: Aquaculture Europe 2016*. Edinburgh: OMICS International.

Parma L, Candela M, Soverini M, Turroni S, Consolandi C, Brigidi P, Mandrioli L, Sirri R, Fontanillas R, Gatta PP, 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 DOI 10.1016/j.anifeedsci.2016.10.022.

Penn MH, Bendiksen EA, Campbell P, Krogdahl AS. 2011. High level of dietary pea protein concentrate induces enteropathy in Atlantic salmon (Salmo salar L.). *Aquaculture* **310(3–4)**:267–273 DOI 10.1016/j.aquaculture.2010.10.040.

Piazzon MC, Calduch-Giner JA, Fouz B, Estensoro I, Simó-Mirabet P, Puyalto M, Karalazos V, Palenzuela O, Sitjà-Bobadilla A, Pérez-Sánchez J. 2017. Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiome* **5**:Article 164 DOI 10.1186/s40168-017-0390-3.

Rimoldi S, Finzi G, Ceccotti C, Girardello R, Grimaldi A, Ascione C, Terova G. 2016. Butyrate and taurine exert a mitigating effect on the inflamed distal intestine of European sea bass fed with a high percentage of soybean meal. *Fisheries and Aquatic Sciences* **19(1)**:1–14 DOI 10.1186/s41240-016-0002-3.

Rimoldi S, Terova G, Ascione C, Giannico R, Brambilla F. 2018. Next generation sequencing for gut microbiome characterization in rainbow trout (*Oncorhynchus mykiss*) fed animal byproduct meals as an alternative to fishmeal protein sources. *PLOS ONE* **13(3)**:e0193652 DOI 10.1371/journal.pone.0193652.

Ringø E, Gatesoupe FJ. 1998. Lactic acid bacteria in fish: a review. *Aquaculture* **160(3–4)**:177–203.

Ringø E, Zhou Z, Vecino JLG, Wadsworth S, Romero J, Krogdahl Å, Olsen RE, Dimitroglou A, Foey A, Davies S, Owen M, Lauzon HL, Martinsen LL, Schryver DE, Bossier P, Sperstad S, Merrifield DL. 2016. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquaculture Nutrition* **22**:219–282 DOI 10.1111/anu.12346.

Robb DHF, Crampton VO. 2013. On-farm feeding and feed management: perspectives from the fish feed industry. On-farm feeding and feed management in aquaculture. In: Hasan MR, New MB, eds. *FAO Fisheries and Aquaculture Technical Paper No. 583*. Rome: FAO, 489–518.

Robles R, Lozano AB, Sevilla A, Márquez L, Nuez-Ortín W, Moyano F. 2013. Effect of partially protected butyrate used as feed additive on growth and intestinal

metabolism in sea bream (*Sparus aurata*). *Fish Physiology and Biochemistry* **39(6)**:1567–1580 DOI 10.1007/s10695-013-9809-3.

Salsali H, Parker WJ, Sattar SA. 2008. The effect of volatile fatty acids on the inactivation of Clostridium perfringens in anaerobic digestion. *World Journal of Microbiology and Biotechnology* **24(5)**:659–665 DOI 10.1007/s11274-007-9514-4.

Sampugna J, Quinn JG, Pitas RE, Carpenter DL, Jensen RG. 1967. Digestion of butyrate glycerides by pancreatic lipase. *Lipids* **2(5)**:397–402 DOI 10.1007/BF02531853.

Santigosa E, García-Meilañ I, Valentín MV, Navarro I, Pérez-Sańchez J, Gallardo MA. 2011. Plant oils' inclusion in high fish meal-substituted diets: effect on digestion and nutrient absorption in gilthead sea bream (*Sparus aurata* L.). *Aquaculture Research* **42**:962–974 DOI 10.1111/j.1365-2109.2010.02679.x.

Savas S, Kubilay A, Basmaz N. 2005. Effect of bacterial load in feeds on intestinal microflora of seabream (*Sparus aurata*) larvae and juveniles. *Israeli Journal of Aquaculture* **57**:3–9.

Simó-Mirabet P, Piazzon MC, Calduch-Giner JA, Ortiz Á, Puyalto M, Sitjà-Bobadilla A, Pérez Sánchez J. 2017. Sodium salt medium-chain fatty acids and Bacillus-based probiotic strategies to improve growth and intestinal health of gilthead sea bream (*Sparus aurata*). *PeerJ* **5**:e4001 DOI 10.7717/peerj.4001.

Skřivanová E, Molatová Z, Skřivanová V, Marounek M. 2009. Inhibitory activity of rabbit milk and medium-chain fatty acids against enteropathogenic Escherichia coli O128. *Veterinary Microbiology* **135(3–4)**:358–362 DOI 10.1016/j.vetmic.2008.09.083.

Sullam KE, Essinger SD, Lozupone CA, O'Connor MP, Rosen GL, Knight R, Kilham SS, Russell JA. 2012. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Molecular Ecology* **21**:3363–3378 DOI 10.1111/j.1365-294X.2012.05552.x.

Sun CQ, O'Connor CJ, Roberton AM. 2003. Antibacterial actions of fatty acids and monoglycerides against Helicobacter pylori. *FEMS Immunology and Medical Microbiology* **36**:9–17 DOI 10.1016/S0928-8244(03)00008-7.

Tacon AGJ, Metian M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: trends and future prospects. *Aquaculture* **285(1–4)**:146–158 DOI 10.1016/j.aquaculture.2008.08.015.

Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. 2014. Development of a prokaryotic universal primer for simultaneous analysis of bacteria and archaea using next-generation sequencing. *PLOS ONE* **9(8)**:e105592 DOI 10.1371/journal.pone.0105592.

Terova G, Díaz N, Rimoldi S, Ceccotti C, Gliozheni E, Piferrer F. 2016. Effects of sodium butyrate treatment on histone modifications and the expression of genes related to epigenetic regulatory mechanisms and immune response in European Sea Bass (*Dicentrarchus Labrax*) fed a plant-based diet. *PLOS ONE* **11(7)**:1–20.

Thormar H, Hilmarsson H, Bergsson G. 2006. Stable concentrated emulsions of the 1-monoglyceride of capric acid (monocaprin) with microbial activities against the food-borne bacteria *Campylobacter jejuni*, *Salmonella spp.* and *Escherichia coli*. *Applied and Environmental Microbiology* **72**:522–526 DOI 10.1128/AEM.72.1.522-526.2006.

Tian L, Zhou X-Q, Jiang W-D, Liu Y, Wu P, Jiang J, Kuang S-Y, Tang L, Tang xx, Zhang Y-A, Xie F, Feng L. 2017. Sodium butyrate improved intestinal immune function associated with NF-kB and p38MAPK signaling pathways in young grass carp (*Ctenopharyngodon idella*). *Fish & Shellfish Immunology* **66**:548–563 DOI 10.1016/j.fsi.2017.05.049.

