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Fish health and fillet quality following substitution of fishmeal (FM) and fish oil (FO) with vegetable meal (VM) and oil (VO) in the modern aquaculture feeds

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1. ABSTRACT

Plant proteins in vegetable meals (VM) and oils are used in an increasing percentage in diets for carnivorous fish to replace fish meal (FM) and fish oil (FO) in order to reduce production costs and to face the limitation of marine resources and to be sustainable in respect to the protection of oceanic biodiversity. However, several studies report that fish respond to such replacements by reducing growth rate, feed conversion and fillet quality. It may be due to a deficiency or poor availability in VM ingredients of some essential amino acids, but also to the occurrence of anti-nutritionals and inflammatory compounds acting on the fish intestine. This can lead to economic losses for fish farmers, increased fish diseases occurrence, and negative environmental impact. This study focuses on European sea bass and Rainbow trout, as two among the most commercially important fish species in Italy. Aims of the study are to improve feed formulation and optimize feed additives such taurine, as well as butyrate, salt of a short chain fatty acid (SCFA), to protect fish intestine and evaluate the effects on fish fillet quality of the modern commercial diets where FM is partially substitute with VM.

In that context, our experimental study investigated butyrate and taurine in the diet, as nutraceutics able to reduce or minimize the negative effects of VM inclusion in fish performances and health.

After improvement of analytical methods for butyrate titration in fecal samples, the possibility that a butyric fermentation occurs in fish intestine has been experimentally evaluated. The protective effect of butyrate on fish intestine has been studied by following approaches including histology with light and electronic microscopy, then a molecular approach to study inflammatory cytokines, as well as the epigenetic action of this SCFA.

In spite, dietary butyrate showed no significant differences in weight gain or SGR (specific growth rate) of sea bass, the protective effect on the intestine walls was highlighted, together with its epigenetic effects on hiperacetylation histone H4 at lysine 8, while no effects has been found on the histone H3 at Lys9. A butyrate effect on the liver, as an increased transcriptional expression of the genes Dicer1, ehmt2, and hdac11 and il-10 have been assessed, suggesting an anti-inflammatory as well as an antiviral role of butyrate in seabass receiving a 2% of Na-butyrate as diet supplement. The effect of a dietary supplementation of the sulphonic aminoacid taurine, almost absent in the VM, was studied on seabass growth performances and on its potential role as antioxidant. Seabass fed with 1.5% taurine addition

showed an increase of the fish growth performance and a marked reduction of the ROS production under resting conditions that was enhanced following forced swimming performances.
The synergistic effect of butyrate and taurine as feed additives was also explored in seabass feeding diet containing an elevate concentration of a soy protein. Different cytological protective mechanisms have been observed, indicating the existence of a synergistic action of the two nutraceutical compounds.

The quality of fish fillet in trout fed with 6 different commercial feed where FM was differently substituted by VM, has been studied by assessing the fillet lipidograms. Trout was utilized in this study as is know for the capacity to elongate and desaturate the n-3 long chain fatty acid EPA in DHA. The EPA/DHA ratio in feed and in fillet were expected to be modified by the fish, with an increased restitution of DHA as consequence of a reduction in EPA. Actually, such a dynamic was observed in fish fed with only 4 of the challenged commercial feed and that was supposed to be due to an effect on the fatty acids metabolism, following an unbalancement of the essential aminoacids in the diets where higher substitution of FM with VM was operated.

In conclusion, a number of the expected negative effects due the FM substitution with VM have been actually assessed. On the same time, the possibility to mitigate such negative performances with diet manipulation have been documented as well. Beside some practical results immediately applicable by the fish farming industry, indications for further researches are emerging from this study.

2. INTRODUCTION

2.1 Feeding the fish and the resources

A pivotal problem concerning the pressure of animal production on biodiversity resources is represented by the need of nutrients for feed production when aquaculture is concerning, oceanic biodiversity may be threatened by the fishery supplying fishmeal and oil to feed the farmed fish.

In fact, one of the criticisms of aquaculture has been the need for using significant amounts of fishmeal and other marine protein sources from wild caught fishery in prepared diets for the cultured species. Unfortunately the availability of oceanic resources utilized to produce fish feed, cannot further be utilized to cover the need of a worldwide increasing aquaculture production (Hardy, 2010). Therefore, since more than a decade their price on the world markets are showing an increased trend, so feed costs became a major concern for the aquaculture industry worldwide. After years of progressive growth, fishmeal prices increased rapidly in the second half of 2014 and expected to remain high through 2016 and on, also after recent news coming from Peru that El Niño climate phenomenon has begun to impact on the anchovy biomass. Fishmeal (FM) prices remained in a narrow trading range from 1980 until 2007 when they doubled in price due to massive purchases from China. In 2012, China imported 1.2 million metric tons, almost one-third of global fishmeal production that is traded among countries and 60% of Peru's production. China will continue to import fishmeal, meaning that prices will remain high.

As a consequence, commercial feed producers have been trying to replace FM using more sustainable feed with a larger portion of proteins originated from plant-based products (PP) being mainly soybean meal (Rolland *et al.*, 2015b; Gatlin III *et al.*, 2007; Delbert *et al*., 2007). Moreover, distal intestine enteritis were described in association to elevate substitutions with PP (Beaverfiord & Krogdahl, 1996; Uran *et al.*, 2008; Baeza-Ariño *et al.*, 2014; Gai *et al.*, 2012). Utilization of PP undoubtedly reduces pressures on the natural biodiversity, nevertheless PP tipically have a low content of certain essential amino acids (EAAs), specially lysine (Lys) and methionine (Met), as well as the sulphonate neutral ß-amino acid taurine (Tau). Crystalline amino acids (CAAs) are applied to such diets, but despite supplementation, PP based diets do not perform as well as FM based diets and that

may be due to the plasma concentration of the supplemented amino acids (AAs). The hypothesis that supplemented CAAs could reach the plasma earlier than the protein-bound AAs, resulting in a temporal mismatch in availability at the sites of protein has been reported (Rolland et al., 2015a; 2015; Powell et al., 2015), and it has been recently demonstrated by Hardy's group, whose data are reported in a publication in preparation (Hardy et al., 2016, in prep.).

Alternative raw protein material only recently allowed, as protein sources for fish diets in Europe and in Italy, are terrestrial animals meals, in respect to the 2013 EC act "Commission Regulation (EU) No 56/2013" of 16 January 2013 of the European Parliament. Such regulation opens a possibility for using as aquaculture feed ingredients few processed animal proteins (PAP) as poultry and pig. So, by-product poultry meals (PBP) are more and more applied in either partial or total substitution of FM and PP meals, regrettably by following an empiric approach, not supported by solid scientific data. At the horizon, other alternative protein sources are appearing, as insect meals (IM), supported by some experimental findings and farm experiences, in spite being still missing, in the EU legislation, a rule opening to this latter opportunity (Salze & Davis, 2015; Rossi & Davis, 2014; IPIFF, 2014; Barroso *et al*., 2014; Henry *et al.,* 2015). Other potential protein sources, as for instance cultivated algae proteins, are still long to be economically profitable for the aquaculture industry, while at the moment only promising seem to be proteins from bacterial grown on natural gases as methane.

Lowering fishmeal levels in feeds by 50% while maintaining growth rates was not especially difficult, but in some farmed fish species, increased feed conversion ratios and an uptick in survival to harvest were seen. The largest reductions in the percent of fishmeal had been in salmon and trout feeds (Burr *et al.*, 2012) as well as in feed for Mediterranean marine species as European seabass and Gilthead sea bream.

Today, some commercial fish feeds are produced with less than 10% fishmeal, but performance of fish fed these feeds is reduced and . In Italy, a number of feed producers and fish growers have reverted to higher fishmeal feeds (up to 30%). They report that although these feeds are more expensive than low-fishmeal feeds, feed conversion ratios are better, a better diseases control is allowed and fish reach earlier harvest size, resulting in a higher economic return that more that outweighs the increase in feed cost. Nevertheless, such a trend cannot be expected to continue further, due to the fluctuation of the FM prices and the strong market competition with the imported fish products. Beside the lack of EAAs, a main problem with replacing fishmeal with plant proteins (PP) is associated with distal intestinal

enteritis due to the presence of anti-nutritional factors in plant meals, for instance when soybean meal is used above 20%.

Moreover, recent researches utilizing the Next Generation Sequencing (NGS) technologies, indicating the effects of the diet on the gut microbioma, also highlight intestine bacteria affecting fish innate immunology and diseases resistance (Ingerslev et al., 2014b), distal intestine inflammation (Reveco et al., 2014), intestinal nutrient absorption (Fuentes-Appelgren et al., 2014; Ingerslev et al., 2014a; Wong et al., 2013; Rurangwa et al., 2015). Normal gut microflora contribute nutrients to fish and help to maintain normal gut function. Altering the gut microflora by reducing fishmeal and increasing PP in the feed alters gut function leading to stimulation of inflammatory responses that diminish feed efficiency.

A number of yet unpublished researches, among which studies carried at the University of Insubria in Italy and University of Idaho in the USA, have revealed that sub-clinical signs of enteritis appear within 10 days or less when high soy diets are fed to rainbow trout and seabass, but it may take longer time, as over three to six months, for the condition to become clinically evident. This finding explains in part why high soybean fish diets result in lower fish growth and protein retention. Although the distal enteritis condition is not fully developed, it affects gut function and nutrient absorption by diverting feed energy and nutrients away from muscle growth and towards inflammation and cytokines stimulation, as it may be assessed also with the study of intestinal transporters activity (PepT1) (Terova et al., 2009; Rimoldi et al., 2015a). Besides, the reduced feed conversion efficiency is at the bases of water pollution, with organic releases containing P, N and oxygen demanding compounds (BOD). Moreover, while it is possible to replace fish meal and fish oil components in the diet with terrestrial ingredients, this strategy may significantly increase the pressure on freshwater resources, due to water consumption and pollution in crop production for aquafeed. Thus, for the aquaculture sector to grow sustainably, freshwater consumption and pollution due to aquafeed need to also be taken into account (Pahlow et al. 2015).The main gap that this project intend to fill is essentially to find valid protein sources to substitute FM in the feed for seabass and trout farmed in Italy. The ways to afford such a gap have been individuated in 1) finding solutions for time-matching of EAAs and protein-bound AAs availability at the site of protein syntheses; 2) finding effective diet corrections to mitigate soybean and/or other plant sources induced enteritis; 3) challenging alternative protein sources than PP, as PBP and IM; 4) exploring the possibility to switch toward trout genotypes elsewhere selected for PP based feed. All that, in a frame of environmental and economic sustainability, as well as fish quality. The research consortium is multidisciplinary and strictly

complementary, skilled to produce innovation to be immediately applied to the industry as well as to improve the needed basic knowledge, indispensable to launch innovative processes.

2.2 Essential Aminoacids: lysine and methionine

Despite supplementation with Lys and Met in crystalline form (CAA), when PP based diets is utilized in the diet, fish do not perform as well as FM based diets. The supplemented CAAs (free) reach the plasma earlier than the ones originated by proteins, resulting in a temporal mismatch in availability at the sites of protein synthesis and possibly resulting in an excessive aminoacids catabolism (Rønnestad *et al.*, 2000, 2001; Rojas-Garcia & Rønnestad, 2003; Rolland, 2015). Coated Lys and Met were positively experienced in warm-blooded terrestrial animals as well as in some fish species among which tilapia, Chinese sucker, cobia and Black sea bream (Yuan *et al.*, 2011; Leng *et al.*, 2013; Chi *et al*., 2014; Lu *et al.*, 2014). In Rolland *et al*. (2015) a positive relationship among dietary Met, plasma Met and all the EAAs was demonstrated in spite, in our opinion, the real advantages of coated Met versus the crystalline form needs further studies, together with the performance of 2-hydroxy4-(methyltio)butanoic acid (HMTBa). The latter, an organic acid acting as precursor of Met, has been reported to be more efficient than L-Met in turbot's growth, also improving immunitary response (Ma *et al*., 2013). HMTBa was also reported safe for the same species when supplemented less than 5% in the diet, but causing a decrease in growth performance when supplemented at 10% (Hu et al., 2015). In spite other authors reporting to have lower efficiency than L-Met and DL-Met in rainbow trout (Powell et al., 2015), HBMTa could result in other interesting properties, as promotion of the antioxidant defences in fish intestine and hepatopancreas (Feng et al., 2011). A robust definition of the actual need in Lys and Met addition in the diets for trout and seabass is due and will be part of this project, also in consideration of their relevant and growing market costs, being now close to 1,500€/ton for Lys, 5,000€/ton for DL-Met and 5,200€/tons for HMTBa.

2.3 Taurine

A comprehensive review on the beneficial effects of supplementation with the sulphonate neutral ß-aminoacid taurine (Tau) in the diet for fish and shrimp, is reported by El-Sayed (2014). The indispensability of Tau in the fish diet is now generally accepted and it changes with the species and onthogenetic development (Pinto et al., 2013) as well as with the

environmental conditions, while the effect of diet matrix and other environmental parameters have received limited attention (Salze and Davis, 2015). As FM is a significant source of essential minerals, it was hypothesized that the reduced fish growth when fed with low FM diets, even after addition of lys and met in crystal form, were due to a low mineral bioavailability and that Tau acted as an organic acid improving minerals bioavailability. Taurine may interact mitigating the diet antinutritional factors, as it has been illustrated by the restorative effect of cholytaurine on saponin- and lectin-induced enteritis in trout (Iwashita et al., 2008, 2009) and in a recently submitted study carried at University of Insubria (Rimoldi et al, submitted 2015b). A role of water salinity is evident, that increases Tau's need in hyperosmotic environment. Being water-soluble, Tau is transported through intestinal epithelium by a specific Na+/Cl- transporter (TauT) that may be inhibited by environmental and antinutritional factors in the diet (Bedford et al., 2000; Pinto et al., 2012). The role of dietary Tau in regulating Tau transporter, has been reported in Atlantic salmon by Zarate and Bradley (2007), resulting more important during the life in saltwater (smolt) than in freshwater (parr). However apparent contradicting studies exist, as for instance rainbow trout fed in freshwater with a diet FM-free, improves growth and feed efficiency with Tau supplementation in feed (Gaylord et al, 2006, 2007), suggesting it to be essential in the nutrition of this species. Different trout strains, selected either in Japan or in the USA, show differences in the biosynthesis capacity and dietary need of Tau, with relevant diversity in the respective growth potential (Gaylord et al., 2007; Jirsa et al., 2014). Moreover, leaching as well as the Maillard reaction during feed extrusion may defeat the objective of its addition in feed, so application of a protected (coated) form may result of advantage (Aragão et al., 2014). In spite of some protected forms of taurine being available on the market, a lack of information exists on its potential utilization in aquaculture feed.

In legislative terms, taurine regulations are different within countries about its use for human and animal feed. In the European Union, the Observed Safe Level (OSL) is estimated to be 100 mg taurine per kg body weight per day for people, and synthetic taurine is considered efficacious in cats, dogs, and carnivorous fish diets (EFSA, 2012). In China, taurine is authorized for fish feed in all species, and listed as a nutrition enhancer for children. In Japan, taurine is considered a feed additive; therefore it can be used in fish and other livestock, although quantities are not regulated. In USA, taurine is not considered a safe substance, but a feed additive or drug. In fish feed, taurine presence is permitted only by increasing fish or krill meal, naturally rich in taurine (Salze & Davis, 2015). In animals that need for taurine,

USA permits its supplementation as for chicks up to 0.054% and particularly for cats, where the addition is vital.

2.4 Nutraceuticals for mitigation of intestine damages

A number of nutraceuticals have been reported in literature, possibility having positive effect in the mitigation of soy-derived intestine inflammation. Short Chain fatty Acid (SCA) and in particular *butyric acid*, generated the interest of scientists and feed producers. The alternative form tributyrin, has the characteristics to become a valid alternative. At the intestinal level, butyrate plays a regulatory role on transepithelial fluid transport, ameliorate mucosal inflammation and oxidative status, reinforce the epithelial defense barrier and modulate visceral sensitivity and intestinal motility (Berni Canani et al., 2011). Its application in aqua-feed was patented by an international feed company, meaning to mitigate the negative effects on fish intestine, due to substitution of FM with soy products. Na-butyrate has been reported to mitigate the inflammation damages induced in seabass distal intestine by soy products in the diet, resulting in a strong protective action documented by histological observations of the intestine and with a molecular approach on the transcriptomic of the oligopeptide transportes (PepT1) (Scollo *et al*., 2012). In sea bream, coated butyrate has been reported to protect intestine Estensoro et al. (2014), to facilitate the action of the nutrient transport through the intestine (Robles et al., 2013), while Liu et al. (2014) reported it as a dietary supplement to repair intestinal damage in carp fed oxidised soy-bean oil. In a study on seabass, Rimoldi et al. (2013; 2015c) showed significant changes in the expression of 4 out of 7 target genes related to butyrate supplementation to the diet and a significantly increased the acetylation state of histone H4 and H3, as well as the expression of a gene called Dicer1 in the liver. Dicer1 is a very important gene which is known to participate in the innate defenses, with an RNA silencing-based action that generate antiviral immunity (Aliyary and Ding, 2009; Chiappinelli et al., 2012). Nevertheless, in spite of a benefit of butyrate additions in feed for aquaculture seems to be sustained by some basic approaches, when farmers and feed producers are inquired on results observed from butyrate additions in feed, their responses sound sometime contradictory. One of the explications for that may come from studies reporting that butyrate may have either a promotive or a protective apoptosis action in enterocytes, due to the different ratio of ω3/ω6 Long-chain polyunsaturated Fatty Acids (LCFAs) associate in the diet (Crim et al., 2008; Turk *et al.*, 2011). More realistically, doubts exist about the concentration adopted by the feed industry, because of its dose-dependent

action and limited biological half-life. Therefore, such apparent paradox suggests the need of further studies on the effect of butyrate in protecting fish intestine, and possibly on immunitary defenses. Miller (2004) reports tributyrin, an esterification of 3 molecules of butyric acid with glycerol, being even more effective than butyrate, at least in mammalians. The authors also report effective concentration in vitro being over 0.05 mM butyrate but its action requests at least 0.3 mM to act on neoplastic cells. Information about the plasmatic half-life of butyrate in fish are missing, while in human resulting 6' after intravenous injection. Longer half-life being observed after ingestion of tributyrine with the diet (40') while even longer has been postulated following an intake repeated during the day. Tributyrin is now offered on the market to be supplemented to farmed animal feed, at the price of 5,000€/ton and nowadays no data seem to be available reporting its effects on fish. This price, higher than Na-butyrate (2,700€/ton as sodium butyrate, rising up to 3,200€/ton when coated), could be recovered by a higher efficiency of this molecule. Moreover, because of its supplementary actions expected on the immune system, possibly due to its longer persistence, new applications could be hypothesized for tributyrine utilization in aquaculture. Nevertheless information about tributyrine effectiveness to mitigate the inflammation damages caused by soy products, are so far completely missing. Therefore, focused studies on the possible benefits that any form of butyrate may ensure to seabass and trout are suitable, after an appropriated tuning under laboratory conditions. Moreover, an induced production of butyrate by the intestine microbiota could sound an interesting solution in the case it will result realistic as indicated by our preliminary results. While in warm-blooded animals commensal clostridium bacteria are specialized in producing butyrate by anaerobic fermentation of poorly digestible polysaccharides (inulin, pectin, others), unlikely the clostridium activity at the lower temperature of the fish intestine, is limited or none. So, alternative pathways should be activated, where other bacterial streams, possibly *Vibrionaceae* but not only, could indirectly produce it. Therefore, new information will be obtained by studying which kind of intestinal microbiota may be induced by the different diets.

2.5 Diet, gut microbiota and fish health. An approach to the study

Intestine of fish has distinct segments with specific physiological roles and anatomy. Each different intestinal regions may be characterized by distinct microenvironments, which could

harbor unique microbiota profiles. Adherent intestinal bacteria live in intimate contact with their host and play crucial roles in many aspects of host development, metabolism and immunity, as described in a review by Ghanbari et al., (2015). In the past few years, the role of microbiota in human and animal health and nutrition has become a leading topic in nutritional research. The role of the intestinal microbiota of fish (zebrafish) (Fuentes-Appelgren et al., 2014) has recently been explored and was found to be involved in key processes related to the stimulation of nutrient metabolism, innate immune response, intestinal epithelial cell proliferation and overall fish growth and health. A few studies have reported to explore the effect of plant versus fishmeal diets on gut microbiota (Ingerslev *et al.*, 2014a; Rurangwa *et al*., 2015; Reveco *et al.*, 2014). The results of these studies showed that in both the species, rainbow trout and zebrafish, gut microbiota were significantly different when fish fed different protein sources were compared. The role of gut microbiota in development of distal enteritis in fish has been little investigated but recent studies in humans and livestock, made possible by high-throughput technology to identify the entire microbiome using culture-independent methods, pointing to a major role of gut microbiota in many medical conditions. The arising question concerns the possibility to tune the microbiota composition through the diet, inducing bacterial colonies that may fight against pathogens hosted in intestine, as well as producing (via anaerobic fermentation) molecules with nutraceutic potential and/or protective for the intestine (i.e. SCFAs). A number of nutraceutical additive being reported in literature having beneficial effects on fish intestine and/or liver, an example is represented by MOS®, a mannan-oligosaccharide, chitin and chitosan (Torrecillas et al., 2007; Staykov et al., 2007; Akter et al., 2015; Esteban et al., 2001; Wang et al., 2005; Kono et al., 1987; Ringø et al., 2012) or probiotics (Ramos et al., 2015). The knowledge of microbiota association with the diet could represent a tool on the way of its tuning in the intestine. Some facts stimulate the hypothesis of a possible protective manipulation of the gut microbiota, extending beneficial effects in intestine and liver, as reported by Torrecillas et al. (2007). Similar figure has been reported by Scollo et al (2012) following butyrate addition in the diet, and by the preliminary results found by Hardy et al. (2015, in prep.), the latter utilizing the Illumina NGS to afford studies of the gut microbioma in two trout strains fed either FM or PP as protein sources.

2.6 Poultry by-product meal (PBM), as substitute protein source

PBM have been recently introduced in fish feed, frequently by following an empiric approach instead robust scientific data. Published studies related to the utilization of PBM in substituting FM in aquaculture feed are limited to few fish species and results are sometimes in contrast. Sealey *et al.* (2011), utilized 3 different qualities of poultry meal to feed during 8 weeks rainbow trout fingerlings under laboratory conditions, challenging with an infective test. Results indicated that adopted diets did not lower growth, neither disease resistance. Rossi and Davis (2012; 2014), utilizing PBP to feed pompano, reported similar results, underlining the need of a taurine supplementation. Differently, substituting FM with PM to feed Black Sea turbot in a 60 days experiment, Yigit *et al.* (2006) concluded that up to 20% of FM may actually be substituted, while higher percentages lie to reduced growth rates, nitrogen retention and feed conversion efficiency. Again, Gonzàlez-Rodrìguez *et al.*, (2013), reported reduced growth rate and increased malformations in tench fed feather meal.

2.7 Insect meal as substitute protein source

Insect meal could be a promising alternative to fishmeal and to the high demand of food in the next decades.

The possibility of rearing insects on bio-waste or organic compounds made insect meal on a large-scale production to satisfy the crude protein requirement of animal feeds (Makkar *et al.*, 2014).

The composition of IM may result variable according to the insect species and life stages (Finke, 2015), while the lipidic content is generally too high for the extrusion processes and the meals must be de-fatted, the lipidogram not resulting sufficiently rich in precious lipidic sources (Makkar *et al*., 2014). The insects EAA composition is of interest in spite the problem of digestibility needing to be afforded. The elevate composition in chitin should be evaluated for any possible interference with the intestine microbial activity (Henry *et al.*, 2015).

Some studies reported practical consequences of mealworm diet inclusion. In *Dicentrarchus labrax* a 25% of fishmeal substitution with mealworm did not lead to negative effects on weight gain, while at 50% level of the mealworm induced reduction in specific growth rate and feed consumption ratio. Then, lipid body composition was influenced by 50% of

Tenebrio molitor meal with decreasing in EPA contents (from 395.07 to 164mg/ 100g) and DHA (from 619.88 to 203.26 mg/100g) (Gasco *et al.*, 2014a). While, in rainbow trout the levels of that meal can reach up to 50% without a growth performance reduction in feedstuffs, leading to a saving on fishmeal protein (Gasco *et al.*, 2014b).

2.8 Preserving fish quality in view of increasing fishmeal substitution

Fish quality includes a mixture of organoleptic properties, such as odour, appearance, texture and flavour. Not perceived directly, the consumer, due to polyunsatured fatty acids (PUFA), vitamins, carotenoids and proteins contents, considers the fish nutritional value essential.
In general, dietary requirement of PUFA are necessary for many species and differences among them are about the amount of fatty acids and the bioconversion and elongation capability of fatty acids. For instance, in fish n-3 PUFAs, especially eicosapentaenoic acid EPA (20:5 *n*-3) and docosahexaenoic acid DHA (22:6 *n*-3) are found in high concentrations in the phosphoglycerides of cellular membranes, and DHA is particularly abundant in the retina and brain, where has a crucial role maintaining the structure and function of the excitable membranes of these tissues (Lauritzen *et al.*, 2001). Accordingly, the fish nutritional value should be preserved to supply a quality product together a protective role against coronary heart diseases attributed to n-3 fatty acids and to their cardioprotective action (Grigorakis *et al.*, 2007). Research about fish nutrition and feed documented the correlation between the dietary fatty acid composition and fatty acid composition of seabass and sea bream muscle (Nicolosi Asmundo *et al.*, 1993; Krajnovic-Ozretic *et al.*, 1994; Grigorakis et al., 2002; Izquierdo et al., 2003, 2005; Montero et al., 2005; Mnari *et al.*, 2007). Regarding this issue, the substitution of fishmeal (FM) and fish oil (FO) must be controlled because EPA/DHA ratio may be altered or reduced by plant sources introduction. As seen in seabass and sea bream fed with soybean oil, linseed oil and rapeseed oil, a reduction of the above-mentioned ratio was detected. Arachidonic acid (ARA) significantly decreased in all the cases of soybean, linseed and rapeseed substitutions, while linoleic and linolenic acid of plant origin increased in fish muscle (Izquierdo *et al.*, 2003, 2005; Montero *et al.*, 2005). If FO substitutions reduce fatty acids muscle composition (Izquierdo *et al.*, 2003, 2005), replacements of FM involve reduction of growth performances, probably linked to a combination of lower feed intake and lower feed efficiency in European seabass, sea bream and in rainbow trout.

An interesting study in rainbow trout (Lazzarotto *et al.*, 2015) reared on a plant-based diet from first feeding to reproduction were capable of synthesizing sufficient amounts of n-6 and n-3 LC-PUFAs from precursors to survive, reproduce and provide a viable offspring. Although the presence of diet devoid of long chain PUFAs, female rainbow trout gave a viable progeny linked to the fact that were remarkably able to synthesize ARA, EPA and DHA from dietary precursors.

In conclusion, many factor influence the fatty acids requirement, as diet, seasonality, sexual development and finally the fish species, therefore feed must be carefully formulated depending on nutritional requirements.

2.9 Main farmed fish species in Italy

Italy is among the four largest finfish producers EU27, together with the UK, Greece, and Spain. According to FAO (2012a,b), Italy is also one of the six largest finfish producer among the non-EU and EU members, together with Norway, the UK, Turkey, Greece, and Spain.

The national fish production is about 232,000 tons, but Italy needs to import more than 900,000 tons of fish to meet internal fish demand, because of the inability of the aquaculture and fisheries chain to sustain the consumption of 21 kg $year^{-1}$ per capita of fish and seafood products (ISMEA, 2012).

Rainbow trout is the most important species (55.5 %), followed by European sea bass (13.6 %), gilthead sea bream (12.2 %), gray mullet (5.3 %), and sturgeon (2 %). Marine fish production has increased significantly over the last 20 years, and the two leading species are presently contributing 25.8 % of the total finfish production (Parisi et al., 2014).

European seabass

The European (or common) seabass, Dicentrarchus labrax (Linnaeus, 1758), one of six members of the Moronidae family, belongs to the order Perciformes (perch-like fish), which is the largest order of vertebrates, containing about 40 % of all bony fish. The European sea bass is primarily a marine fish, but it is sometimes found in brackish and freshwater; in fact it is highly euryhaline (0.5–36 ‰ salinity) and able to adapt at a broad temperature range (2-32 °C).

The European sea bass has a silvery, elongated body (depth of body is 22–27 % of total length), with two clearly differentiated dorsal fins, and a rather high tail (Pickett & Pawson, 1994). Large, regular scales cover the body and its color varies considerably ranging from dark grey, blue or green on the back to a white or pale yellow belly. The maximum length of European sea bass is over 1 m and weight is over 12 kg (Pickett & Pawson, 1994).
The European sea bass is a gonochoric species and a seasonal breeder, spawning from winter through early spring, with some differences in timing according to the specific location.

Nutrition

At larval stage, sea bass are fed with artificial diet and live feeds, usually in the form of rotifers (the rotifer *Brachionus plicatilis* fed on unicellular algae) and brine shrimp (*Artemia salina*).
Feeding on live prey usually lasts 40–50 days, according to water temperature, and the rearing
protocol. Then fish are switched to an artificial diet (weaning). The timing of the first dry food supply has been continuously moved forward to an earlier date in recent years due to new, more elaborate artificial diets, including vitamins and immunostimulants, which better fit the larval requirements in terms of composition, size, buoyancy, and flavor (Moretti et al., 1999).
The young fish actually receive the first feeding with inert feed at the very early age of 17–19 days, but dry, compounded feed does not become their only source of nutrition until much later. Young fish are weaned in a dedicated section of the hatchery where the metamorphosed fish (about 45 days old) can grow to a size of 2–3 g. At this stage, they are called fingerlings or juveniles and must have assumed the adult appearance. When a size of 2–5 g is reached, weaned fry leave the hatchery and are stocked in the on-growing/fattening facilities (Moretti et al., 1999).

The commercial value is high for both for fish captured from wild stocks and from aquaculture production. All Mediterranean countries produce European seabass and also Gilthead sea bream in cages. The leading countries, according to production volume in 2004, were Greece, Turkey, Spain, Italy, Croatia, and France. According to the latest available data (FAO, 2014), in Italy with a production of 6700 tons represents the fifth world producer of European sea bass, after Turkey (65.512 tons), Greece (42.500 tons), Spain (14.455 tons) and

Egypt (13.798 tons). Among the Italian regions, Sicily plays a significant role and covers about 20% of Italian euryhaline fish production (Santulli & Modica, 2009).

Rainbow trout

The rainbow trout, Oncorhynchus mykiss, is a species of salmonid native to the Pacific Ocean in Asia and North America including the central, western, eastern, and especially the northern portions of the United States. It has also been introduced into New Zealand, Australia, South America, Africa, Japan, southern Asia, Europe and Hawaii.
Rainbow trout possesses the well-known stream-lined (salmonid form) body shape. They have a pinkish or reddish lateral stripe, sometimes with lavendar or orange overtones, from the gill cover running the length of the fish to the tail. The caudal fin has rows of small dark spots, and there are more small blackish spots sprinkled on the head and sides, and spotting on the dorsal and adipose fins. The belly is whitish. The lower fins are pale-pink without spots. At spawning time, males become deeply colored with an intensely red side stripe.

Nutrition

At first feeding, the digestive tract of rainbow trout is well developed. Rainbow trout fry begin feeding in streams, consuming terrestrial and aquatic invertebrates (zooplankton and insects). Insects remain their dominant food source throughout life, although they are opportunistic piscivores. The anadromous form of rainbow trout, called the steelhead trout, migrates to the ocean at 1–3 years of age and feeds on krill, small fish, shrimp and squid (FAO, 2015). The most important food is freshwater shrimp, which contains the carotenoid pigments responsible for the orange-pink color in the flesh. In aquaculture, this pink coloration can be produced by including the synthetic pigments astaxanthin and canthaxanthin in aquafeeds (FAO, 2012c).

Monoculture is the most common practice for cultivating rainbow trout in Italy, and intensive systems are considered necessary in most situations to make the operation economically attractive.
A variety of grow out facilities are used, ranging from flow-through systems to cages in lakes. The majority of Italian trout farms use flow-through systems, which consist of raceways or concrete tanks with continuously flowing water. Alternative on-growing systems

for trout include cage culture production systems, where fish are held in floating cages, ensuring a good water supply and sufficient dissolved oxygen.

One of the basic, vital factors influencing the growth of rainbow trout is the quality of water. Rainbow trout is a typical cold-water fish: the appetite of rainbow trout is optimal in the water temperature range of about 7–18°C; above 18°C, fish appetite sharply decreases and feed intake stops. The water temperature in which the trout make the best growth out of the consumed feed varies from 13 to 15°C (Molony, 2000).

2.10 Goals

The main goal of this study was to evidentiate some of the bottleneck problems arising when vegetable sources of proteins are substituting FM, in order to emerge new indicators and to address future researches.

In view of the need to substitute FM with alternatives protein sources in the feed for farmed carnivorous fish, problems could emerge linked to fish health and quality.

So, our study approach was to investigate on the possible nutraceutical corrections that could mitigate negative effects of PM substituting fishmeal. In details, we focused the attention on the possible role of butyrate and of taurine in protecting the fish intestine versus inflammations caused by the vegetable contents of the diet.

Moreover, the fish fillet quality was investigated in fish being fed with different commercial feeds, focusing on the content of long chain fatty acids (*n*-3 LCFA), as EPA and DHA.

Because of their commercial importance in Italy, Rainbow trout and European seabass were utilized in this study.

3. *MATERIALS AND METHODS*

The experimental design will consider:

1. the study of butyrate effect in European seabass, particularly in the relation of diet and its potential production by gut microbiota and its regulatory role in the expression of a suite of genes related to epigenetic mechanisms;
2. the study of taurine effect in European seabass through the monitoring of metabolic parameters related to oxygen consumption, critical swimming speed and ROS level;
3. the comparative study of taurine and butyrate effects in European seabass through histological analysis and molecular analysis related to immunity response;
4. the quality analysis of commercial feeds and meat in Rainbow trout.

3.1 Butyrate

The possibility to induce a butyrate production by the intestine microbiota has been studied on seabass, by manipulating the diet. The intent was to induce a butyrate fermentation in fish microbiota that would eventually protect the digestive structure versus inflammation caused by the soy meal included in the diet. In order to afford such study, appropriate analytical methodologies have been improved for GC-MS and for HPLC applications. The butyrate residual in fecal products for seabass have then been analyzed following feeding the fish with 4 different diets. The microbiota colonized the same fecal products has been assessed with a DGGE approach. Further have been fed with additions of prebiotics expected to induce a butyric fermentation. Effects of the same prebiotics on the immunity defenses has been studied as well. A further study has been carried out on the protective mechanisms of butyrate in the intestine and on its possible epigenetic action.

The butyrate effects on seabass will be investigated through several technique approaches.
Faecal samples of seabass feeding with four diets different in vegetable meals content have been analysed for butyric acid presence by GC-MS. Due to insufficient quantification of butyric acid, we have tested another analytic approach, HPLC, to quantify butyric acid in faeces.