Van Immerseel F, De Buck J, Pasmansa F, Velge P, Bottreau E, Fievez V, Haesebrouck F, Ducatelle R. 2003. Invasion of Salmonella enteritidis in avian intestinal epithelial cells in vitro is influenced by short-chain fatty acids. *International Journal of Food Microbiology* **85**:237–248 DOI 10.1016/S0168-1605(02)00542-1.

Van Immerseel F, Fievez V, De Buck J, Pasmans F, Martel A, Haesebrouck F, Ducatelle R. 2004. Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with salmonella enteritidis in young chickens. *Poultry Science* **83**:69–74 DOI 10.1093/ps/83.1.69.

Vinolo MAR, Rodrigues HG, Nachbar RT, Curi R. 2011. Regulation of inflammation by short chain fatty acids. *Nutrients* **3(10)**:858–876 DOI 10.3390/nu3100858.

Wang J, Wu X, Simonavicius N, Tian H, Ling L. 2006. Medium-chain fatty acids as ligands for orphan G protein-coupled receptor GPR84. *Journal of Biological Chemistry* **281**:34457–34464 DOI 10.1074/jbc.M608019200.

Wong JMW, De Souza R, Kendall CWC, Emam A, Jenkins DJA. 2006. Colonic health: fermentation and short chain fatty acids. *Journal of Clinical Gastroenterology* **40(3)**:235–243 DOI 10.1097/00004836-200603000-00015.

Yang X, Yin F, Yang Y, Lepp D, Yu H, Ruan ZH, Yang CH, Yin Y, Hou Y, Leeson S, Gong J. 2018. Dietary butyrate glycerides modulate intestinal microbiota composition and serum metabolites in broilers. *Scientific Reports* **8**:4940 DOI 10.1038/s41598-018-22565-6.

Yoon BK, Jackman JA, Valle-González ER, Cho N-J. 2018. Antibacterial free fatty acids and monoglycerides: biological activities, experimental testing, and therapeutic applications. *International Journal of Molecular Sciences* **19(4)**:Article 1114 DOI 10.3390/ijms19041114.

Zhang J-X, Guo L-Y, Feng L, Jiang W-D, Kuang S-Y, Liu Y, Hu K, Jiang J, Shu-Hong L, Tang L, Xiap-Qiu Z. 2013. Soybean b-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish. *PLOS ONE* **8(3)**:e58115 DOI 10.1371/journal.pone.0058115.

Zhou Z, Liu Y, He S, Shi P, Gao X, Yao B, Ringo E. 2009. Effects of dietary potassium diformate (KDF) on growth diformate (KDF) on growth performance, feed conversion and intestinal bacterial community of hybrid tilapia (Oreochromis niloticus x O. aureus). *Aquaculture* **291**:89–94 DOI 10.1016/j.aquaculture.2009.02.043.

3. DISCUSSION

The finfish and crustacean aquaculture sectors are still highly dependent upon marine capture fisheries for sourcing key dietary nutrient inputs, including fish meal (FM) and fish oil (FO). Based on increasing global FM and FO costs, it is predicted that dietary FM and FO inclusion levels within compound aquafeeds will decrease in the long term. FM and FO are thus being increasingly targeted as a high value specialty feed ingredients for use within higher value starter, finisher and broodstock feeds. Commercial feed producers have been trying to replace FM by using alternative protein sources such as vegetable proteins meals (VMs) (Tacon et al., 2008). Even though VMs can replace a substantial part of the FM, they have several limitations due to unbalanced amino acid profiles, high fiber and antinutritional factors content, and competition with use for human consumption (Hardy, 2010). Anti-nutritional factors, such as saponins, lectins, phytate, trypsin inhibitors, phenols, and tannins could damage the intestinal tract thus reducing nutrient absorption and fish growth. Indeed, studies on Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhychus mykiss*), common carp (*Cyprinus carpio*), and gilthead sea bream (*Sparus aurata*) have indicated that the inclusion of less refined plant products such as soybean meal (SBM) in the diet triggers an inflammation process in the distal intestine, referred to as SBM- induced enteropathy (Baeverfjord and Krogdahl, 1996; Olli and Krogdahl, 1994; van den Ingh et al., 1991; Knudsen et al., 2008; Urán, et al., 2008a,b; Venou et al., 2006). Although the provoking mechanism remains to be established, the SBM-induced enteritis is believed to be caused by a disruption of the intestinal barrier, with subsequent exposure of otherwise shielded layers of the mucosa to luminal components, including food-derived and microbial antigens (Romarheim et al., 2011). The typical signs of such inflammation are a

shortening of the primary and secondary intestinal mucosal folds, an increase in the number of Goblet cells, and the infiltration of inflammatory cells, particularly macrophages and eosinophilic granulocytes into the lamina propria. This reduces the capacity of the enterocytes lining the epithelium to absorb nutrients (Baeverfjord and Krogdahl, 1996; van den Ingh et al., 1991; Buttle et al., 2001). These effects proved to be dose–dependent in Atlantic salmon; the most prevalent symptoms were observed at the highest SBM inclusion level (30%), but even diets containing as low as 7.6% SBM induced morphological changes in the intestine (Krogdahl, 2003).

It has been documented that the inflammatory effects caused by SBM are not derived from the soy protein but from other components present in the SBM, such as saponin, in combination with at least one more unidentified components (van den Ingh et al., 1991; Baeverfjord and Krogdahl 1996; Bakke-Mckellep et al., 2000; Krogdahl et al., 2003; Knudsen et al., 2008). The effects of replacement of FM with VM, often accompanied by reduced fish performance, are not restricted to SBM inclusion solely, but have been observed after inclusion of many other plant protein sources in several teleost species such as gilthead sea bream, turbot, Atlantic cod, and parrot fish (Gomez-Requeni et al., 2004; Sitja-Bobadilla et al., 2005; Yun et al., 2011; Hansen et al. 2007; Lim and Lee 2009). Baeza-Ariño et al. (2014) described liver and gut alterations of gilthead sea bream, *S. aurata* L., fed diets in which FM was replaced by a mixture of rice and pea protein concentrates. The results of the histological analysis showed significant changes in the case of the 90% substitution in parameters such as thickness of the gut layers, number of Goblet cells and villi's length and thickness, whereas the integrity of the gut structure was not significantly affected by a diet with up to 60% of replacement. In some cases, severe vacuolization

was encountered, which consequently deformed enterocytes and displaced the nucleus.

The short chain fatty acid of butyrate, may promote the healing of inflamed intestine through its major role in enhancing epithelial cell proliferation and differentiation and in improving the intestinal absorptive function (Canani et al., 2012; Gálfi and Neogrády 2002; Wong et al., 2006).