The possibility of a butyric acid endogenous production was confirmed and DGGE analysis of microbiota focused on identifying fish gut's bacteria able to synthesize butyric acid.
Different prebiotics have been tested in seabass to stimulate the microflora on synthesising butyric acid. Faecal samples have been processed by HPLC analysis to determine which prebiotic high butyric acid was. Finally, the prebiotic effects on immunity state was investigate, as well.

3.1a Analytical methods

3.1a.1 GC-MS analysis

Butyric acid quantification was performed in a Thermo Scientific GC/DQII and equipped with a DB-WAX column (Agilent Technologies, Santa Clara, CA) projected for food, flavor and fragrance applications. The volatility of butyric acid allowed us to apply the headspace method reporting in Valero *et al.* (2000).
400 mg of feces were introduced in a vial and acidified with 800uL of 3.5 M sulphuric acid. Different temperatures of evaporation (25°C, 60°C, 100°C) were tested to saturate the vial headspace with butyric acid. 250uL of vial headspace was injected into machine. Helium was used as carrier gas through all system. The initial oven temperature was 60°C until reaching 135°C at 25°C/min and then the temperature was reached 180°C at the rate of 15°C/min. The butyric acid retention time was recorded at 9.04min.
Unfortunately, this method was resulted weak to quantify the butyric acid in stool samples due to its low concentration. Therefore, we decided to turn into another kind of analysis and trying to detect butyric acid by HPLC-UV.

3.1a.2 HPLC-UV analysis

The HPLC-UV system consisted of a PU-980 pump connected to a DG-1580-53 degasser, an AS-950-10 autosampler and an UV-1575 UV/Vis detector, all from Jasco-Europe S.R.L.
Chromatographic separation was tested on TC-C18 column (150mm X 4.6mm i.d.) with particle sizes of 5µm (Agilent Technologies, USA). The HPLC column was protected by a guard column of the same type. The column was thermostatized at 30°C. The mobile phase consisted in HPLC water plus 3% of CH_3CN (Carlo Erba reagents). An isocratic elution was

performed to identify butyric acid at 1.25mL/min flow rate with a run of 15 minutes. The UV detector was set at a wavelength of 210 nm. Data processing was performed using the ChromNAV (Jasco-Europe).

The limit of detection (LOD) and the limit of quantification (LOQ) were also reported as sign of method validation. Blank sample was run for three times and the parameters were calculated as followed:

$$\mathbf{LOD} = 10\ \frac{\sigma a}{b}$$

$$\mathbf{LOQ} = 3.3\ \frac{\sigma a}{b}$$

where σa is the standard deviation of the blank response and b is the slope of calibration curve.

3.1b Experimental design

3.1b.1 Animals and diets

Four experimental diets were tested in duplicate circular tanks where 150 seabass were stocked in each tank. Animals were nourished twice a day *ad libitum*. The water marine parameters were monitored: temperature was about 24.7±0.6°C with 4/5 hours-water turnover, pH was 7.9±0.4 unit and dissolved oxygen was around 100% of saturation.

Table 3.1 reported the ingredients of iso-proteic, iso-energetic and iso-lipidic diets with 45% protein and 18.5% of fat inclusion.

Table 3.1 Ingredients of four diets with different level of vegetable meals and fishmeal

Raw materials	Diet A (%)	Diet B (%)	Diet C (%)	Diet D (%)
Fishmeal	35.00	27.00	21.00	15.00
Vegetable meals*	48.14	57.49	52.70	68.37
Refined vegetable meals**	1.76	0.53	1.00	1.09
Haemoglobin			9.00	
Fish oil	4.05	4.05	4.50	4.20
Soybean oil	9.45	9.45	10.31	9.80
Vitamins and minerals	0.51	0.52	0.51	0.52
Antioxidant	0.40	0.40	0.40	0.40
Others	0.60	0.60	0.58	0.62

*Soybean, wheat, pea, corn gluten, ** Soybean and pea protein concentrate

3.1b.2 Faecal samples collection

Seabass stool samples were collected by squeezing the fish abdomen or sacrificing the animal. The main problem of collecting fish stool was the reduced quantity obtained by squeezing the animal, may be a stressful animal, and the consequence was the fish killing to collect a good amount of stool.

3.1b.3 DNA isolation from feces and PCR- denaturing gradient gel electrophoresis (DGGE)

Beside the effort of quantifying butyric acid in feces, the goal was also to detect bacteria able to produce butyric acid in fish gut.

In order to ensure the detection of non-cultivable bacteria, DNA was purified directly from fecal matter. Total genomic DNA was obtained from 1 g of bulk feces from each diet tank and extracted by UltraClean® Fecal DNA Kit isolation (MO BIO Laboratories Inc). The extracted genomic DNA was used as a PCR template with the primers for the V6–V8 variable regions of the 16S rRNA gene, F-968-GC (5′-AACGCGAAGAACCTTAC-3′ with a GC clamp at the 5′ end) and R-1401 (5′-CGGTGTGTACAAGACCC-3′; Nübel et al., 1996). The PCR was performed in a final volume containing 50 ng μL−1 of genomic DNA, 0.025 U μL−1 Taq DNA polymerase (MP Biomedicals), 0.2 mM of each dNTP (Eurogentec premix), 0.4 μM of each primer and 2 mM $MgCl_2$. The PCR conditions in the T100 thermal cycler (Bio-Rad) were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles including denaturation at 95 °C for 1 min, annealing for 1 min and a 2-min elongation at 72 °C. A final extension cycle was performed at 72 °C for 10 min. The amplification products were visualized by electrophoresis on a 1.5% agarose gel containing ethidium bromide.

DGGE was performed using the Dcode Universal Mutation Detection System (Bio-Rad). A volume of 25 μL of PCR products was loaded onto an 8% polyacrylamide gel (16 cm × 16 cm × 1.5 mm) prepared from 40% w/v acrylamide-N,N′-methylenebisacrylamide stock, 37.5 : 1 (Bio-Rad). The denaturing gradient was prepared by mixing 40% and 80% solutions of 7 M urea and 40% formamide. The electrophoresis was run in 1 × Tris/Acetic acid/EDTA (TAE) buffer diluted from 50 × TAE buffer stock solution (Bio-Rad). The migration was performed at 90 V (constant voltage) for 16 h, with a temperature gradient of 60 °C. After

electrophoresis, the gel was stained for 30 min by 200 mL of TAE solution with SYBR Green (0.1 µL mL−1; Sigma). After rinsing, the gel was scanned on Gel Doc (Bio-Rad).
Some well-separated bands with a high peak intensity were excised from the gels, and left overnight soaking in 100-µL sterile milli-Q water at 4 °C. The infusion was then used as a template for a second PCR-DGGE similar to the first round, for purity control. The purified bands were again excised and soaked for PCR with primers F-968 without a GC clamp and R-1401 (initial denaturation at 95 °C for 3 min, followed by 30 cycles including denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and a 1-min elongation at 72 °C; a final extension cycle was performed at 72 °C for 5 min). The products were purified using a MiniElute PCR purification kit (Qiagen) and sent for sequencing. Sequences were aligned by BLASTn 16S ribosomal RNA sequences (Bacteria and Archea) to determine the species.

3.1c Prebiotic effects on butyric acid production and on fish immunity state

3.1c.1 Animals and diets

Seabass (*Dicentrarchus labrax*) were reared in two rectangular tanks of 2500L, each tank was divided in two portions to constitute 4 feeding groups (Novelose330, chitosan, inulin, and betaglucan), and the diets are showed in Table 3.2.
All samples were stocked at -80°C until being processing.

3.1c.2 Butyric acid calibration standard curve

The calibration standard curve has been composed of four concentrations (Sigma Aldrich-Italy) prepared from working solution of butyric acid (0.5M): 0.1mM, 0.125mM, 0.25mM, 0.5mM. Each solution was prepared in vial and diluted in HPLC water in a total volume of 1000uL. Three replicates for each points have been made and the calibration standard has resulted with R^2 =0.99.

3.1c.3 Anaerobic growth of feces

1g of collected faeces of each treatments (insect meal, Novelose330, chitosan, inulin betaglucan and negative control) were cultured in 20mL of thioglycolate broth (Scharlau, Scharlab S.L., Spain) for anaerobic growth at 40°C and incubated for 48h.

After the anaerobic growth, 3mL of broth have been processed and the pH was checked with NaOH 1M until reaching 7-8. Then 1.5mL of n-Exane (Carlo Erba reagents) was added and the samples were centrifugated at 3.500rpm for 10 minutes to clean the matrix. After the centrifugation, the upper phase was removed and an adequate quantity of HCl (37%) was added to the sample until reaching a range of 2-3 pH. 10uL of the extract was injected in HPLC-UV. The extraction protocol and the detection method of butyric acid were obtained by De Baere *et al.* (2013) with some changes.

Table 3.2 Seabass diets composition with 2% of prebiotics addition.

Raw material	Negative CTRL (%)	Novelose330(%)	Betaglucan(%)	Chitosan (%)	Inulin (%)
Wheat	17.0	17.0	17.0	17.0	17.0
DDGS	8.0	8.0	8.0	8.0	8.0
Wheat gluten meal	4.0	2.0	2.0	2.0	2.0
Mixed oil	10.0	10.0	10.0	10.0	10.0
Vitamins & minerals premix	0.4	0.4	0.4	0.4	0.4
Soybean meal (48%)	10.6	10.6	10.6	10.6	10.6
Fish meal (65%)	50.0	50.0	50.0	50.0	50.0
Novelose330		2			
Betaglucan 85% from *Saccharomyces cerevisiae*			2		
Chitosan				2	
Inulin (90%)					2
Nutrients (%)					
Protein	45.0	45.0	45.0	45.0	45.0
Fat	16.0	16.0	16.0	16.0	16.0
Fiber	1.8	1.8	1.8	1.8	1.8
Ash	8.2	8.2	8.2	8.2	8.2
Calcium	1.7	1.7	1.7	1.7	1.7
Total Phosphorus	1.3	1.3	1.3	1.3	1.3
Methionine	1.1	1.1	1.1	1.1	1.1
Methionine + cystein	1.6	1.6	1.6	1.6	1.6
Lysine	3.0	3.0	3.0	3.0	3.0

3.1c.4 Respiratory Burst (RB) activity

The RB activity was performed in blood of seabass feeding with novelose330, inulin and chitosan. Not sufficient blood of seabass feeding betaglucan was collected. 0.5ml of blood was collected in a tube containing anticoagulant, then diluted blood with an equal volume of medium Hank's Balanced Salt Solution (HBSS) (1:1). 0.75 ml of Lympholyte®-Mammal was added to the centrifuge tube; centrifuged for 20 minutes at 800g at room temperature. After centrifugation, there will be a well-defined lymphocyte layer at the interface. Carefully, the cells were removed using a Pasteur pipette, from the interface and transferred to a new centrifuge tube. Then, diluting the transferred cell with medium to reduce the density of the solution. Another centrifugation was made at 800g for 10 minutes to pellet the lymphocytes, and then discarded the supernatant.

Induction of the respiratory burst (RB) activity in blood was measured directly from heparinised blood, following the method described by (Nikoskelainen et al., 2005)

100 µl of diluted blood (1:25) in HBSS (Hanks balanced salt solution, pH 7.4) was dispensed in white flat-bottomed 96-wells, and incubated with 100 µl of a freshly prepared luminol suspension (2 mM luminol in 0.2 M borate buffer pH 9.0) with 1 µg ml-1 phorbol myristate acetate (PMA, Sigma) for 1h at 24–25°C. Luminol-amplified chemiluminescence was measured every 3 min with a plate luminescence reader for generation of kinetic curves. Each sample was run by duplicate and read against a blank in which neither blood and nor PMA was added (Guardiola *et al.*, 2014). The integral luminescence in relative light units (RLU) was calculated.

3.1c.5 Statistical analysis

Differences between respiratory burst values of three diet (chitosan, inulin and novelose) were subjected to GLM procedure. Significance was accepted at $P<0.001$.

Analysis was carried out SAS software (SAS Institute Ins., SAS, Version 8, Cary, NC: SAS Institute Inc., 2000).

3.1d Butyrate effect at epigenetic level

Butyrate's anti-inflammatory effects on mucosal and its regulatory role in immunity system will be investigated by molecular analysis. Moreover, the epigenetic effects of dietary butyrate in sea bass will be detected by proteomic analysis of the acetylation state of hepatic core histones and by hepatic and intestinal expression of genes related to epigenetic modifications.

3.1d.1 Fish and experimental set up

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

After removing fish deviating from the average weight of approximately 15 g, we distributed fish into six experimental tanks of 600 L each at a density of 35 fish per tank (3 replicates) and let them to acclimate over a period of 1 week. There were no significant differences in fish weight between the experimental tanks at the onset of the experiment ($P>0.05$).

3.1d.2 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 fiberglass tanks of 600L and four rectangular tanks of 2500 liters, connected to a central main biofilter of 350L. The light source was the natural photoperiod enhanced with fluorescent 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.

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

3.1d.3 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. In addition to being used as is, this diet was supplemented with 2 g/kg (2%) of sodium butyrate to produce the experimental butyrate diet. Information about each diet composition is presented in Table 3.3. Diets were prepared using small-scale machinery for mixing ingredients and preparing 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 duplicate (2 tanks/diet). Fish were fed twice a day and feeding rates were restricted to 3.0% of biomass during the feeding experiment based on four-weekly fish weight measurements to adjust the feed ration to a similar percentage of fish biomass in both treatments. The feeding trial lasted 8 weeks. Fish specific growth rate (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).

Table 3.3 Composition of the diets in g/100g on a dry 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	0.45	0.45
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	2.00
Na-butyrate	-	2.00
Total	100.00	100.00

3.1d.4 Proteomic analysis

Preparation of liver nuclear protein fraction

Liver nuclear protein extracts were prepared from six fish per group using 3 ml/g of tissue of an extraction buffer containing: 10 mM Tris/HCl, pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM Pefabloc® (SIGMA-ALDRICH®), 0.5 mM DTT, 1 mM Na_3VO_4, 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 −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, histones were eluted 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 according to the instructions of the Acetyl Histone Antibody Sampler Kit (Cell Signaling) and the protocol applied by Mátis *et al*. (2013). Histone proteins were diluted by 2x SDS and β-mercaptoethanol containing loading buffer (supplemented with 50 mM DTT), sonicated for 15 sec, 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 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 check 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, secondary antibodies in PBST containing 5% fat-free milk. Signals were detected using an enhanced chemiluminescence system (SuperSignal®west Dura Extended Duration Substrate, Thermo Scientific) and then exposing them 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).

3.1d.5 Molecular analysis

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 quality 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)

We applied two strategies for real-time PCR primer design: 1) 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); 2) for the genes not cloned yet, exon sequences from other fish species (stickelback or tilapia) were taken from the Ensembl Genome Browser (http://www.ensembl.org/) and blasted against the European sea bass genome database (Tine et al., 2014). Only when the match was annotated in the sea bass genome the exon was considered for primer design (Table 3.4). Primer efficiency was checked by analyzing the slope of a linear regression from six different dilutions. Efficiencies ranged from 1.8 to 2.4. In addition, the correct binding of the primers was checked by adding a melting-curve analysis (95ºC for 15 s, 60ºC for 15 s and 95ºC 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: 50ºC for 2 min; initial activation step: 95ºC for 10 min; 40 cycles of 15 s denaturation at 95ºC and 1 min annealing/extension at 60ºC). A final dissociation step was also added (95ºC for 15 s and 60ºC 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.

Table 3.4 Quantitative real time PCR primer characteristics

Gene	Forward	Reverse	Product size (bp)	Efficiency	Gene bank accession number
il1β	GCTCGGACAGATGCAAAGTC	CTTTGTCCACCACCTCCAGA	165	2.3	AJ269472.1
il6	ACTTCCAAAACATGCCCTGA	CCGCTGGTCAGTCTAAGGAG	176	2.0	AM490062.1
il8	GTCTGAGAAGCCTGGGAGTG	GCAATGGGAGTTAGCAGGAA	154	2.0	AM490063.1
il10	CGACCAGCTCAAGAGTGATG	AGAGGCTGCATGGTTTCTGT	198	1.8	AM268529.1
irf1	GTCATGCTCACACTGACCCA	AGTTTCTTCTGGCGCTGACT	197	2.4	*
muc2	TCCATTTCCACCTACAACACAG	ACATCAAGCTCCAATCCAAAGT	183	2.0	*
tnfα	CTCAACACAGCGGATATGGA	TGGGGATCTGTTTTCTCAGC	171	2.0	DQ070246.1
18S	Diaz and Piferrer (unpublished)		160	2.0	AM419038.1
dicer1	Diaz and Piferrer (unpublished)		226	2.0	*
ehmt2	Diaz and Piferrer (unpublished)		212	2.2	*
hdac11	Diaz and Piferrer (unpublished)		237	2.2	*
jarid2a	Diaz and Piferrer (unpublished)		198	2.0	*
pcgf2	Diaz and Piferrer (unpublished)		235	2.1	*

* These genes are not cloned in European sea bass and primers were designed by blasting gene sequences from other close-related fish species against sea bass genome. Only a match with a clear annotation at the sea bass genome was used for primer design.

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 (2008) method.

qRT-PCR statistical analysis

qRT-PCR analyses were performed using 2^ddCt values in IBM SPSS Statistics 19 software. Data were checked for normality and homoscedasticity of variance; outliers were eliminated when needed.

Treated versus control groups, in liver and intestine, were analyzed in two ways: 1) by analyzing fold-change differences with respect to the controls (Schmittgen and Livak, 2008) 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.

3.2 Taurine

The substitution of fishmeal with vegetable meals did not lead only to intestinal damages but also to nutritional deficiencies and consequently reduced performances. Therefore, TAURINE is representing a potential nutraceutic to mitigate poorer performances in carnivorous fish species. Beside this positive effect on growth, taurine possesses antioxidant effect on reactive oxygen species (ROS) production.