Like other short chain or volatile fatty acids (acetic, propionic, valeric, and caproic), butyric acid is produced during the fermentation of dietary fibers by the anaerobic microbiota associated with the epithelium of the animals' digestive tract. In addition to being the main respiratory fuel source of the intestinal cells, and preferred to glucose or glutamine, this four-carbon chain organic acid molecule has potential immunomodulatory and anti-inflammatory properties (Vinolo et al., 2009; Toden et al., 2007; Terova et al., 2016), and exert multiple other beneficial effects on host energy metabolism (Hamer et al., 2008; Den Besten et al., 2013; Liu et al., 2014; da Silva et al., 2016). Although the mechanisms underlying these effects are still enigmatic and subject of intense scrutiny, it is believed that they encompass the complex interplay between diet, gut microbiota, and host energy metabolism.

However, much of the research on butyrate has been focused on terrestrial vertebrates, including humans whilst very few studies have been conducted in fish. In particular, little is known about the effects of butyrate used as a feed additive on fish intestinal integrity. In terrestrial farmed animals such as pig and chicken, butyrate included in the diet has had a positive influence on body weight gain, feed utilization, and composition of intestinal microflora. It exerted trophic effects on the intestinal epithelium through an increase in the villi length and crypt depth, too (Gálfi and Bokori, 1990; Kotunia et al., 2004; Hu and Guo, 2007). In fish, Robles et al. (2013)

reported an effect of butyrate used as a feed additive in increasing the availability of several essential amino acids and nucleotide derivatives, which have been demonstrated to increase fish growth when they were added individually to the diet.

It is widely accepted that butyrate functions as an epigenetic regulator through its histone deacetylase (HDAC) inhibition activity (Myzak et al., 2006, Dashwood et al., 2006). Studies confirmed that butyrate induces profound changes in gene expression related to multiple signal pathways and genomic networks in bovine cells (Li et al., 2007). However, epigenetic modification such as histone acetylation induced by butyrate is part of a multilevel regulatory machinery.

In human, butyrate is also used as a dietary micronutrient and HDAC inhibitor in the challenge of preventing and treating colonorectal tumors (Davie et al., 2003). However, the efficacy of butyrate as a chemotherapeutic agent has been limited by its rapid uptake and metabolism by normal cells (resulting in a half-life of 6 min and peak blood levels below 0.05 mM (Miller et al., 1987) before reaching tumors (Pouillart, 1998). More stable butyrate derivatives such as tributyrin have also not been successful on a consistent basis (Pouillart, 1998).

Li (2006) reported that butyrate induces profound changes in the expression of at least 450 genes in bovine kidney epithelial cells (Li et al. 2006). Such a tremendous effect would only be possible through chromatin remodelling where histones are involved. However, further research is needed to better understand the involvement of histone modifications in the regulation of chromatin structure and gene expression, and to identify the nature of metabolic pathways that are controlled by nutrition through epigenetic mechanisms (Delage et al. 2008).

Currently, there is a strong interest in the use of organic acids and their salts as feed additives since such products seem to have growth-promoting effects in livestock. Their positive effects are well documented in terrestrial livestock production (Hu Z et al. 2007, Øverland M et al. 2000, Øverland M et al. 2008, Lückstädt C. 2008), but some questions remain regarding their efficacy in fish farming, and conflicting reports exist on the subject. Indeed, growth was significantly enhanced in some fish species, such as rainbow trout (*Oncorhynchus mykiss*), when fed an organic acid blend supplement mainly consisting of formate and sorbate (De Wet L. 2005), but not in trout fed other commercial supplements such as lactic acid (Pandey A. et al. 2008) or citric acid (Pandey A. et al. 2008, Vielma J. At al. 1999).

In our experiment, the results of the 8-week-long feeding trial showed no significant differences in weight gain and SGR of sea bass that received 0.2% sodium butyrate supplementation in the diet in comparison to control fish that received a diet without Na-butyrate.

Butyrate in the feed significantly increased the acetylation state of histone H4 at lysine 8, leading to a twofold increase in comparison to the control group, but no changes were found in the acetylation of histone H3 at Lys9. Interestingly, for histone H3 two different isoforms were separated on the immunoblots, which could correspond to H3.1 and H3.2 isoforms previously found in terrestrial animals.

Concerning gene expression, butyrate applied as a nutritional supplement caused significant changes *in vivo* in the expression of genes related to epigenetic regulatory mechanisms such as *hdac11*, *ehmt2*, and *dicer1*. Statistical analysis for these genes showed significant differences due to the butyrate treatment and to the interaction between tissue and treatment. The expression of four (*il1β*, *il8*, *irf1,* and *tnfα*) out of seven target genes

related to mucosal protection and inflammatory response was significantly different between the two analyzed tissues but only for the *il10* gene showed differences associated with the butyrate treatment. Our results in intestine showed a decrease in hdac11 expression and a slight increase in *il10* levels. This suggests that, in butyrate-treated fish, antigen-specific T-cell responses could be impaired, which probably activates immune tolerance. *Hdac11* has also been related to the immune system by down-regulating the expression of *il10* in antigen-presenting cells. Overexpression of *hdac11* is thought to inhibit il10 expression and activate T-cell responses.

Gene transcript abundance analysis in this study clearly showed tissue-dependent differences in the expression of five target genes involved in epigenetic regulatory mechanisms (Piferrer, 2013); the expression was in general, higher in the liver than in the intestine. As previously found in European sea bass reared in different temperatures (Díaz et al. 2015), three of target genes (*dicer1*, *ehmt2*, and *hdac11*) exhibited increased expression in the liver as a consequence of butyrate treatment, suggesting that these genes are involved in physiological processes in charge of coping with external challenges.

The Dicer1 family is known to participate in the innate immune response to pathogens, mainly in RNA silencing-based antiviral immunity (Aliyari et al. 2009, Chiappinelli et al. 2012). Indeed, studies in the past twenty years have established a completely new RNA-based immune system against viruses that is mechanistically related to RNA silencing or RNA interference. This viral immunity begins with recognition of viral double-stranded or structured RNA by the Dicer nuclease family of host immune receptors. Moreover, *dicer1* knockdown experiments showed an increase in the interferon response against pathogens (Chiappinelli et al. 2012).

Although our results showed a slight increase in the expression of *irf1*, a higher expression of *dicer1* was also observed in the liver, suggesting that in butyrate-treated fish dicer 1 was inhibiting an interferon response against the external insult.

In our study, we revealed some of the effects of butyrate supplementation. This information is essential for the development of plant-based diets in the efforts to improve the sustainability of the aquaculture of many species, carnivorous in particular.

In the second part of the study, a specific mix of 1-monoglycerides of short- and medium-chain organic acids (SILOhealth 108Z) was tested in the diet of gilthead sea bream (*Sparus aurata*), to determine the effects on fish intestinal microbiota and growth performance. SILOhealth 108Z is a synergic combination of short and medium chain 1-monoglycerides (from C3 to C12), particularly rich in monobutyrin.Butyrate, despite being the least abundant of the three-primary gastrointestinal SCFAs (acetate, propionate and butyrate), exerts important protective and anti-inflammatory functions in the gut of several fish species, ultimately enhancing gut health and improving fish growth performance (Benedito-Palos et al., 2015; Liu et al., 2014; Terova et al., 2016; Rimoldi et al., 2016). An abundant butyrate-producing gut microbial community is essential for a well-functioning intestine (Van Immerseel et al., 2010).