Our research will be dedicate to the taurine's effects on increase of ROS production due to swimming performance of European seabass. The ROS level will be measured by the monitoring of respiratory burst activity where forced swimming will be an amplifier of stress.

3.2a Animals

Sub-adult seabass (*Dicentrarchus labrax*) sizing 92.57±3.842 g, sizing 19.38±0.34 cm as standard length (21.26±0.35cm as total length), were previously acclimatized in 2.5 m^3 rectangular tanks connected to a water recirculation system, fed with a commercial feed for seabass (Raanan Fish Feed Ltd.). Then two groups of 14 fish were individually tagged and furtherly acclimatized during 30 days in circular tanks sizing 800L connected to the same RAS.

The water parameters were continuously monitored: temperature 20±2°C, pH 8.56, total N-NH-3< 0.2 mg L^{-1}, N-NO^{-2} <0.02 mg/L, salinity 22g L^{-1}. Dissolved oxygen (DO) was maintained 99-100 % of the saturation value, by adding pure O_2 to the system.

After an individual assessment of RB at time zero (t_0), the two groups were fed with different diets, one being still the previous diet (C), while the second group received a 1.5% addition of taurine (T). A second RB assessment on resting fish (t_1) has been carried out after 15 days of different diet supply. Following further 10 days resting, a third RB assessment followed on fish individually swimming (t_2), testing two fish per day. At the end of the t_2 cycle, further 15 days resting were allowed before starting with a second swim test (t_3), sampling the fish in the same order of t_2, to ensure all the seabass the same resting time. When swimming test started, 2 fish per day were challenged, one from the control (C) and the second from the challenged tank (T).

3.2b Diets

Two diets reported in table 3.5 were applied in the two populations of seabass: a control diet (C) and a test group receiving diet with or without 1.5% of taurine (T). Fish were fed once a day every morning (1% body weight/day) with reformulated isoenergetic commercial feeds. All diets were prepared in our laboratory by mixing the ingredients in a blender, then grinding the product into 2-mm wide strains that were cut and dried at 45°C. The taurine used in the experiment was organically produced at Julita Farm (Julita, Sweden).

Table 3.5 Formulation of control (C) diet and taurine 1.5% (T) diet.

Raw material	C (%)	T (%)
Full fat soy	12.8	12.8
SPC	13.6	13.6
Wheat	8.0	8.0
Wheat gluten meal	8.19	8.19
DCP	1.72	1.72
Mixed oil	12	12
Lysine (98%)	0.29	0.29
Vitamins & minerals premix	0.4	0.4
Corn gluten	16.0	14.5
Soybean meal (48%)	16.7	16.7
Fish meal (65%)	10.0	10.0
Anti moulds	0.1	0.1
Protein	45.0	45.0
Fat	16.0	16.0
Fiber	2.3	2.3
Ash	6.4	6.4
Calcium	1.0	1.0
Total Phosphorus	0.95	0.95
Methionine	0.9	0.9
Methionine+cysteine	1.6	1.6
Lysine	2.3	2.3
Taurine	_	1.5

3.2c Duration of the sampling times

The experiment consisted in three interval times (t_0, t_1, t_2 and t_3).

t_0: biometric measurements, blood sampling and fish respiratory burst was individually monitored at t_0.

t_1: after 15 days of feeding, biometrics measurements, blood sampling and RB assay were carried out.

t_2: after resting 10 days, fish have been tested in the swimming chamber and biometrics measurements and RB data were collected.

t_3: after resting 15 days, the second swimming test was performed following the sampling order of t_2. Biometrics and RB data collected.

At the end of experiment, all the seabass were anaesthetized in tricaine methane sulphonate (MS-222; 0.1 g L-1, Sigma) and sampled for further analysis.

3.2d Biometric parameters

At the beginning and at the ending of each time, fish were measured for the weight (g) and for the total and standard length. All fish were tagged to monitor the incremental growth and the swimming performance during all the experiment.

3.2e Critical swimming speed protocol

3.2e.1 Respirometer

An AutoTM Resp, Loligo® Systems (Tjele, Denmark) with a 10L swimming chamber (10 cm×10 cm×40cm) was utilized for swimming test, where individual fish was placed during the experiment. A flush pump allowed exchange of water between the respirometer and the buffer tank, in which physio-chemical characteristics of the water (temperature, salinity, oxygen) were controlled using a thermo regulator (20.0±0.1°C), an air pump and a filter. Water oxygen saturation in the respirometer was measured with an oxymeter (OXY-REG)

interfaced to the PC via an RS port 232, and an oxygen probe (Galvanic oxygen electrode MINI-DO) placed in a sealed measure chamber, which continuously received water from the respirometer. The temperature was measured by one channel of TEMP-4 consisted of four channels instrument for monitoring, recording and controlling water temperature with AutoResp™ or TempCTRL software) and the temperature sensor Pt100/Pt1000 was also placed in a sealed chamber. A data-acquisition system recorded continuously oxygen saturation in the respirometer (mean oxygen saturation±s.e.m. = 98.4± 0.4%).

The swimming training was performed at t_2 and t_3 where each fish was tested in order to assess oxygen consumption (MO_2), critical swimming speed (U_{crit}). The U_{crit} values for both the t_2 (U_{crit1}) and t_3 repeated (U_{crit2}) were calculated using the following formula (Brett 1964; Plaut 2001).

Ucrit = Ui+ [Uii (Ti /T ii)]

Where Ui is the highest velocity maintained for the entire interval (cms-1),

U_{ii} is the velocity increment (cm s-1), t_i is the time elapsed at fatigue velocity (s), and t_{ii} is the prescribed interval time (s). The critical swim speed values (U_{crit}) for each fish were calculated in cm/s and adjusted for standard fish length to present the U_{crit} in body lengths per second (BL s-1). No correction for the solid blocking effect (an animal swimming in a channel obstructs the flow, causing water to run faster past the swimming animal. This results in a fractional error, (i.e. a difference in water velocity depending on the presence and size of flow obstructed by animals) of the fish was considered, as the total cross-sectional area of the fish did not exceed 5% of that of the swimming chamber (Bell & Terhune 1970).

The same recovery time (15 days) was passed between t_2 and t_3 for each fish. The t_3 could be considered a replicate of t_2 and the interesting t_2 results were validated in t_3.

Seabass were transferred to the swimming respirometer, covered with a black net and they were exposed to progressive increments in swimming speed about 20, 40, 60, 80 and then 100 cm s-1 but calibrated on each fish in order to develop 0.7, 1.4, 2.1, 2.8, 3.5 BL s-1 (Dupont-Prinet *et al.*, 2009). Instantaneous oxygen uptake (MO_2, in mg kg−1 h−1) was measured twice every 30 min by intermittent stopped-flow respirometry (Steffensen, 1989). The swimming chamber was automatically set to complete three phases: 7 minutes of flushing, 1 minute of waiting and 7 minutes of measuring oxygen level, hence two loops for each speed increment. Fish were considered to be fatigued when they were unable to remove themselves from the posterior screen of the swimming chamber despite gentle encouragement

by sudden increases in current velocity (Chatelier *et al.,* 2005). After forced swim, fish were undergone taking blood sample and put in another tank recovering 24h. The mean of the two MO_2 values was calculated for each speed until fatigue. No correction for the solid blocking effect of the fish was considered, as the total cross-sectional area of the fish did not exceed 5% of that of the swimming chamber (Bell & Terhune 1970).

3.2f Respiratory Burst activity

0.5ml of blood was collected in a tube containing anticoagulant, then diluted blood with an equal volume of medium Hank's Balanced Salt Solution (HBSS) (1:1). 0.75 ml of Lympholyte®-Mammal was added to the centrifuge tube; centrifuged for 20 minutes at 800g at room temperature. After centrifugation, there will be a well-defined lymphocyte layer at the interface. Carefully, the cells were removed using a Pasteur pipette, from the interface and transferred to a new centrifuge tube. Then, diluting the transferred cell with medium to reduce the density of the solution. Another centrifugation was made at 800g for 10 minutes to pellet the lymphocytes, and then discarded the supernatant.

Induction of the respiratory burst (RB) activity in blood was measured directly from heparinised blood, following the method described by (Nikoskelainen *et al.,* 2005)

100 µl of diluted blood (1:25) in HBSS (Hanks balanced salt solution, pH 7.4) was dispensed in white flat-bottomed 96-wells, and incubated with 100 µl of a freshly prepared luminol suspension (2 mM luminol in 0.2 M borate buffer pH 9.0) with 1 µg ml-1 phorbol myristate acetate (PMA, Sigma) for 1h at 24–25°C. Luminol-amplified chemiluminescence was measured every 3 min with a plate luminescence reader for generation of kinetic curves. Each sample was run by duplicate and read against a blank in which neither blood and nor PMA was added (Guardiola *et al.,* 2014). The integral luminescence in relative light units (RLU) was calculated.

3.2g Statistical analysis

Statistics were performed with GraphPAD PRISM 5.0.

The significance of values of respiratory burst at different times (t_0, t_1, t_2, t_3) was calculated with randomized block ANOVA, considering time as a block. Time and taurine effects with

interaction were performed with two-way ANOVA for body weight, oxygen consumption (MO_2), critical swimming speed (U_{crit}) and following by post-hoc comparison Bonferroni's test.

3.3 Butyrate and taurine

Both feed additives, butyrate and taurine, will be considered in a unique experiment based on the effects on the fish intestine when soybean meal (SBM) is substituted for fishmeal (FM) in European sea bass diet and the mitigating effects that may eventually be obtained by adding either taurine or sodium butyrate to the diet. With this purpose in mind, the intestinal tissue was studied by optical microscopy and transmission electron microscopy (TEM). Moreover, the activity of some genes coding for cytokines in the intestine of European seabass fed with different diets was investigated as well.

The feeding experiments were carried out at the pilot station at the Department of Biotechnology and Life Sciences, University of Insubria, Varese, Italy. A total of 30 European seabass (*Dicentrarchus labrax)* 514 ± 67.4g randomly sampled from 2500-L acclimation tanks were individually biometrized and tagged, then were randomly introduced into 6 circular 750-L tanks, 5 per tank. Fish were fed daily ad libitum, in duplicate, one of the 3 different diets reported in Tab. 1 for a period of 60 days (T60), after which all were sampled. The experimental tanks were connected to a water recirculation system, filled with 20 m3 of water. The entire system was remotely monitored and controlled for water pH, temperature, and dissolved oxygen. Temperature was maintained at 20 ± 0.5°C , salinity at 22 g L-1, pH 7.2, total N-NH3 ≤0.2 mg L-1, N-NO2- ≤0.2 mg L-1, N-NO3- , dissolved oxygen (DO) 8-8.5 mg L-1, and DO saturation over 97%.
Sampled fish were sacrificed with an overdose of tricaine-methasulfonate, (Finquel MS-222 ®, Agent Chemical Laboratories, USA) and weighed. The specific growth rate (SGR) was calculated as follows:
SGR= 100 x [ln (final BW) – ln (initial BW)] /days

Then, either the distal intestine of all fish was sampled and fixed for the histological or immunohistochemical examination in 4% buffered formalin, pH 7.2, or conserved at -80°C for the molecular analyses.

3.3a The experimental feed

All three experimental diets, named C, B, and T, contained the same low percentage of fish meal and elevated percentage of soy proteins, as shown in table 3.6. Diet C was assumed as control, while B and T included 2% of either sodium butyrate or taurine, respectively.

Table 3.6 Diet composition of control diet (C), diet with 2% butyrate supplement diet (B) and diet with 2% taurine supplement (T).

Raw material	C (%)	B (%)	T (%)
Full fat soy	12.8	12.8	12.8
SPC	13.6	13.6	13.6
Wheat	8.0	8.0	8.0
Wheat gluten meal	8.19	8.19	8.19
DCP	1.72	1.72	1.72
Mixed oil	12	10	12
Lysine (98%)	0.29	0.29	0.29
Vitamins & minerals premix	0.4	0.4	0.4
Corn gluten	16.0	16.0	14.0
Soybean meal (48%)	16.7	16.7	16.7
Fish meal (65%)	10.0	10.0	10.0
Anti molds	0.1	0.1	0.1
Protein	45.0	45.0	45.0
Fat	16.0	16.0	16.0
Fiber	2.3	2.3	2.3
Ash	6.4	6.4	6.4
Calcium	1.0	1.0	1.0
Total phosphorus	0.95	0.95	0.95
Methionine	0.9	0.9	0.9
Methionine + cystein	1.6	1.6	1.6
Lysine	2.3	2.3	2.3
Taurine	_	_	2
Butyrate	_	2	_

3.3b Light microscopy

Sampled intestine was fixed by immersion in 4% buffered formalin, dehydrated in a graded ethanol series, embedded in paraffin, and sectioned at 4 µm. After dewaxing and rehydrating the sections, they were consecutively stained with hematoxylyn-eosin. Periodic Acidic Schiff (PAS) and Alcian blue stain at pH 2.5 were used to reveal neutral and acidic mucosubstances, respectively.

3.3c Immunohistochemistry for leukocyte assessment

The indirect immunohistochemical methodology protocol included a primary antibody specific for the leukocyte antigen CD45 and a secondary antibody conjugated with alkaline phosphatase, substrate, and chromogen. The sections (4 µm) of distal intestine were dewaxed and rehydrated according to normal procedures and incubated for 18 hours at 37°C. To avoid false positives due to the chromogen reaction with endogenous alkaline phosphatase, the latter were deactivated with CH_3COOH 20% applied for 15 min at 4°C. To avoid other kinds of false positives due to the antibody reaction with aspecific antigens, the samples were treated with PBS/BSA 2% Tween20 0.1% at environmental temperature for 30 min. Then, the samples were washed 5 x 1 min in phosphate buffer (PBS 1x) before applying the primary antibody (CD45 Antibody, pAb, Rabbit GenScript NJ,USA) at a 1:100 dilution overnight at 4°C. After another 5 x 1-min washing cycles in phosphate buffer, the secondary antibody directed to immunoglobulins belonging to the same species was incubated for 1 h and utilized to produce the primary antibody, anti-rabbit IgG (whole molecule)-alkaline phosphatase antibody produced in goat (SIGMA-ALDRICH). The substrate system (SIGMAFAST BCIP/NBT tablet) was used to develop the reaction, which was monitored at the light microscope. After 15-20 min, a final washing with phosphate buffer was performed to stop the reaction; the glass was mounted with PBS/glycerol 1:1. Negative controls were prepared with samples without applying the primary antibody.

3.3d Electron microscopy

Specimens already fixed in formalin were transferred to a 2% Karnovsky solution at 4°C for 2 hours and then embedded in Epoxy resins. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a TEM Morgagni Philips/FEI electron microscope.

3.3e Semi-quantitative PCR: expression levels of cytokines

Total RNA was extracted from 125 mg of distal intestine (3 fish/ group). Tissue lysis and homogenization were performed in special disposable sterile tubes (gentleMACS M ™, MiltenyiBiotec), in order to minimize the possibility of cross contamination between samples, and using the gentleMACSDissociator (MiltenyiBiotec). After an automated purification process, using the Maxwell® 16 Instrument and Maxwell® 16 Tissue LEV total RNA purification Kit (Promega, Italy) the RNA was isolated. The concentration and purity of total RNA were determined by a spectrophotometer NanoDrop ™ (Thermo Scientific), measuring the absorbance at 260 nm and the absorbance ratio 260/280, respectively. The integrity of RNA was verified by electrophoresis on 1% agarose gel stained with ethidium bromide.

Reverse transcription of 1 µg total RNA from each fish intestine was performed with random decamers in a volume of 20 µl using the High-Capacity cDNA Archive Kit (Life Technologies, Italy) according to the manufacturer's instructions.

Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis was performed in order to quantify the transcript levels of interleukin-1β (IL-1β) (GenBank Acc. No. AJ269472), interleukin-6 (IL-6) (GenBank Acc. No. AM490062), interleukin-8 (IL-8) (GenBank Acc. No. AM490063), interleukin-10 (IL-10) (GenBank Acc. No. AM268529), and tumor necrosis factor α (TNF-α) (GenBank Acc. No. DQ070246) genes. Each PCR reaction was carried out in triplicate using forward and reverse primers selected for target genes and β-actin (GenBank Acc. No. AY148350), which represented the internal normalizer (Tab. 3.7). The reaction mixtures included 1 µl of cDNA, 0.5 L of each specific primer (50 µM), and 12.5 µL of GoTaq 2X Master Mix (Promega) in a final volume of 25 µL. The PCR reactions were incubated at 95 °C for 4 min, followed by 30 cycles of thermal cycling (30 s at

95 °C, 30 s at 55 °C and 35 s at 72 °C). The final cycle was followed by a 5-min extension step at 72 °C.

PCR products were subsequently analyzed on 2% agarose gel, and bands were quantified using Gel Doc acquisition system (Bio-Rad) and QuantityOne software (Bio-Rad). The intensity of each band obtained (INT/ mm2) was normalized based on intensity levels of β-actin. The expression levels of each target gene were documented with reference to expression in control group (C), for which levels were arbitrarily set as equal to 1.

Table 3.7 Nucleotide sequences of the primers utilized for the semi-quantitative PCR.

GENE	GenBank Acc. n°	Nucleotide sequence 5'-3'
IL-1β	AJ269472	F: GCTCGGACAGATGCAAA
		R: CTTTGTCCACCACCTCCAGA
IL-6	AM490062	F: ACATGCCCTGAGAAGTCCAG
		R: CCGCTGGTCAGTCTAAGGAG
IL-8	AM490063	F:GTCTGAGAAGCCTGGGAGTG
		R: CTCGGGGTCCAGGCAAAC
IL-10	AM268529	F: CGACCAGCTCAAGAGTGATG
		R: AGAGGCTGCATGGTTTCTGT
TNF-α	DQ070246	F: CTCAACACAGCGGATATGGA
		R: TGGGGATCTGTTTTCTCAGC
β-ACT	AY148350	F: GAGCGTGGCTACTCCTTCAC
		R: GGTCCTTACGGATGTCAACG

3.3f Statistical analysis

The quantitative data were submitted to ANOVA variance analyses and the Duncan's test used for post hoc analysis, applying the software IBM SPSS Statistics 21. The value $p<0.05$ was considered significant for differences.