Many researches are currently focusing on the gut microbiota in relation to its influence on parameters such as hosts' health status, metabolism and generally a wide range of biological processes (Navarrete et al., 2012; Rawls et al., 2004; Semova et al., 2012).

The recent introduction of next-generation sequencing (NGS) technologies has substantially improved our knowledge of gut microbial ecosystem in fish, in response to a variety of environmental factors, including diet. To

the best of our knowledge, this study represents the first investigation on the effects of dietary 1-monoglycerides of short- and medium-chain fatty acids on fish gut bacterial community.

In agreement with previous metagenomics studies conducted on the same fish species (gilthead sea bream) to investigate the effects of diet on gut microbiome, our results indicated that *Firmicutes* and *Proteobacteria* were the most dominant phyla of the gut microbiome regardless of the diet (Parma et al., 2016; Estruch et al., 2015). Similarly, Piazzon et al, (2017) found a dominance of *Proteobacteria* in the intestine of juvenile sea bream unrelated to the diet; however, in our findings, the relative abundance of *Firmicutes* was 0.5%, much lower in comparison to the previous study where they detected 27.9%. This divergence could be related to the fact that Piazzon and colleagues (2017) investigated only changes in the autochthonous bacterial community, whereas our study considered both the luminal- (allochthonous) and mucosa-associated communities (autochthonous). Indeed, *Firmicutes* are generally the dominant phylum of transient microbial community in the distal intestine with a relative abundance of around 70% (Parma et al., 2016; Estruch et al., 2015).

Although we did not observe an overall effect of 0.5% SILOhealth dietary supplementation on the bacterial richness and diversity, the composition of the gut microbiota in terms of relative abundance (% of bacteria in a specific taxa compared to the total number of detected bacteria) of specific taxa, was significantly influenced by the dietary treatment. This was in line with recent literature reporting that, although the microbiota composition of cultured fish is very resistant to diet changes, dietary variations were associated with changes in the relative abundance of *Lactobacillaceae*, *Streptococcus*, *Staphylococcaceae,* and *Clostridiales* (Wong et al., 2013).

The results of another study by Ingerslev et al., (2014) showed that despite the observed plasticity in the bacterial composition during the period around first feeding, it was evident that on a taxonomic scale, the microbiota was dominated by four phyla; *Bacteroidetes*, *Proteobacteria*, *Firmicutes*, and *Actinobacteria* . In two earlier investigations in rainbow trout, it was indicated that the four phyla constitute the 'core' microbiota after first feeding regardless of whether the feed has a marine or a plant based origin (Desai et al., 2012; Navarrete et al., 2012).

Firmicutes phylum includes different genera of lactic acid bacteria such as *Streptococcus*, *Lactobacillus*, and *Leuconostoc*. They are generally considered to be beneficial microorganisms associated with a healthy intestinal epithelium and are often used as probiotics for fish and other vertebrates; therefore, an increase in their number is mostly considered desirable (Kim et al., 2012; Askarian et al., 2011; Ringø and Gatesoupe, 1998). Additionally, low concentrations of SILOhealth 108Z (from 0.01% to 0.1%) inhibited growth of pathogenic bacteria *in vitro*, without inhibiting the beneficial *Lactobacillus plantarum* and *Lactobacillus acidophilus* (Parini & Paoli, 2016). This *in vitro* test proved that SILOhealth 108Z selectively exerts antibacterial action against *Vibrio parahaemolyticus*, *Vibrio mimicus*, *Aeromonas salmonicida*, *Aeromonas hydrophila*, *Bacillus cereus*, and *Photobacterium damselae*.

Our findings clearly indicated that SILOhealth 108Z positively modulated the fish intestinal microbiota by increasing the relative abundance of beneficial lactic acid bacteria, namely, *Lactobacillus*. Therefore, the specific composition of 1-monoglycerides of short- and medium-chain fatty acids contained in SILOhealth 108Z has great potential as a feed additive in aquaculture.

4. CONCLUSIONS

In the present study, we reveal some of the effects of butyrate supplementation. Results of the 8-week-long feeding trial showed no significant differences in weight gain and SGR of sea bass that received 0.2% sodium butyrate supplementation in the diet in comparison to control fish that received a diet without Na-butyrate. Butyrate in the feed significantly increased the acetylation state of histone H4 at lysine 8, leading to a twofold increase in comparison to the control group, but no changes were found in the acetylation of histone H3 at Lys9. Interestingly, for histone H3 two different isoforms were separated on the immunoblots, which could correspond to H3.1 and H3.2 isoforms previously found in terrestrial animals. Concerning gene expression, butyrate applied as a nutritional supplement caused significant changes *in vivo* in the expression of genes related to epigenetic regulatory mechanisms such as hdac11, ehmt2, and dicer1. Statistical analysis by two-way ANOVA for these genes showed significant differences due to the butyrate treatment (P=0.002) and to the interaction between tissue and treatment (P=0.010). The expression of four (il1β, il8, irf1, and tnfα) out of seven target genes related to mucosal protection and inflammatory response was significantly different between the two analyzed tissues but only for the il10 gene the differences observed in the expression (p=0.003) were due to the butyrate treatment. This information is essential for the development of substitution diets in the efforts to improve the sustainability of the aquaculture of carnivorous species. Moreover, the present study indicated that there were no differences in growth performance between gilthead sea bream fed the diet supplemented with 0.5% of SILOhealth 108Z and fish fed the control diet. Economic feed conversion ratio (eFCR) was, instead, significantly improved by dietary administration of 1-monoglycerides. Our findings

clearly indicated that SILOhealth 108Z positively modulated the fish intestinal microbiota by increasing the relative abundance of beneficial lactic acid bacteria, namely, *Lactobacillus*. Therefore, the specific composition of 1-monoglycerides of short- and medium-chain fatty acid contained in SILOhealth 108Z has great potential as a feed additive in aquaculture. The present study provides a further confirmation that it possible through diet manipulation to obtain positive effects on gut microbiota, which is known to have a very important role in growth performance, feed conversion, and disease resistance of farmed fish. However, further experiments are needed to elucidate which feed ingredients have the highest impact on changes in the gut microbiota and how these changes can interact with host metabolism.

ACKNOWLEDGEMENTS

Firstly and most of all, I would like to thank my supervisor Professor Genciana Terova, for her expertise, assistance, guidance and patience through my three years of Phd up to the process of writing this thesis.

I would also like to thank my colleague and brilliant scientist Simona Rimoldi for the scientific support, suggestions and the precious help in laboratory experiments.

I would like to extend my sincere gratitude to the exceptional team of Animal Biotechnology and Aquaculture lab.