3.4 Commercial feeds and product quality

Continuous quality monitoring of feed is a tool to foresee a good final product that is fish fillet. On the other hand, the forecasts on final product are not enough to ascertain the real fish quality because depend on the fish species ability to in vivo fatty acid bioconversion. Indeed, the fillet lipid profile gives us the fish nutritional value. Concerning our interest in quality, we will focus on the fatty acids profile of fillets and feeds of rainbow trout feeding with six commercial diets of 3 fish feed factories, monitoring the possible correlation between the content of fatty acids in fillet and feed. Besides that, amino acid profile of feeds has been performed to detect eventually methionine and lysine imbalances due to dietary plant protein supplementation.

3.4a Animals

The trial was conducted at the Agricultural Company "Fattoria del Pesce s.r.l." located in Cerano (Novara, Italy) where six commercial diets were tested in six tanks reared with rainbow trout (*Onchorynchus mykiss*). Fish, sizing 683 ± 25.75g (body weight ± s.e.m.), were stocked in 5m^3 rectangular tanks and feeding rates were fixed to 1% of biomass. The water temperature was in a wide range of 7°C-17°C.

Four rainbow trout/ tank were chosen to perform fatty acid fillet analysis. Muscle was prepared, immediately frozen and kept at -20°C until analysis.

3.4b Biochemical analysis

3.4b.1 Total lipids extraction

The total lipids extraction was performed according to the procedure of Folch et al. (1957). 5g of rainbow trout fillet was homogenized in 40 mL of chloroform-methanol mixture (2:1 v/v) by Ultra-Turrax homogenizer (T25-IKA-Werke, Staufen, Germany). The sample was subsequently filtered under vacuum through a Whatman No. 1820-047 filter paper and diatomaceous silica. The extract was washed with 33-34 mL of 0.73% (w/v) NaCl, mixed vigorously for 60s, and then left overnight at room temperature. The organic phase was separated and the solvents were evaporated under vacuum at 40 °C.

3.4b.2 Fatty acids saponification and methylation

Fatty acid methyl esters (FAME) were prepared using methanolic KOH. 100 mg of crude extract were mixed with 6 mL of KOH in small sample bottles. The mixture was heated at 150°C for 20 minutes and then cooled to room temperature. 10 mL of BF_3 was added to each samples and heated for 30-45 minutes. The FAME was extracted through a centrifugation at 3000 rpm for 5 minutes with the addition of 10ml deionized water and 5 ml hexane. The lower phase was discarded and 1 mL of hexane and Na_2SO_4 added. The FAME extract was collected in vials.

The FAME was separated in a Thermo Scientific GC/DQII and equipped with a 30m CP Sil-5 CB (0.25 mm i.d., film thickness 1 μm; Varian, Inc., Palo Alto, CA). Helium was used as a carrier gas at a flow rate of 1.4 mL/min. The initial oven temperature was 60°C held for 1 min, which was raised to 150°C at a rate of 30°C/min and further to 235°C at a rate of 5°C/min held for 8 min. The third ramp was raised at the temperature 280°C, at a rate of 4°C/min held for 1 min. The injector and detector temperature were set at 250°C and 270°C, respectively. A sample volume of 1 uL was injected.

The fatty acids methyl esters were identified by comparing the retention time of the samples and appropriate external standard FAME mix (Supelco 37-component FAME mix; Sigma-Aldrich, Milan, Italy).

3.4b.3 Determination of total amino acids feed composition

Samples preparation

Acid hydrolysis was used for amino acids except cysteine (Cys) and methionine (Met) for which performic acid oxidation followed by acid hydrolysis was used and for tryptophan for which alkaline hydrolysis was performed.

Acid hydrolysis (AAT)

About 100 mg of sample was accurately weighed into a pyrex glass tube fitted with teflon-lined screw cap. Ten millilitres of HCl 6M was added and the tube was flushed with nitrogen for 1 min to remove air. Hydrolysis was then carried out at 110°C for 22-24 h; after letting the tubes cool at room temperature, 10 ml of internal standard (L-α-amino-n-butyric acid, AAbA, 2.5 mM in HCl 0.1 M) was added and the content was collected into a 200 ml

volumetric flask, then diluted with Milli Q water. The solution was filtered with 0.45 µm pore size filter and collected into vials for the derivatization.

Performic acid oxidation (AAS)

About 50-70 mg of sample was weighed into a pyrex glass tube fitted with teflon-lined screw cap and put into ice bath for 30 min. After adding 2 ml of performic acid, samples were kept in an ice bath for 16 h. Then, 0.3 ml of hydrobromic acid were added to remove excess of performic acid and after 30 minutes a vacuum system was used to remove the bromine formed during the reaction. Oxidized sample was then hydrolyzed with HCl 6M (Moore, 1963)

3.4b.4 Derivatization

Ten microliters of filtered hydrolysated sample (and standard) were transferred to a 1.5 ml vial with teflon-lined screw cap; 70 µl of borate buffer were added, because the optimal pH range for derivatization is 8.2 – 9.7, and the solution was briefly vortexed. Then, 20 µl of reconstituted AccQ.Fluor reagent (3 mg/ml in acetonitrile) was added and the mixture was immediately vortexed for several seconds. The vial was closed and left to stand for one minute at room temperature. Then, it was heated in a heating block at 55°C for 10 min; derivatives were stable at room temperature for up to 1 week.

3.4b.5 High performance liquid chromatography (HPLC) and chromathography conditions.

The HPLC analysis has been performed with a ternary elution system. Eluent A was sodium acetate buffer adjusted at pH 5.05 with dilute phosphoric acid or sodium hydroxide. Eluent B was HPLC-grade acetonitrile, and eluent C was Milli-Q water. Detection was accomplished by fluorescence with excitation at 250 nm and emission at 395 nm. The output of the signals was adjusted by setting the gain value of the detector at 10. Injections were made every 45 min, using an injection volume of 10 µl.

HPLC analysis for amino acids was performed with an LC 200 Perkin Elmer pump fitted with an ISS-100 auto sampler with 20 µl loop and a spectrofluorimetric detector (Perkin Elmer, Norwalk, Connecticut, USA).

Turbochrom software with two NCI 900 PE Nelson Perkin Elmer interfaces were used for the data collection and integration. Separation was achieved by using one AccQ Tag Amino Acid Analysis column (Waters Corporation Milford, MA, USA) and one Waters pre-column filter. The column temperature was set at 37°C for total amino acids (AAT) analyses and at 31°C for sulphur amino acids (AAS) and tryptophan analyses with a column block-heater manufactured by Waters.

A thermoblock SICAL was used in the derivatization step.

A vacuum system B-721 Buchi (Labortechnic AG, Switzerland) was used for the sample preparation step in the determination of methionine and cysteine.

All solvents were filtered using a Millipore (Milford) system with 0.22 μm pore size membrane filter, instead the samples with 0.45 μm (VWR, International, West Chester, Pennsylvania, USA). An air-circulation drying oven (Heraus, Hanau, Germany) was used in the sample hydrolysis.

During HPLC analysis, the mobile phases were degassed by bubbling helium.

Chromatographic separation was carried out in a Waters AccQ Tag Amino Acid Analysis column fitted with an In-line filter pre-column. The column was thermostated at 37°C (AAT) or 31°C (AAS) and the flow rate was 0.8 ml/min. The injection volume was 20 μl.

Mobile phase A consisted of acetate-phosphate aqueous buffer, mobile phase B was acetonitrile 100% and C was Milli-Q water. Gradient conditions were shown in table 3.8. Before beginning the gradient, the column was equilibrated in 100% A for 10 min. After the last analysis of the day, the column was washed for 30 min with 100% Milli Q water, then conditioned for 15-20 min with acetonitrile:water (60:40). If the column has to be stored for more than 72 h, it was kept in 100% of acetonitrile. Detection was carried out by fluorescence (λ EX 250 nm, λ EM 395 nm).

Table 3.8 Gradient table for amino acids elution

STEP	MIN	A (%)	B (%)	C (%)
0	In.(10)	100	0	0
1	0.5	99	1	0
2	18	95	5	0
3	25	92	8	0
4	38	82	18	0
5	48	81	19	0
6	50	0	60	40
7	55	0	60	40
8	56	100	0	0

3.4c Statistical analysis

During the experimental test 24 rainbow trout were fed with six different animal feeds (4 fish tested each group). The amount of fatty acids (C14:0, C16:0, C16:1, C18:0, C18:1n9, C18:2n6, C20:0, C18:3n-6, C20:1, C18:3n-3, C20:2n6, C20:3n6, C22:1n9, C20:4n6, C20:5n3, C22:6n3, C18:4n3, C22:5n3, C20:4n3) found in the 24 rainbow trout fillets were analysed by GLM procedure using the SAS software (SAS Institute Inc., SAS, Version 8, Cary, NC: SAS Institute Inc., 2000).

The six animal feeds were considered as sources of variation according to the following linear model:

$$y_{ij} = \mu + A_i + e_{ij}$$

where :

y_{ij} = dependent variable (fatty acids of rainbow trout fillets);

« = overall mean;

A_i = fixed effect of the animal feed ($i = 1,6$);

e_{ij} = residual random effect of each observation.

A Pearson's correlation was estimated between fatty acids content in animal feeds and in rainbow trout fillets in order to ascertain their association; moreover, to observe if the quantity of a single fatty acid could have some effect on this association a Spearman's correlation was calculated between the rank of the six Pearson coefficients separately estimated in the six animal feeds and the corresponding rank of each fatty acid content. The correlations were performed by PROC CORR (SAS Institute Inc., SAS, Version 8, Cary, NC: SAS Institute Inc., 2000).

4. RESULTS

4.1 Butyrate

4.1a Analytical methods

4.1a.1 GC-MS analysis

The headspace method in GC-MS resulted insufficient to detect the low amount of butyric acid in fecal samples of seabass feeding with 4 diets different in vegetable meals content.

4.1a.2 HPLC analysis of butyric acid quantification

The HPLC resulted efficient to quantify butyric acid in culture medium of feces.
The method was validated on TC-C18 column (150mm x 4.6 mm i.d.) with particle sizes of 5µm because a good selectivity combined to a reasonable run time (15 minutes).
LOD and LOQ values of butyric acid were 0.005 mM and 0.015 mM, respectively.

4.1b DGGE of fish gut microbiota

DGGE (Fig.4.1) showed the biodiversity of gastrointestinal microbiota of seabass feeding with 4 different diets. All four microbiota reported two bacteria species *Aliivibrio sp.* and *Vibrio sp.* (bands 2 and 3) able to produce butyric acid. *Uncultured spirochete* appeared (bands 4 and 5) in all diets as well as *Enterobacteriaceae* (Table 4.1).
Photobacterium aquimaris (band 8) were retrieved in diet A and B, while in the others 2 diets seemed low displayed.

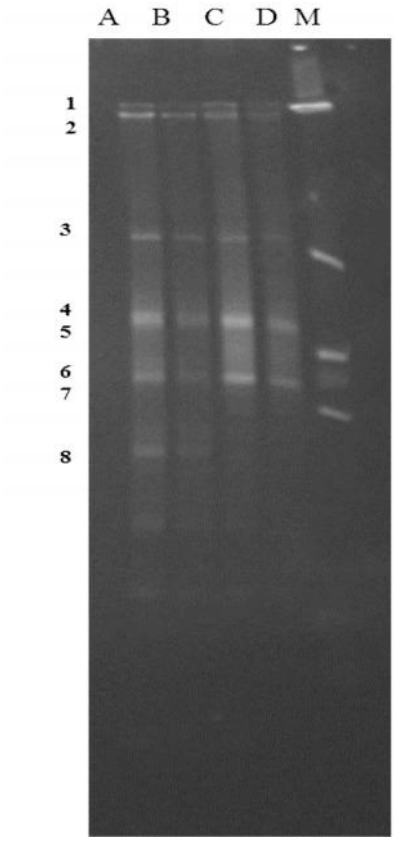

Fig 4.1 PCR-DGGE profiles from 16rRNA gene of V6-V8 variable regions, representing biodiversity of fecal content seabass. Some taxa were identified by sequencing bands 2 and 3: *Vibrio spp.*

Table 4.1 Bacteria found in seabass fecal samples, according to their homology to 16SrRNA partial sequences in Genebank.

	Bacteria	NCBI ACC. N°	Butyrate synthesis
1	*Enterobacteriaceae bacterium SR5*	JF495478.2	
2	*Aliivibrio sp.*	FR744854.1	YES
3	*Vibrio sp.*	JF792689.1	YES
4	*Uncultured spirochete*	DQ340184.1	NO
5	*Uncultured spirochete*	DQ340184.1	NO
6	*Photobacterium aquimaris*	AB428877.1	
7	*Photobacterium aquimaris*	AB428877.1	
8	*Photobacterium aquimaris*	AB428877.1	

4.1c Prebiotic effects on butyric acid production and on fish immunity state

Quantification of butyric acid derived from anaerobic growth of fecal samples is reported in figure 4.2.

Chitosan and novelose330 seemed to be good substrates to fermentation with 0.3mM and 0.288mM of butyric acid, respectively. Nevertheless, negative control (no prebiotics addition) recorded 0.286 mM of butyric acid.

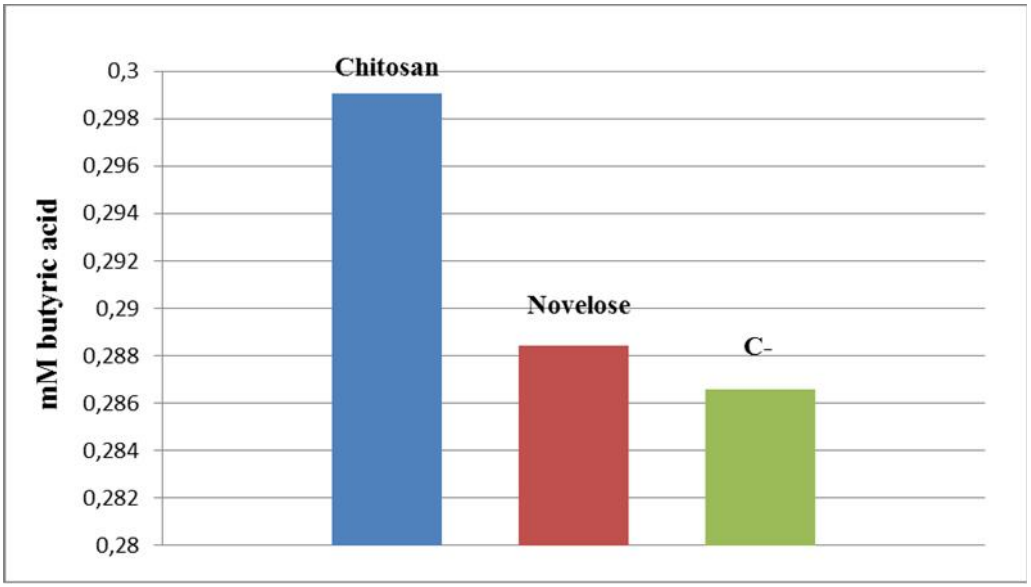

Figure 4.2 Butyric acid quantification in cultured feces of seabass fed with chitosan, novelose and of rainbow trout fed with insect meal (chitin) and without it (no chitin). The negative control represented fish feces without prebiotic.

4.1c.1 Anaerobic growth of feces

In figure 4.3, feces samples of seabass cultured on prebiotics substrates were processed to quantify butyric acid.

Chitosan resulted the prebiotic more efficient to induce butyric acid production (0.4 mM) respect to the others substrates. This *in vitro* condition confirmed the previous growth result about chitosan.

Betaglucan and the negative control gave the lowest butyric acid production; novelose and sucrose could be considered medium butyric acid producers. Inulin reported an increased amount of butyric acid (0.31 mM) respect to the low and medium butyric acid producers.
No statistical analysis could be performed because not enough samples were available.

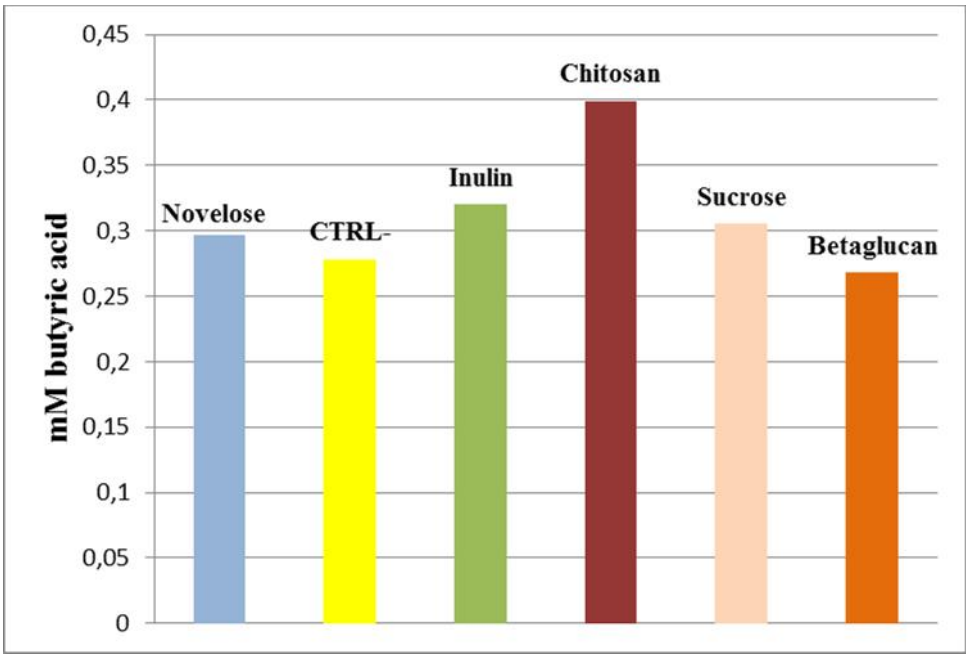

Figure 4.3 Butyric acid detection in feces cultured with different prebiotics substrates. Not enough samples to determine the significance.