I would like to address particular thanks to my father who encouraged, believed in me and helped contribute to this important research project. Without his support, this adventure would not have been possible.

Last of all, thanks to my close friends for keeping me company along the way.

REFERENCES

Aliyari R., Ding S.W., 2009. RNA-based viral immunity initiated by the Dicer family of host immune receptors. Immunology Review. 227(1):176–188.

Allen R.G., Pereira L.S., Raes D., Smith M., 1998. Crop evapotranspiration —guidelines for computing crop water requirements. FAO Irrigation and drainage paper 56. Food and Agriculture Organization, Rome.

Anuta J.D, Buentello A., Patnaik S., Kemp M.C., December 2011. Effect of dietary supplementation of acidic calcium sulfate (vitoxal) on growth, survival, immune response and gut microbiota of the pacific white shrimp, litopenaeus vannamei. Journal of the World Acquaculture Society. Vol. 42, No. 6

Askarian F., Kousha A., Salma W., Ringo E., 2011. The effect of lactic acid bacteria administration on growth, digestive enzyme activity and gut microbiota in Persian sturgeon (*Acipenser persicus*) and beluga (*Huso huso*) fry. Aquaculture Nutrition 17, 488–497.

Auchterlonie N., 2018. The continuing importance of fishmeal and fishoil in aquafeeds. Aquafarm Conference. Pordenone, Italy.

Baeverfjord G., Krogdahl A., 1996. Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar*, distal intestine: a comparison with the intestines of fasted fish. Journal of Fish Diseases. 19 (5), 375–87.

Baeza-Ariño R., Silvia Martínez-Llorens S., Nogales-Mérida S., Jover-Cerda M., Tomás-Vidal. A., 2014. Study of liver and gut

alterations in sea bream, *Sparus aurata*, fed a mixture of vegetable protein concentrates. Aquaculture Research. 1–12.

Bakke-Mckellep M., Mcl Press C., Baeverfjord G., Krogdahl A., Landsverk T., 2000. Changes in immune and enzyme histochemical phenotypes of cell in the intestinal mucosa of Atlantic salmon, *Salmo salar L.,* with soybean meal-induced enteritis. Journal of Fish Diseases. 23, 115–127.

Benedito-Palos, Laura & Ballester-Lozano, Gabriel & Simó, Paula & Karalazos, Vasileios & Ortiz, Alvaro & Calduch-Giner, Josep & Pérez-Sánchez, Jaume., 2015. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). Aquaculture. 454.

Bergman EN., 1990. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Reviews. 70:567-590.

Biancotto C., Frigè G., Minucci S., 2010. Histone modification therapy of cancer. Advances in genetics. 70:341–386.

Bjerkeng B., Storebakken T., Wathne E., 1999. 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. Aquaculture Nutrition. 5:181–191.

Brien J.D., Daffis S., Lazear H.M., Cho H., Suthar M.S., 2011. Interferon regulatory factor-1 (IRF-1) shapes both innate and $CD8^+$ T cell immune responses against west nile virus infection. PLOS Pathogens. 7(9): e1002230.

Brim H., Kumar K., Nazarian J., Hathout Y., Jafarian A., 2011. SLC5A8 gene, a transporter of butyrate: a gut flora metabolite, is frequently methylated in african american colon adenomas. Plos ONE. 6(6): e20216.

Buttle, L. G., Burrells, A.C., Good, J.E., Williams, P.D., Southgate, P.J., Burrells, C., 2001. The binding of soybean agglutinin (SBA) to the intestinal epithelium of Atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss*, fed high levels of soybean meal. Veterinary Immunology and Immunopathology. 80, 237e44.

Canani R.B., Di Costanzo M., Leone L., Pedata M., Meli R., Calignano A., 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World Journal of Gastroenterology 17(12): 1519–1528.

Canani R.B., Di Costanzo M., Leone L., 2012. The epigenetic effects of butyrate: potential therapeutic implications for clinical practice. Clinical Epigenetics. 4(1), 4.

Chiappinelli K.B., Haynes B.C., Brent M.R., Goodfellow P.J., 2012. Reduced DICER1 elicits an interferon response in endometrial cancer cells. Molecular Cancer Research. 10: 316–325.

Cummings J.H., Macfarlane G.T., 1991. The control and consequences of bacterial fermentation in the human colon. Journal of Applied Bacteriology. 70(6):443–459.

da Silva B.C., Vieira F. do N., Mouriño J.L.P., Ferreira G.S., Seiffert W.Q., 2013. Salts of organic acids selection by multiple characteristics for marine shrimp nutrition. Aquaculture. 384–387:104–110.

da Silva B.C., Vieira F.N., Mourino J. L.P., Bolivar N., Seiffert W.Q. 2016. Butyrate and propionate improve the growth performance of *Litopenaeus vannamei*. Aquaculture Research. 47, 612–623.

Dashwood R.H., Myzak M.C., Ho E., 2006. Dietary HDAC inhibitors: time to rethink weak ligands in cancer chemoprevention? Carcinogenesis. 27(2):344–9.

Davie J.R., 2003. Inhibition of histone deacetylase activity by butyrate, The Journal of Nutrition. Volume 133, Issue 7, Pages 2485S–2493S.

De Schryver P., Sinha A.K., Kunwar P.S., Baruah K., Verstraete W., Boon N., Bossier P., 2010. Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus labrax*. Applied microbiology and biotechnology. 86(5), 1535-1541.

De Wet L., 2005. Organic acids as performance enhancers. Aqua Feeds: Formulation and Beyond. 2:12–14.

Delag, B., Dashwood R. H., 2008. Dietary manipulation of histone structure and function. Annual Review of Nutrition. 28, 347–366.

Den Besten G., Van Eunen K., Groen A. K., Venema K., Reijngoud D. J., Bakker B. M., 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of Lipid Research. 54:2325–2340.

Desai, A.R., Links, M.G., Collins, S.A., Manseld, G.S., Drew, M.D., Van Kessel, A.G., Hill, J.E., 2012. Effects of plant-based diets on the

distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 350, 134–142.

Díaz N., Piferrer F., 2015. Lasting effects of early exposure to temperature on the gonadal transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed genetic and environmental sex determination. BMC Genomics. Sep 4;16(1):679.

Emenaker N.J., Calaf G.M., Cox D., Basson M.D., Qureshi N., 2001. Short-chain fatty acids inhibit invasive human colon cancer by modulating uPA, TIMP-1, TIMP-2, mutant p53, Bcl-2, Bax, p21 and PCNA protein expression in an in vitro cell culture model. Journal of Nutrition. 131 (suppl. 11): 3041S–3046S.

Encarnacao, P., 2008. Natural growth promoters: a sustainable solution to improve gut health and performance in aquaculture species. Fish Technology Update. February 2008.

Estruch G., Collado M.C., Peñaranda D.S., Tomás Vidal A., Jover Cerdá M., Pérez Martínez 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.