4.1c.2 Respiratory burst activity

Respiratory burst (RB) activity (Fig.4.4) was performed in blood samples of seabass feeding with chitosan, inulin and novelose 330.
Seabass feeding chitosan showed low values of RB with a significance $P \leq 0.001$ respect to seabass feeding inulin and novelose 330.

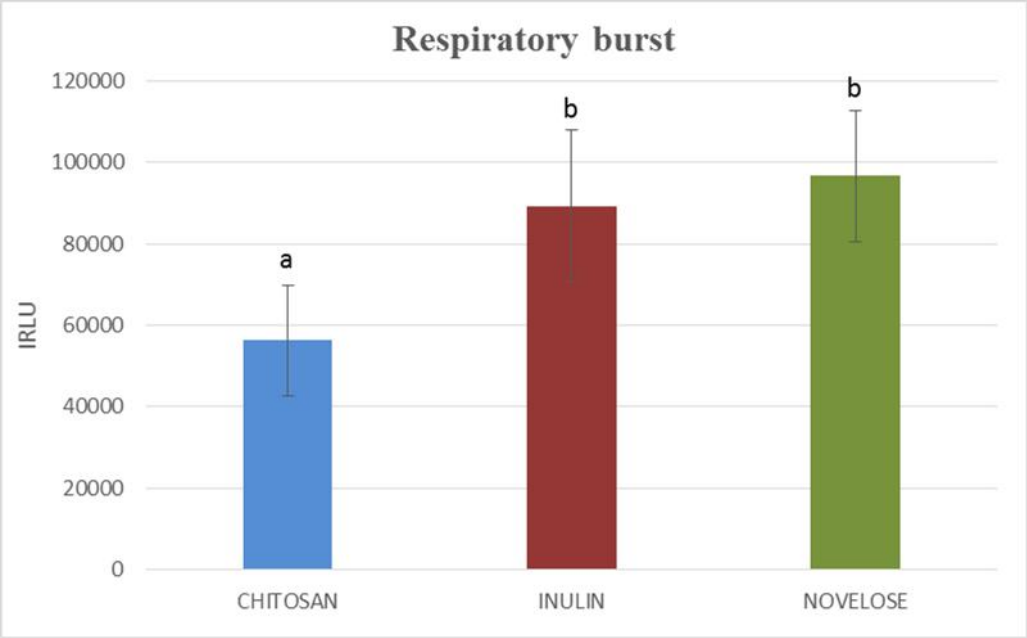

Figure 4.4 The graphic shows the respiratory burst activity after PMA stimulation of lymphocytes in chitosan group (n=5), inulin (n=6) and novelose group (n=6).

Letters a,b mean a significance differences for P<0.001.

4.1d Butyrate effect at epigenetic level

Effect of butyrate on growth performance

The initial weight of 14.91±1.73 g of the control fish group (Figure 4.5) increased to 20.63±4.17 g after 4 weeks of feeding and to 30.22±5.61 g after 8 weeks of feeding. The difference with respect to time zero's mean body weight became significant only at the end of the feeding experiment. Fish of the other group 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. In this group, too, the increase in body weight became significantly different from time zero value only at the end of the fattening trial. However, the differences in fish growth performance between treatments did not reach statistical significance (Figure 4.7). Survival was high (around 95%) with no significant differences between the fish groups 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.

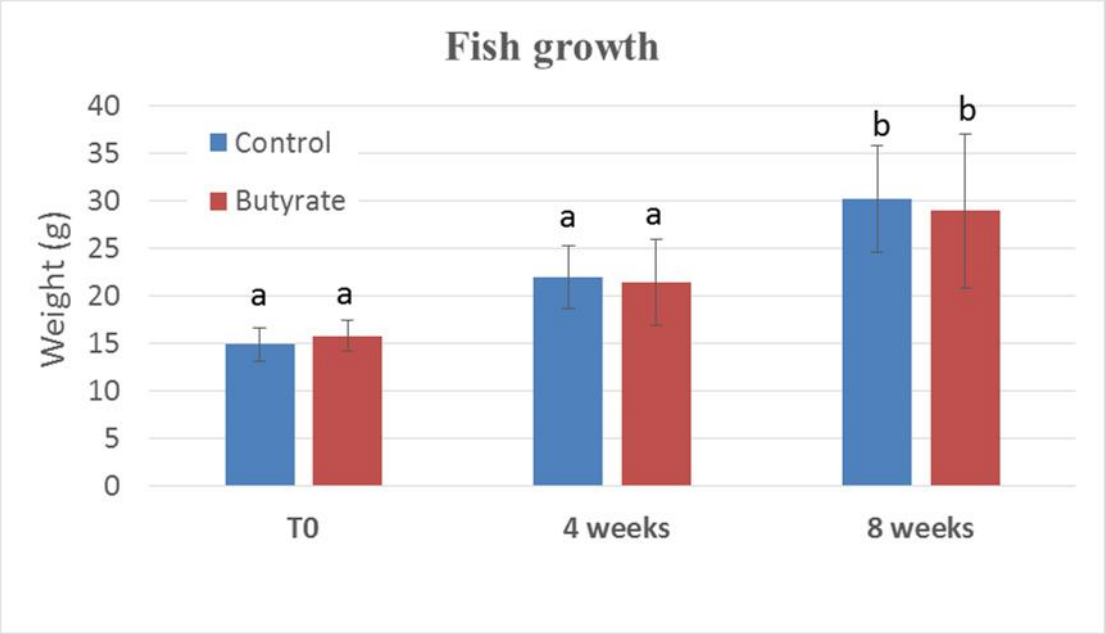

Figure 4.5 Effects of dietary butyrate on fish growth.

*The data were tested by ANOVA followed by Tukey's HSD test to determine whether there were any significant differences between the means of different groups. Different letters indicate significant differences ($P < 0.05$).

4.1d.1 Proteomic analysis

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. The result of this analysis is presented in Figure 4.6, whereas the intensity values (OD*mm^2) of each band are reported in Table 4.2. Among the primary antibodies furnished by the Acetyl-Histone Antibody Sampler Kit (Cell Signaling Technology) only anti-H2A (not acetylated form), anti-H3 (acetylated and not), and anti-AcH4 (acetylated form) worked correctly in sea bass. In contrast, anti-H2B, anti-AcH2B, anti Ac-H2A, and anti-H4 antibodies did not recognize any epitope in sea bass. The kit manufacturer guaranteed cross-reactivity with fish (zebrafish) only for anti-H3 (acetylated or non-acetylated forms) and anti-H4 (non-acetylated) antibodies, but only the former worked properly in our species.

Immunoblotting on hepatocyte core histone extracts (Table 4.2) 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 acetylation of histone H4 at lysine 8 ($P<0.05$), leading to an approximately threefold increase in comparison to the control group (no butyrate) (Table 4.2). 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 (Matis *et al.*, 2013).

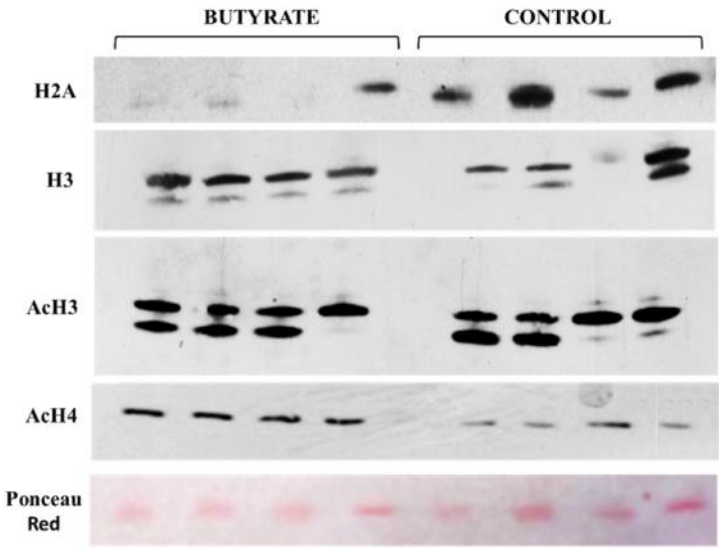

Figure 4.6 Effects of butyrate on acetylation state of isolated hepatocyte histones in sea bass*

*One–dimensional immune-blotting analysis of H2A, H3, and H3, H4 acetylated histones are shown. At histone H3, the upper band represents the H3.1 isoform and the lower the H3.2. Ponceau staining was used as loading control.

Table 4.2 Quantification of core histone protein expression (Vol OD*mm^2)

(*) indicates significant difference between two fish groups ($P < 0.05$).

	Vol OD*mm^2	
	BUTYRATE	**CONTROL**
H2A	1.12 ± 1.10*****	5.46 ± 2.62
H3	5.85 ± 2.19	8.03 ± 0.40
AcH3	6.42 ± 1.33	8.36 ± 1.20
AcH4	1.51 ± 0.23*****	0.51 ± 0.31

4.1d.2 Molecular analysis

Genes related to epigenetic regulatory mechanisms

Regardless of treatment, a 2-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 4.3), 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, maybe due to high variability found between one fish and the other. Furthermore, regardless of tissue, *ehmt2* showed significant differences due to the 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 4.7 A-C.

Table 4.3 Statistical analysis of the expression of genes related to epigenetic regulatory mechanisms

Gene	Intestine	Liver	Intestine		Liver		2- way ANOVA	
	FC ± SEM^{+}	FC ± SEM^{+}	Student t-test		Student t-test			
dicer 1	2.40 ± 1.864	5.88 ± 3.393	t	0.548	t	1.068	F (Ts)	14.661 (*P*=0.001)***
			p-value	0.596	p-value	0.313	F (Tr)	0.025 (*P*=0.875)
							F (Ts x Tr)	2.219 (*P*=0.050)*
ehmt 2	1.18 ± 0.869	2.52 ± 1.617	t	0.498	t	1.421	F (Ts)	61.878 (*P*=0.000)***
			p-value	0.629	p-value	0.169	F (Tr)	13.426 (*P*=0.002)**
							F (Ts x Tr)	8.093 (*P*=0.010)**
pcgf2	1.29± 0.932	14.75 ± 9.485	t	0.114	t	-0.15	F (Ts)	7.211 (*P*=0.014)*
			p-value	0.911	p-value	0.886	F (Tr)	0.003 (*P*=0.096)
							F (Ts x Tr)	0.024 (*P*=0.878)
jarid 2a	2.66 ± 2.186	11.06 ± 4.028	t	0.893	t	0.898	F (Ts)	6.159 (*P*=0.022)*
			p-value	0.395	p-value	0.378	F (Tr)	0.825 (*P*=0.374)
							F (Ts x Tr)	0.385 (*P*=0.542)
hdac 11	0.49 ± 0.200	1.67 ± 0.774	t	-1.388	t	1.791	F (Ts)	45.051 (*P*=0.000)***
			p-value	0.19	p-value	0.099	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). +qRT-PCR gene expression fold change (butyrate-treated versus control fish

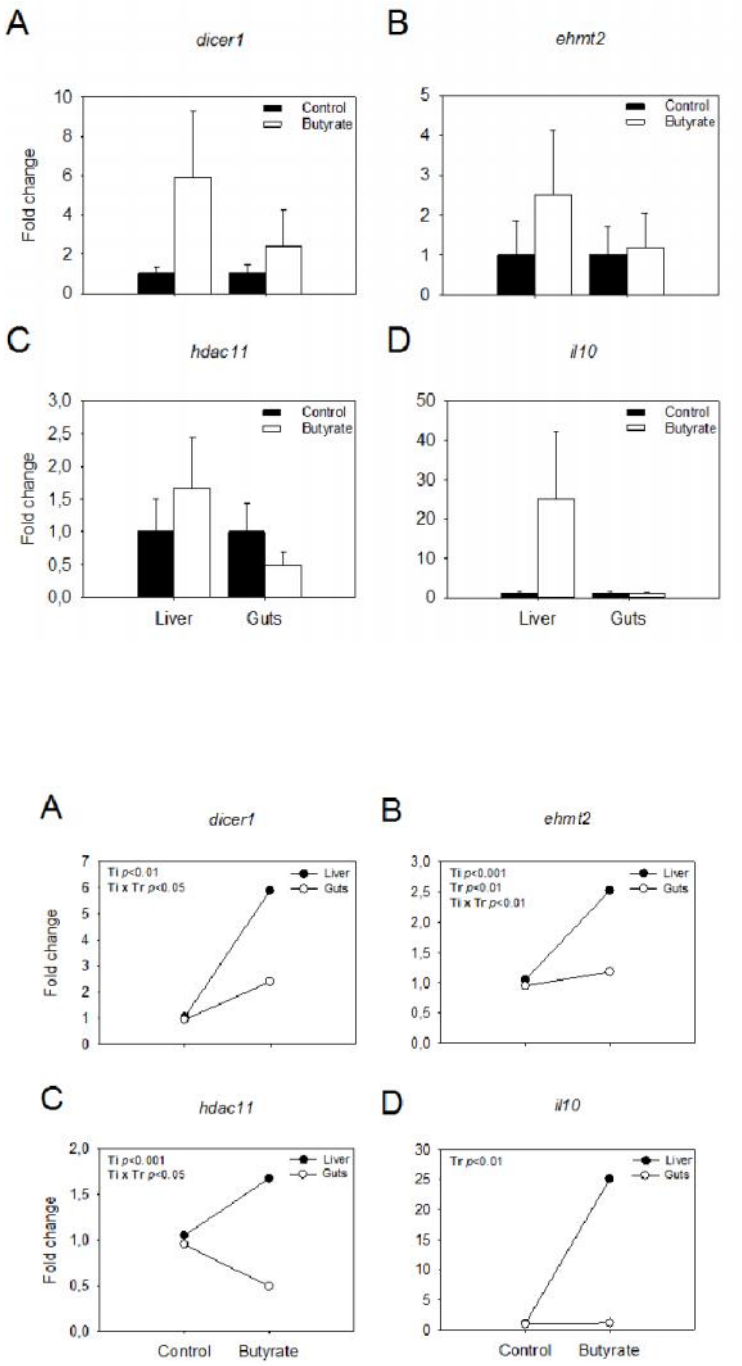

Figure 4.7 Effects of dietary butyrate on gene expression in two tissues of the European sea bass: liver and intestine, as determined by qRT-PCR analysis. Only those genes that showed statistical differences for the interaction between tissue and treatment (A: dicer1, B: ehmt2 and C: hdac11), or differences in expression solely due to the treatment (D: il10) are depicted.

Genes related to mucosal protection and inflammatory response

Statistical analysis by 2-way ANOVA revealed that the expression of four (*il1β*, *il8*, *irf1*, and *tnfα*) out of seven target genes related to inflammatory response and immune system was 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 4.4). This effect was also demonstrated with pairwise comparisons using Student's *t*-test (P=0.002). In contrast to what was observed with the epigenetically related genes and with the exception of *il10* in the liver (fold change 25.09±17.18; Fig. 4.7D), the magnitude of fold change in the other two genes (*il6*, *muc2*) was lower (range 0.01-4.74). Furthermore, in contrast to the epigenetically 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.

Table 4.4 Statistical analysis of the expression of genes related to inflammatory response, mucosal protection and immune homeostasis.

Gene	Intestine	Liver	Intestine		Liver		2- way ANOVA	
			Student t-test		Student t-test			
	FC ± SEM+	FC ± SEM+						
il1β	1.83 ± 0.693	0.07 ± 0.035	t	1.208	t	-2.155	F (Ts)	11.368 (P=0.003)**
			p-value	0.255	p-value	0.083	F (Tr)	0.000 (P=1.000)
							F (Ts x Tr)	0.000 (P=1.000)
il6	0.47 ± 0.113	0.01 ± 0.006	t	0.109	t	-1.071	F (Ts)	2.068 (P=0.165)
			p-value	0.915	p-value	0.309	F (Tr)	1.126 (P=0.301)
							F (Ts x Tr)	0.949 (P=0.341)
il8	1.77 ± 0.907	0.69 ± 0.342	t	0.874	t	-0.603	F (Ts)	8.129 (P=0.009)**
			p-value	0.411	p-value	0.560	F (Tr)	0.632 (P=0.435)
							F (Ts x Tr)	0.660 (P=0.425)
il10	1.13 ± 0.242	25.09 ± 17.176	t	1.792	t	3.361	F (Ts)	0.036 (P=0.851)
			p-value	0.098	p-value	0.002**	F (Tr)	10.881 (P=0.003)**
							F (Ts x Tr)	1.007 (P=0.326)
irf1	0.17 ± 0.034	4.74 ± 2.777	t	1.398	t	1.329	F (Ts)	48.930 (P=0.000)***
			p-value	0.19	p-value	0.211	F (Tr)	2.401 (P=0.136)
							F (Ts x Tr)	1.505 (P=0.233)
tnfα	1.54 ± 0.303	0.34 ± 0.323	t	1.761	t	-1.144	F (Ts)	55.649 (P=0.000)***
			p-value	0.109	p-value	0.282	F (Tr)	0.000 (P=1.000)
							F (Ts x Tr)	0.000 (P=1.000)
muc 2	0.74 ± 0.226	0.22 ± 0.146	t	0.553	t	-1.820	F (Ts)	4.241 (P=0.059)
			p-value	0.591	p-value	0.291	F (Tr)	0.148 (P=0.706)
							F (Ts x Tr)	0.070 (P=0.795)

Note: Asterisks mark statistical differences (*P<0.05; ** P<0.01; *** P<0.001). +qRT-PCR gene expression fold change (butyrate versus control)

4.2 Taurine

4.2a Specific growth rate (SGR) and body weight

In table 4.5, the specific growth rates of seabass fed for 75 days were reported as mean (± standard deviation) and resulted not statistically significant ($p > 0.05$).
In Table 4.6, the mean (± standard deviation) of body weight was represented for control group and taurine 1.5% group. An increase in body weight, it was showed in taurine 1.5% group along the times, even though data were not statistically significant ($p>0.05$).