FAO. 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Rome.

Francis G., Makkar H.P.S., Becker K., 2001. Antinutritional factors present in plant-derived alternative fish feed ingredients and their effect in fish. Aquaculture. 199 ,197-227

Gálfi P., Neogrády S., 2002. The pH-dependent inhibitory action of n-butyrate on gastrointestinal epithelial cell division. Food Research International. 34 ,581–586.

Gálfi, P., Bokori, J., 1990. Feeding trial in pigs with a diet containing sodium n-butyrate. Acta Veterinaria Hungarica. 38(1–2), 3–17.

Ganapathy V., Thangaraju M., Gopal E., Martin P.M., Itagaki S., 2008. Sodium-coupled monocarboxylate transporters in normal tissues and in cancer. American Association of Pharmaceutical Scientists Journal. 10: 193–199.

Gao Y., Storebakken T., Shearer K.D., Penn M., Øverland M., 2011. Supplementation of fishmeal and plant protein-based diets for rainbow trout with a mixture of sodium formate and butyrate. Aquaculture, 311(1–4):233–240.

Gatlin III D.M., Barrows F.T., Brown P., Dabrowski K., Gaylord T.G., Hardy R.W., Herman E., Hu G., Krogdahl S., Nelson R., Overturf K., Rust M., Sealey W., Skonberg D., Souza E.J., Stone D., Wilson R., Wurtele E., 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquaculture Research. 38, 551-579

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.

Gómez G.D., Balcázar J.L., 2008. A review on the interactions between gut microbiota and innate immunity of fish. FEMS Medical Microbiology and Immunology**.** 52, 145–154.

Gomez-Requeni, P., Mingarro, M., Calduch-Giner, J. A., Médale, F., Martin, S. A. M., Houlihan, D. F., Kaushik, S., Pérez-Sánchez, J., 2004. Protein growth performance, amino acid utilization and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture. 232, 493–510.

Guilloteau P., Martin L., Eeckhaut V., Ducatelle R., Zabielski R., Van Immerseel F., 2010. From the gut to the peripheral tissues: the multiple effects of butyrate**.** Nutrition Research Reviews. 23:366–384.

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. Alimentary Pharmacology & Therapeutics. 27:104–119.

Hansen, A. C., Rosenlund, G., Karlsen, O., Koppe, W., Hemre, G. I., 2007. Total replacement of fish meal with plant proteins in diets for Atlantic cod (*Gadus morhua L.*) I – effects on growth and protein retention. Aquaculture. 272, 599–611.

Hardy R.W., 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. Aquacult. Research. 41: 770–776.

Hinnebusch B.F., Meng S., Wu J.T., Archer S.Y., Hodin R.A., 2002. The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. The Journal of Nutrition 132: 1012–1017.

Hismiogullari S.E., Hismiogullari A.A., Sahin F., Toksoy O.E., Yenice S., Karasartova D., 2008. Investigation of antibacterial and cytotoxic effects of organic acids including ascorbic acid, lactic acid and acetic acids on mammalian cells. Journal of Animal and Veterinary Advances. 7(6):681–684.

Hoseinifar S.H., Sun Y.Z., Caipang C.M., 2017. Short-chain fatty acids as feed supplements for sustainable aquaculture: an updated view. Aquaculture Research. 48(4):1380–1391.

Hu Z., Guo Y., 2007. Effects of dietary sodium butyrate supplementation on the intestinal morphological structure, absorptive function and gut flora in chickens. Animal Feed Science and Technology. 132:240–249.

Ingerslev H-C., von Gersdorff Jørgensen L., Strube M.L., Larsen N., Dalsgaard I., Boye M., Madsen L., 2014a. The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type. Aquaculture, 424-425: 24-34.

Jackson and Newton, 2016. Project to model the use of fisheries byproduct in the production of marine ingredients, with special reference to the omega 3 fatty acids EPA and DHA. Institute of Aquaculture, University of Stirling, UK and IFFO, The Marine Ingredients Organisation.

Kim S.-J., Mendis Eresha. 2006. Bioactive compounds from marine processing byproducts - A review. Food Research International. 39. 383-393.

Kim S.K., Bhatnagar I., Kang K.H., 2012. Development of marine probiotics: prospects and approach. Adv. Food & Nutrition Research. 65, 353–362.

Knudsen D., Jutfelt F., Sundh H., Sundell K., Koppe W., Frøkiær H., 2008. Dietary soya saponins increase gut permeability and play a key role in the onset of soyabean-induced enteritis in Atlantic salmon (*Salmo salar L.*). British Journal of Nutrition. 100 (01).

Kotunia A., Wolinski J., Laubitz D., Jurkowska M., Rome V., Guilloteau P., Zabielski R., 2004. Effect of sodium butyrate on the small intestine development in neonatal piglets fed correction of feed by artificial sow. Journal of physiology and pharmacology 55(2), 59–68.

Krogdahl Å., Bakke-McKellep A. M., 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.). Aquaculture Nutrition. 9, 361–371.

Kruh, J. (1982) Effects of sodium butyrate, a new pharmacological agent, on cells in culture. Molecular and Cellular Biochemistry. 42: 65–82.

Leeson S., Namkung H., Antongiovanni M., Lee E.H., 2005. Effect of butyric acid on the performance and carcass yield of broiler chickens. Poultry Science. 84: 1418-1422.

Li C.J., Li R.W., Wang Y.H., Elsasser T.H., 2007. Pathway analysis identifies perturbation of genetic networks induced by butyrate in a bovine kidney epithelial cell line. Functional & Integrative Genomics. 7(3):193–205

Li C.J., Li R.W., Elsasser TH.H., 2010. MicroRNA (miRNA) expression is regulated by butyrate-induced epigenetic modulation of gene expression in bovine cells. Genetics & Epigenetics. 2010:3 23–32

Li H., Gilbert E.R., Zhang Y., Crasta O., Emmerson D., 2008. Expression profiling of the solute carrier gene family in chicken intestine from the late embryonic to early post-hatch stages. Animal Genetics. 39: 407–424.

Li R.W., Li C., 2006. Butyrate induces profound changes in gene expression related to multiple signal pathways in bovine kidney epithelial cells. BMC Genomics. 7: 234.

Lim, C., Lückstädt, C., Webster, C.D., Kesius, P., 2015. Organic acids and their salts. In: Lee, C.S., Lim, C., Gatlin, D.M., Webster, C. (Eds.), Dietary Nutrients, Additives, and Fish Health. Willey-Blackwell, Hoboken, NJ, USA, pp. 305–320.

Lim S.J., Lee K.J., 2009. Partial replacement of fish meal by cottonseed meal and soybean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus*. Aquaculture. 290, 283–289.

Liu W., Yang Y., Zhang J., Gatlin D.M., Ringø E., Zhou Z., 2014. Effects of dietary microencapsulated sodium butyrate on growth, intestinal mucosal morphology, immune response and adhesive bacteria in juvenile common carp (*Cyprinus carpio*) pre-fed with or without oxidized oil. British Journal of Nutrition. 112:15–29.

Llewellyn M.S., Boutin S., Hoseinifar S.H., Derome N., 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Frontiers in Microbiology. 5: 1-17.

Locksley R.M., Killeen N., Lenardo M.J., 2001. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell. 104(4):487–501.

Louis P., Flint H.J., 2009. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiology Letters. 294: 1-8.

Lückstädt C., 2008. The use of acidifiers in fish nutrition. CAB Reviews: Perspectives in Agriculture Veterinary Science. Nutrition and Natural Resources. 3(044):1–8.

Macfarlane G.T., Macfarlane S., 2011. Fermentation in the human large intestine: its physiologic consequences and the potential contribution of prebiotics. The Journal of Clinical Gastroenterology. Nov;45 Suppl:S120-7.

Mallo J.J., Balfagón A., Gracia M.I., Honrubia P., Puyalto M., 2012. Evaluation of different protections of butyric acid aiming for release in the last part of the gastrointestinal tract of piglets. Journal of Animal Science. 90 Suppl 4:227-9.

Maslowski K.M., Mackay C.R., 2010. Diet, gut microbiota and immune responses. Nature Immunology. 12: 5–9.

Mátis G., Neogrády Z., Csikó G., Kulcsár A., Kenéz Á., Huber K., 2013. Effects of orally applied butyrate bolus on histone acetylation and cytochrome P450 enzyme activity in the liver of chicken–a randomized controlled trial. Nutrition & Metabolism. 10:12.

Miller A.A., Kurschel E., Osieka R., Schmidt C.G., 1987. Clinical pharmacology of sodium butyrate in patients with acute leukemia,

European Journal of Cancer and Clinical Oncology. Volume 23, Issue 9, Pages 1283-1287, ISSN 0277-5379.

Miller S.J., 2004. Cellular and physiological effects of short-chain fatty acids. Mini-Reviews in Medicinal Chemistry. 4(8):839–845.

Myzak M.C., Ho E., Dashwood R.H., 2006. Dietary agents as histone deacetylase inhibitors. Molecular Carcinogenesis. 45(6):443–6.

Namkung H., Yu H., Gong J., Leeson S., 2011. Antimicrobial activity of butyrate glycerides toward *Salmonella Typhimurium* and *Clostridium perfringens*. Poultry Science. 90: 2217–2222

Navarrete, P., Magne, F., Araneda, C., Fuentes, P., Barros, L., Opazo, R., Espejo, R., Romero, J., 2012. PCR-TTGE analysis of 16S rRNA from rainbow trout (*Oncorhynchus mykiss*) gut microbiota reveals host-specifc communities of active bacteria. PLoS One. e31335, 1–10.

Nayak S.K., 2010. Role of gastrointestinal microbiota in fish. Aquaculture Research**.** 41: 1553–1573.

Ng W.K., Koh C.B., 2017. The utilization and mode of action of organic acids in the feeds of cultured aquatic animals. Reviews in Aquaculture. 9:342–368.

Olli J.J., Krogdahl Å., 1994. Nutritive value of 4 soybean products as protein sources in diets for rainbow trout (*Oncorhynchus mykiss*, Walbaum) reared in fresh-water. Acta Agriculturae Scandinavica A– Journal of Animal Science. 44, 185–192.

Øverland M., Granli T., Kjos N.P., Fjetland O., Steien S.H., Stokstad M., 2000. Effect of dietary formates on growth performance, carcass traits, sensory quality, intestinal microflora, and stomach

alterations in growing–finishing pigs. Journal of Animal Science. 78:1875–1884.

Øverland M., Kjos N.P., Borg M., Skjerve E., Sørum H., 2008. Organic acids in diets for entire male pigs: effect on skatole level, microbiota in digesta, and growth performance. Livestock Science. 115:169–178.

Owen M.A.G., Waines P., Bradley G., Davies S., 2006. The effect of dietary supplementation of sodium butyrate on the growth and microflora of *Clarias gariepinus* (Burchell, 1822). XII international symposium fish nutrition and feeding. Book of abstracts: pp 149.

Pandey A., Satoh S., 2008. Effects of organic acids on growth and phosphorus utilization in rainbow trout *Oncorhynchus mykiss*. Fisheries Science. 74:867–874.

Parini P., Paoli A., 2016. Selective antibacterial action of specific composition of short- and medium-chain 1-monoglycerides. Aquaculture Asia Pacific. Feed Technology. Vol 12. Nr 3.

Park J.Y., Helm J.F., Zheng W., Ly Q.P., Hodul P.J., 2008. Silencing of the candidate tumor suppressor gene solute carrier family 5 member 8 (SLC5A8) in human pancreatic cancer. Pancreas 36: e32–39.

Park J.Y., Zheng W., Kim D., Cheng J.Q., Kumar N., 2007. Candidate tumo suppressor gene SLC5A8 is frequently down-regulated by promoter hypermethylation in prostate tumor. Cancer Detection and Prevention. 31: 359–365.

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.

Penn M.H., Bendiksen E.Å., Campbell P., Krogdahl Å., 2011. High level of dietary pea protein concentrate induces enteropathy in Atlantic salmon (*Salmo salar L.*). Aquaculture. 310, 267-273.

Piazzon M.C., Calduch-Giner J.A., Fouz B., Estensoro I., Simó-Mirabet P., Puyalto M., Karalazos V., Palenzuela O., Sitjà-Bobadilla A., Pérez-Sánchez J., 2017. Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. Microbiome. 5:164

Piferrer F. 2013. Epigenetics of sex determination and gonadogenesis. Developmental Dynamics. 242:360–370.

Pouillart, P.R., 1998. Role of butyric acid and its derivatives in the treatment of colorectal cancer and hemoglobinopathies. Life Sciences. 63, 1739–1760.

Prasad K.N., Sinha P.K., 1976. Effect of sodium butyrate on mammalian cells in culture: a review. In Vitro. 12: 125–132.

Rada-Iglesias A., Enroth S., Ameur A., 2007. Butyrate mediates decrease of histone acetylation centered on transcription start sites and down-regulation of associated genes. Genome Research. 17(6):708–19.

Rawls J.F., Samuel B.S., Gordon J.I., 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proceedings of the National Academy of Sciences. U. S. A. 101, 4596–4601.

Rimoldi S., Finzi G., Ceccotti C., Girardello R., Grimaldi A., Ascione C., Terova G., 2016. Butyrate and taurine exert a mitigating effect on the inflamed distal intestine of European sea bass fed with a high percentage of soybean meal. Fisheries and Aquatic Sciences. 19(1), 40.

Ringø E., Gatesoupe F.J., 1998. Lactic acid bacteria in fish: a review. Aquaculture. 160:177e203.