Table 4.5 Specific growth rate (SGR) of seabass fed with control and 1.5% taurine for 75 days.

SGR (%)± SD	t_1	t_2	t_3
CTRL	1.13±0.42	0.78±1.25	0.67±0.60
Taurine 1.5%	1.17±0.97	0.85±1.42	1.17±0.38

($p>0.05$ as assessed by Bonferroni's test post-hoc to two way ANOVA.)

Table 4.6 Means (± S.D.; N=14) of fish body weight at different times *

	t_0	t_1	t_2	t_3
Control	86.43±15.61[a]	102.6±19.83[ab]	104.1±17.47[ab]	108.4±18.72[ab]
Taurine (1.5%)	98.71±23.10[a]	116.4±29.18[b]	118.5±28.08[b]	130.6±34.73[b]

*Letters mean the significance between control group and 1.5% taurine group at the same time level ($p>0.05$ as assessed by Bonferroni's test post-hoc to two way ANOVA.)

4.2b Oxygen consumption (M_{O2})

Fig.4.8 shows the effect of exercise on MO_2 at t_2 where no differences were reported in seabass fed with control and with taurine (1.5%), although a final increment was noted in control group ($P>0.05$). In fig. 4.9a the two lines of two groups are much overlapped, with a slightly separation at final speeds.

In Fig.4.8, at t_3, the level of oxygen consumption was more pronounced in control group while the 1.5% taurine group consumed less oxygen during exercise, even though data were not significant ($P>0.05$). In Fig. 4.9b, it is worth noting the net distinction of two groups, where fish fed with 1.5% taurine achieved a better oxygen consumption performance, in contrast with control diet.

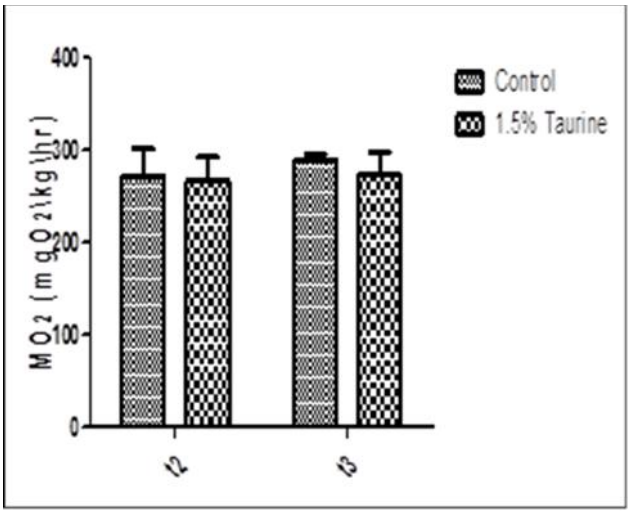

Figure 4.8 The effects of swimming speed on oxygen consumption (MO_2) in control group and 1.5% taurine group.).*

*At each swimming speed, values are means ± s.d. (N=14). ($p>0.05$ as assessed by Bonferroni's test post-hoc to two way ANOVA

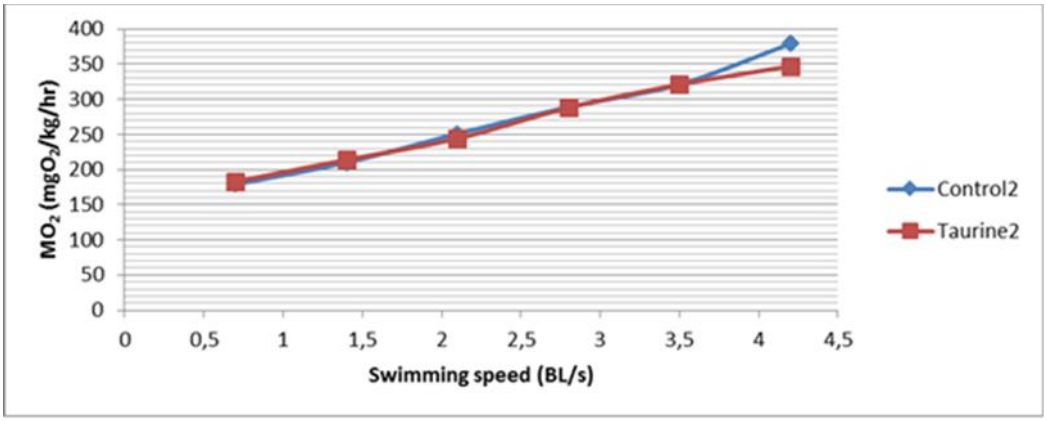

Figure 4.9a Oxygen consumption (MO2) at t2 in seabass fed commercial diet with and without 1.5% taurine.

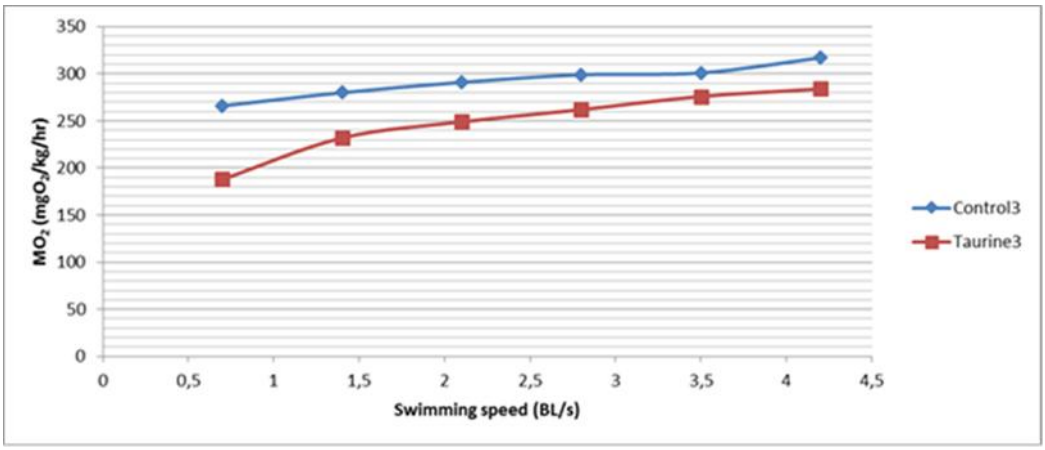

Figure 4.9b Oxygen consumption (MO2) at t3 in seabass fed commercial diet with and without 1.5% taurine.

4.2c Critical swimming speed

In fig. 4.10, at t_2 seabass fed with control diet and the group fed with 1.5% of taurine, achieved respectively a mean (± s.d.) U_{crit} of 3.97± 0.30 and U_{crit} of 4.16± 0.29 BL s^{-1}.

At t_3 the seabass group fed with the addition of 1.5% taurine achieved a significantly mean (± s.d.) U_{crit} 4.60±0.12 BL s^{-1}, whereas the group fed without taurine achieved U_{crit} 4.27±0.07 ($p<0.01$). At the second swimming performance (t_3), both groups of fish showed an increase of U_{crit} means, more displayed in fig. 4.11.

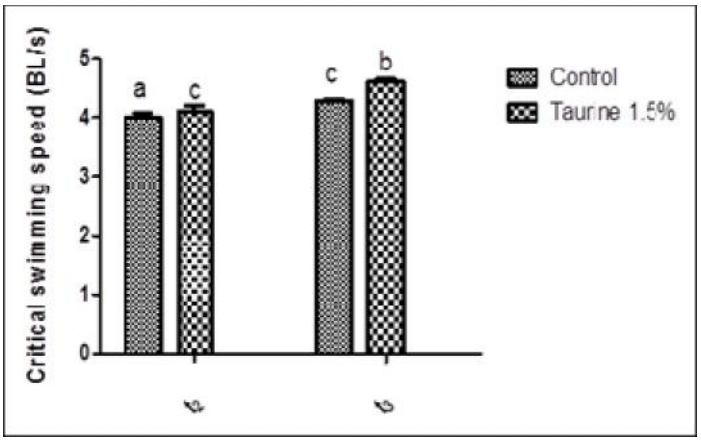

Figure 4.10 Critical swimming speed (U_{crit}) expressed in mean ± s.d. (N=14). Letters mean the significance*

* At t_2, $p>0.05$ as assessed by Bonferroni's test post-hoc to two way ANOVA, at t_3 $p<0.01$ as assessed by Bonferroni's test post-hoc to two way ANOVA.

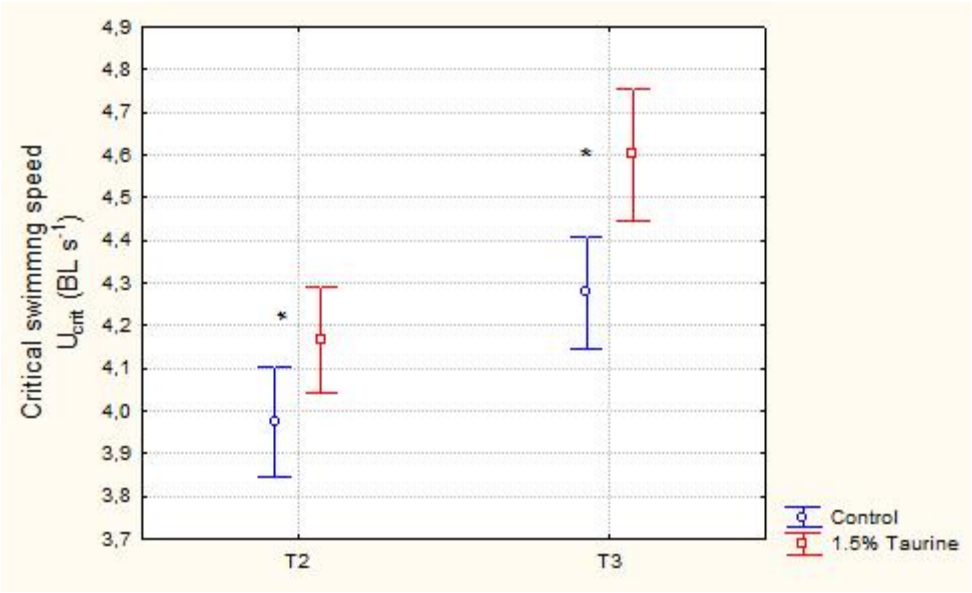

Figure 4.11 Critical swimming speed (U_{crit}) expressed in mean ± s.d. (N=14)*

*Stars mean the significance ($p<0.05$).

4.2d Respiratory burst

Fig. 4.12 shows the respiratory burst activity in the control diet and in the 1.5% of taurine addition diet in different sampling times. No statistical differences were detected between times (days), so fish recovered in the same period. At t_1, after 15 days of taurine administration, a difference between fish fed with control diet and the group fed with the addition of 1.5% taurine was revealed.

Subsequently, at t_2 and t_3, fish were involved in the swimming performance and the assay of respiratory burst was performed immediately after the swim exercise. The respiratory burst activity of lymphocytes, after PMA stimulation, at t_2 and t_3 was lower in fish fed with 1.5% of taurine than in fish group fed with control diet. In fact, seabass fed with taurine showed a constant trend of ROS production, through the time. Furthermore, in control group, the swimming performance increased the respiratory burst activity from t_1 to t_2 and further incremental increase of respiratory burst activity was showed at t_3. Finally the difference of the respiratory burst activity between control group and 1.5% taurine group is statistically significant ($p<0.05$).

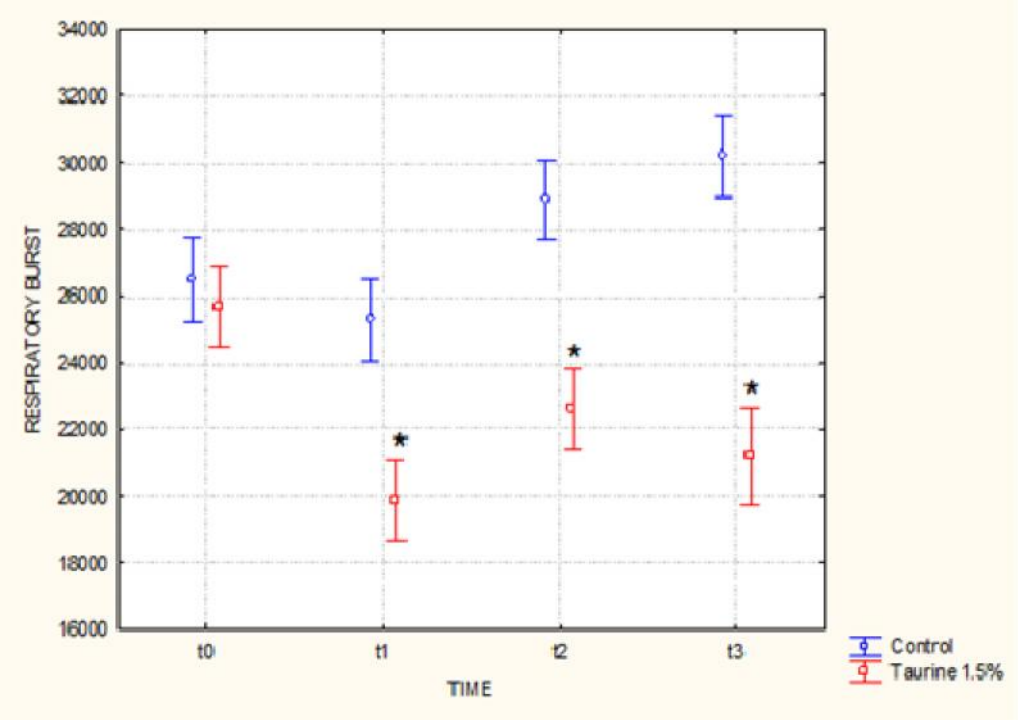

Figure 4.12 The graphic shows the respiratory burst activity after PMA stimulation of lymphocytes in control group and 1.5% taurine group*

*Stars mean statistically different for $p<0.05$

4.3 Butyrate and taurine

4.3a Body weight

Growth performances were found to be significantly different ($P<0.05$) among the fish fed diet T as compared to diets C and B (Tab. 4.7)

Table 4.7 Growth performances of European seabass fed ad libitum for 60 days with a 45% protein diet, containing 10% as FM and 35% as VM.*

	T_0	Diets at T_{60}		
		C	B	T
Biomass (g) ± S.D.	514 ± 67.40	546.8 ± 61.14	540.2 ± 60.95	641.2 ± 60.95
SGR (%)	-	0.12	0.12	0.32

*The differences in SGR were significant ($P<0.05$) only when diet T was compared to each of the other 2 diets.

4.3b Light microscopy and immunohistochemistry

Histologically, the intestinal wall of seabass consists of serosa, muscle layer, submucosa, and mucosa from the outside to the inner lumen. By light microscopy we evaluated different aspects of the various anatomical portions of the distal intestine. The thickness of the muscle and serous layers were evaluated. The shape and length of the folds of the intestinal mucosa and the thickness of the lamina propria of the folds were observed. In addition, attention was paid to the presence and distribution of mucous cells and the presence, size, and content of supranuclear vacuoles of enterocytes. The presence and extent of any inflammatory infiltrates was also examined.

The distal intestines of sea bass fed with feed C had very developed serous and muscular layers. The average length of villi were characterized by simple morphology with areas of flaking and discontinuity of the mucosa (Fig. 4.13a). The lamina propria was thickened with respect to the physiological condition. In enterocytes we observed abundant vacuoles of various sizes not regularly aligned, eosinophils, and PAS-positive areas occupying much of the cytoplasm and forcing the nucleus at the base of the cell (Fig. 4.13b, 4.13c). We observed a few muciparous cells that were uniformly distributed and examined them by using a specific staining procedure for

mucins, Alcian Blue PAS (Fig. 4.13c). An important leukocyte infiltrate was found between the mucosal epithelial cells (Fig. 4.13b, 4.13c yellow arrows) at the level of the submucosa and also at the level of the serous and muscle layer, as further shown by immunohistochemistry (Fig.4.13d).

In the intestines of sea bass receiving the diet in which 2% of butyrate was added, feed B, the thickness of the serous and muscular layers was normal (Fig.4.14a); the intestinal plicae, long, were characterized by a complex structure and the lamina propria appeared slightly thickened, particularly in those areas that corresponded to the branching points of the structure of the fold (Fig. 4.14c). Supranuclear vacuoles were plentiful and diverse in form, size, and content (Fig. 4.14b, red arrow). Numerous muciparous cells were found that were well distributed along the folds (Fig. 4.14b, the green arrow), which was demonstrated specifically by Alcian blue PAS staining (Fig. 4.14c). A hyperproliferative epithelium was observed in areas where the typical single layer showed an abnormal pluristratification (blue arrow Fig. 4.14c). The inflammatory infiltrate was identified between the epithelial cells in the lamina propria and in the submucosa (yellow arrow in Fig. 4.14b), and corroborated by immunohistochemistry (Fig. 4.14d yellow arrows).