Robles R., Lozano A.B., Sevilla A., Márquez L., Nuez-Ortín W., Moyano F.J., 2013. Effect of partially protected butyrate used as feed additive on growth and intestinal metabolism in sea bream (*Sparus aurata*). Fish Physiology and Biochemistry. 39: 1567–1580.

Romarheim O.H., Øverland M., Mydland L.T., Skrede A., Landsverk T., 2011. Bacteria grown on natural gas prevent soybean meal-induced enteritis in Atlantic salmon. Journal of Nutrition. 141, 124–130.

Salsali H., Parker W.J., Sattar S.A., 2008. The effect of volatile fatty acids on the inactivation of Clostridium perfringens in anaerobic digestion. World Journal of Microbiology and Biotechnology. 24(5):659–665.

Sampugna J., Quinn J.G., Pitas R.E., Carpenter D.L., Jensen R.G., 1967 Sep; Digestion of butyrate glycerides by pancreatic lipase. Lipids. 2(5):397-402.

Santigosa E., García-Meilań I., Valentín M.V., Navarro I., Pérez-Sańchez J., Gallardo M.A., 2011. Plant oils' inclusion in high fish meal-substituted diets: effect on digestion and nutrient absorption in gilthead sea bream (*Sparus aurata L.*). Aquaculture Research. 42:962-974.

Semova I., Carten J.D., Stombaugh J., Mackey L.C., Knight R., Farber S.A., Rawls J.F., 2012. Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host Microbe. 12, 277–288.

Silva B.C., Nolasco-Soria H., Magallón-Barajas F., Civera-Cerecedo R., Casillas-Hernández R., Seiffert W., 2016. Improved digestion and initial performance of whiteleg shrimp using organic salt supplements. Aquaculture Nutrition. 22(5): 997-1005.

Silva B.C., Vieira F.N., Mouriño J.L.P., Ferreira G.S., Seiffert W., 2013. Organic acid salts selection by multiple characteristics for marine shrimp nutrition. Aquaculture. 384–387, 104–110.

Sitja-Bobadilla A., Pena-Llopis, S., Gomez-Requeni, P., Medale, F., Kaushik, S., Pérez-Sánchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defense mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture. 249, 387–400.

Tacon, A. G., & Metian, M., 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. Aquaculture. 285(1), 146-158.

Terova G., Rimoldi S., Díaz N., Ceccotti C., Gliozheni E., Piferrer F., 2016. Effects of sodium butyrate treatment on histone

modifications and the expression of genes related to epigenetic regulatory mechanisms and immune response in European sea bass (*Dicentrarchus labrax*) fed a plant-based diet. Plos ONE. 11(7): e0160332.

Thangaraju M., Cresci G., Itagaki S., Mellinger J., Browning D.D., 2008. Sodium-Coupled Transport of the Short Chain Fatty Acid Butyrate by SLC5A8 and Its Relevance to Colon Cancer. The Journal of Gastrointestinal Surgery.

Tian L., Zhou X.Q., Jiang W.D., Liu Y., Wu P., Jiang J., 2017. Sodium butyrate improved intestinal immune function associated with NF-κB and p38MAPK signalling pathways in young grass carp (*Ctenopharyngodon idella*). Fish & Shellfish Immunology. 66:548-563.

Toden S., Bird A.R., Topping D.L., Conlon M.A., 2007. Dose-dependent reduction of dietary protein-induced colonocyte DNA damage by resistant starch in rats correlates more highly with caecal butyrate than with other short chain fatty acids. Cancer Biology & Therapy. 6(2), e1–e6.

Urán P.A., Goncalves A.A., Taverne-Thiele J.J., Schrama J.W., Verreth J.A.J., Rombout J.H.W.M., 2008a. Soybean meal induces intestinal inflammation in common carp (*Cyprinus carpio*). Fish and Shellfish Immunology. 25, 751–760.

Urán P.A., Schrama J.W., Obach A., Jensen L., Koppe W., Verreth J.A.J., 2008b. Soybean meal-induced enteritis in Atlantic salmon (*Salmo salar* L.) at different temperatures. Aquaculture Nutrition. 14, 324–330.

Van den Ingh T.S.G.A.M., Krogdahl A., Olli J.J., Hendriks H.G.C.J.M., Koninkx J.G.J. F., 1991. Effects of soybean-containing diets on the proximal and distal intestine in Atlantic salmon (*Salmo salar*): a morphological study. Aquaculture. 94(4), 397–305.

Van Immerseel F., Ducatelle R., de Vos M., Boon N., van De Wiele T., Verbeke K., Rutgeerts P., Sas B., Louis P., Flint H.J. 2010. Butyric acid-producing anaerobic bacteria as a novel probiotic treatment approach for inflammatory bowel disease. Journal of Medical Microbiology. 59:141–143.

Venou B., Alexis M.N., Fountoulaki E., Haralabous J., 2006. Effects of extrusion and inclusion level of soybean meal on diet digestibility, performance and nutrient utilization of gilthead sea bream (*Sparus aurata).* Aquaculture. 261, 343–356.

Vielma J., Ruohonen K., Lall S.P., 1999. Supplemental citric acid and particle size of fish bone-meal influence the availability of minerals in rainbow trout *Onchorynchus mykiss* (Walbaum). Aquaculture Nutrition. 5:65–71.

Vinolo M.A., Hatanaka E., Lambertucci R.H., Curi R., 2009. Effects of short chain fatty acids on effector mechanisms of neutrophils. Cell Biochemistry and Function. 27(1), 48–55.

Wong J.M.W.R.D., de Souza R.R.D., Kendall C.W.C., Emam A., Jenkins D.J.A. 2006. Colonic Health: Fermentation and Short Chain Fatty Acids. Journal of Clinical Gastroenterology. 40(3):235–243.

Wong, S., Waldrop, T., Summerfelt, S., Davidson, J., Barrows, F., Kenney, P.B., Welch, T., Wiens, G.D., Snekvik, K., Rawls, J.F., Good, C., 2013. .Aquacultured rainbow trout (*Oncorhynchus*

mykiss) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density. Applied and Environmental Microbiology. 79, 4974–4984.

Yin F., Yu H., Lepp D., Shi X., Yang X., Hu J., Gong J., 2016. Transcriptome analysis reveals regulation of gene expression for lipid catabolism in young broilers by butyrate glycerides. PloS ONE. 11(8), e0160751.

Yun B.A., Mai K.S., Zhang W.B., Xu Wei., 2011, Effects of dietary cholesterol on growth performance, feed intake and cholesterol metabolism in juvenile turbot (*Scophthalmus maximus L.*) fed high plant protein diets. Aquaculture. 319, 105–110.

Zhang J.X., Guo L.Y., Feng L., Jiang W.D., Kuang S.Y., Liu Y., Hu K., Jiang J., Shu-Hong L., Tang L., Xiap-Qiu Z., 2013. Soybean b-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish. Plos ONE. 8(3): e58115.