The seabass fed with an addition of 2% of taurine, feed T, presented a well-developed muscle layer (Fig. 4.15a). The long folds tended to be simple and showed the lamina propria to be slightly thickened in some places, but generally thin. Muciparous cells were abundant, preferentially concentrated in the medium-apical fold (Fig. 4.15c). The supranuclear vacuoles were characterized by a heterogeneous content (Fig.4.15b red arrow). A mild overgrowth of epithelium was observed (blue arrow Fig. 4.15c). The inflammatory infiltrate was detected in the submucosa and in the mucosa, both in the lamina propria and between epithelial cells, which was confirmed by immunohistochemistry (Fig. 4.15d yellow arrows).

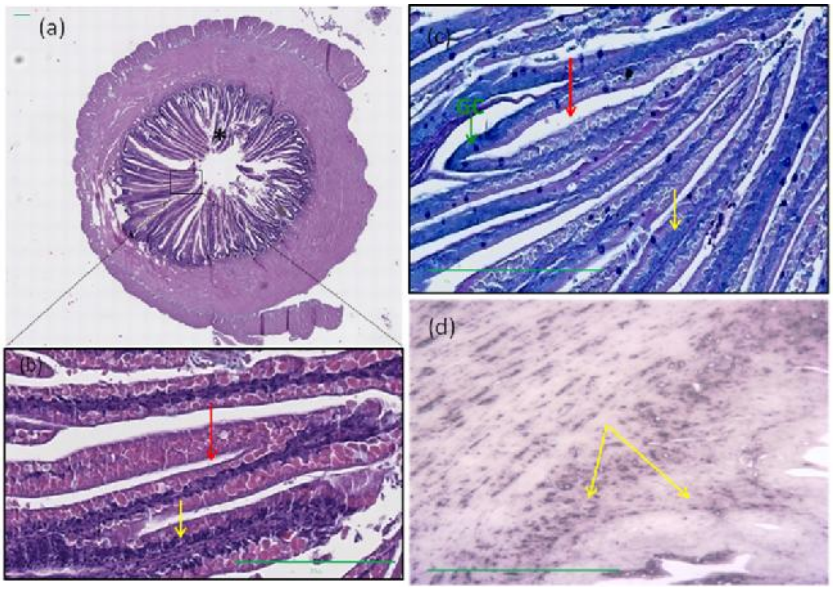

Figure 4.13 Light microscope images obtained from distal intestine of sea bass fed diet C*

* (a) Morphology of a complete section at 1x, bar = 100 µm, EE stain, (*) indicates a discontinuous area, with folding exfoliation; (b) visible at higher magnification are vacuoles in eosinophils (indicated by the red arrow) and leukocyte infiltrate in the intraepithelial skinfold (indicated by the yellow arrow), 40x magnification, bar 25 µm, stain EE; (c) Evaluation of mucus cells or goblet cells (GC), Alcian Blue staining and PAS in mucus cells (green arrow), the vacuoles in the eosinophils (red arrow), also show PAS Positive staining Alcian Blue P.A.S. and leukocytes (yellow arrow), 20x magnification, bar 50 µm; (d) Observation of leukocytes highlighted anti-CD45 immunohistochemical analysis, image magnification 10x, bar 100 µm;

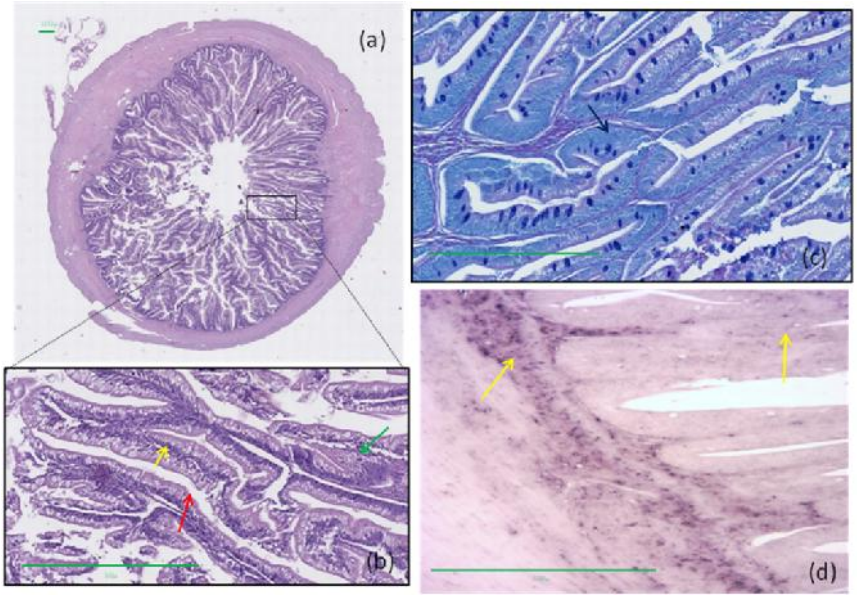

Fig. 4.14 Light microscope images obtained from distal intestine of sea bass fed diet B*

*(a) Complete morphology of the section, observed at a magnification 1x, bar 100 µm, stain EE, (b) at higher magnification intraepithelial lymphocytes (yellow arrow), the vacuoles (red arrow) and mucus cells (green arrow)are visible by EE staining, magnification 20x, bar 50µm; (c) Alcian Blue staining for PAS evaluation of mucus cells, visible also a hyperproliferative epithelium (blue arrow), 20x magnification, bar 50µm; (d) anti-CD45 immunohistochemistry, magnification 10x, bar 100 µm.

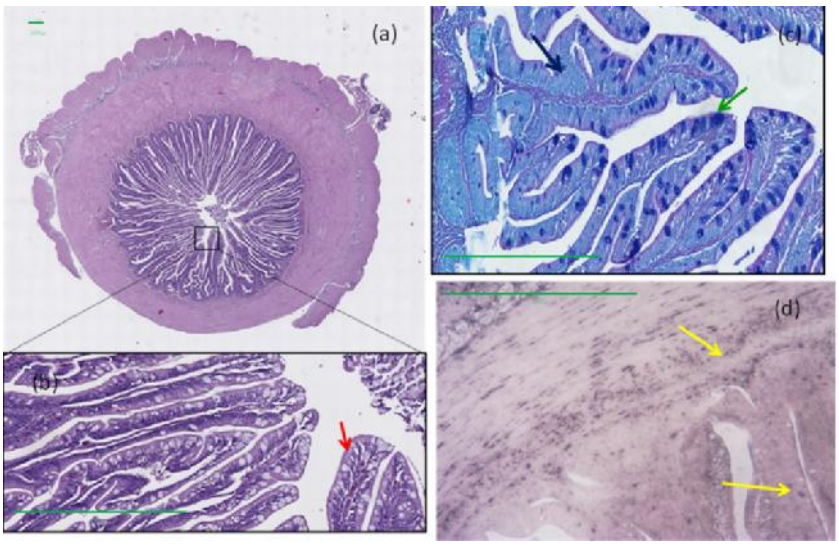

Fig. 4.15 Light microscope images obtained from distal intestine of sea bass fed diet T.*

* (a) Full morphology observed at a magnification 1x, bar 100 µm, stain EE; (b) at higher magnification 20x, bar 50µm, stain EE (red arrow indicating the vacuoles); (c) staining Alcian Blue P.A.S., which highlights the many muciparous cells (green arrow); the blue arrow indicates an area where it was possible to detect an overgrowth of enterocytes, 20x magnification, bar 50 µm; (d) Immunohistochemistry anti-CD45, (yellow arrows indicate the signal of the inflammatory infiltrate).

4.3c Electron Microscopy

Further investigation by using transmission electron microscopy allowed us to highlight the cell ultrastructure in the three types of samples. As in most cells with absorbing properties, enterocytes are characterized in the apical plasmamembrane by the presence of microvilli and numerous invaginations, in apical cytoplasm by many pinocytotic vesicles and tubules (tubulo-vescicular system), and in the supranuclear zone by vacuoles. Different aspects of the enterocytes were investigated. From the lumen to the lamina propria of the fold we considered: the brush border, the tubulovesicular system occupying the apical area of enterocytes, the supranuclear vacuoles, the nuclei, and infiltrating inflammatory cells .

The distal intestines of seabass receiving feed C showed brush border areas with microvilli that appeared irregular and damaged (Fig. 4.16a). The content of the numerous large supranuclear vacuoles was different, from homogeneous electrondense to granular (Fig. 4.16a, 4.16b). In some cases some vacuoles had fused into a single structure (Fig. 4.16b). Numerous leukocytes infiltrating the epithelial cells could be observed (Fig. 4.16c) and, in some instances, the enterocytes nuclei appeared pyknotic.

In the intestines of fish fed with feed B, the microvilli were often interrupted for alteration of the apical plasma membrane (Fig. 4.17a). In the apical cytoplasm, a developed tubulo-vescicular

system was observed, and in the supranuclear cytoplasm numerous characteristic vacuoles were evident that had an irregular shape and heterogeneous content, with clear areas mixed with dense material (Fig. 4.17b, 4.17c). Some enterocytes showed marked cytoplasmic degeneration (Fig.4.17a), and some leukocytes had infiltrated the mucosa.

Sections of distal intestines from fish fed with feed T presented long and well-shaped microvilli, sometimes topped by a layer of mucus (Fig 4.18c). A tubulo-vesicular system was observed in the apical cytoplasm, and in the supranuclear cytoplasm there were numerous vacuoles with a heterogeneous content showing lamellar profiles (Fig.4.18b). Numerous muciparous Goblet cells (Fig. 4.18a) and leukocytes infiltrating the mucosa were detected (Fig. 4.18a).

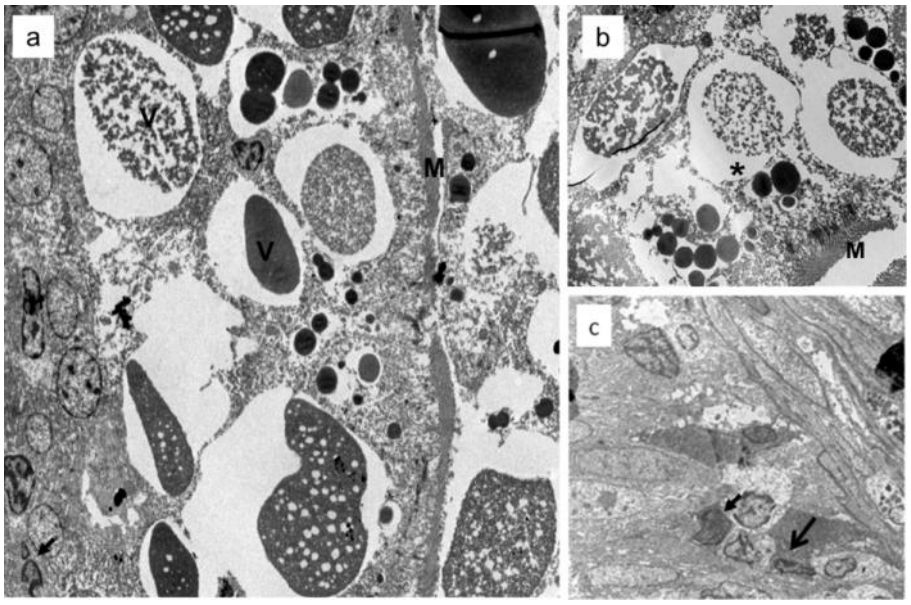

Fig. 4.16 Electron microscope images obtained from distal intestine of sea bass fed diet C*

*(a) a portion of epithelium, with an advanced degeneration and enterocytes with incipient leukocyte infiltration ( ), a focal alteration of the microvilli, and vacuoles with an electrondense content very close to bigger vacuoles with a granular content, magnification 2800x. (b) at higher magnification (5600x) is visible a vacuole (*) that pours its contents into a larger one; (c) pyknotic nuclei of enterocytes (indicated by arrow) magnification 4400x. V = vacuoles; , M= microvilli

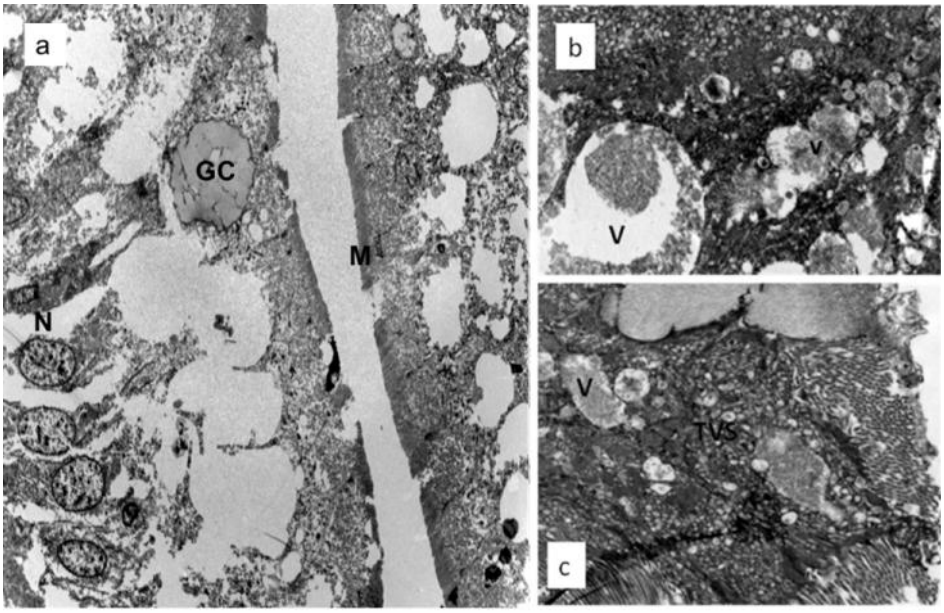

Figure 4.17 Electron microscope images obtained from distal intestine of sea bass fed diet B.*

*(a) Epithelium of two adjacent folds. At the base of the fold, nuclei of enterocytes are visible; in the median portion, intracellular spaces are present that originate from degenerated large vacuoles; in the apex portion partially damaged microvilli are present; the cytoplasm of a Goblet cell is observed, 1800x magnification; (b) vacuoles at higher magnification of 8900x, showing a heterogeneous content; (c) apical portion of a cytoplasm in which there are tubulo-vesicular system and vacuoles with heterogeneous content, 8900x magnification. N =enterocytes nucleus; V = vacuoles; TVS tubulo-vesicular system; GC =goblet cell, M=microvilli.

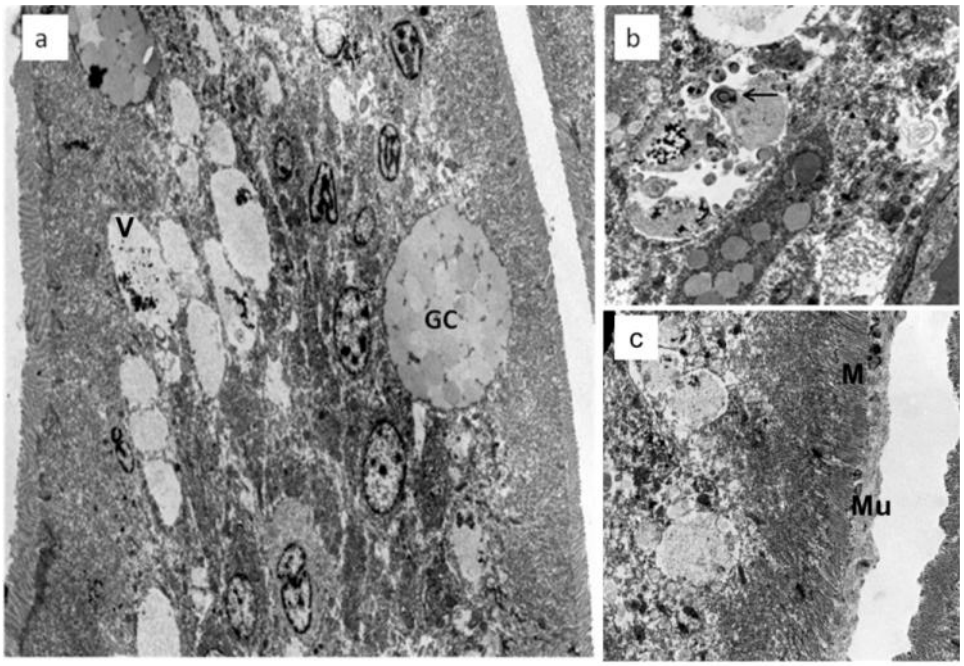

Figure 4.18 Electron microscope images obtained from distal intestine of sea bass diet T.*

* a) the epithelium of a fold with rather well-preserved cells including a muciparous cell is observed. Enterocytes show numerous vacuoles, 2200x magnification; (b) a vacuole containing lamellar body (indicated by arrow) is visible 7100x magnification; (c) epithelium with well developed microvilli dominated by mucus, 5600x magnification; V= vacuoles, M= microvilli, GC =goblet cell; Mu = mucus.

4.3d Molecular Gene Expression Analysis

The expression levels of the proinflammatory interleukins IL-1β, IL-6, IL-8, and TNF and the anti-inflammatory interleukin IL-10 genes were evaluated in the distal portion of sea bass intestine by using semi-quantitative RT-PCR. The results of agarose gel electrophoresis of amplified PCR products are reported in figure 4.19. The semi-quantitative PCR analysis, based on the intensity quantification of the bands, showed that the expression of analyzed genes in intestine was deeply influenced by the type of feed eaten by fish. Although IL-1β was expressed at low levels in all fish analyzed, sea bass fed C diet displayed the highest expression level of IL-1β gene in comparison to other fish groups. Indeed, only one of three fish analyzed for group B and T expressed quantifiable levels of IL-1β transcript (fig. 4.20). In fish fed the T diet, or diet supplemented with taurine, expression of both IL-8 and IL-10 genes was significantly lower ($p < 0.05$) than in those receiving diet C or B (fig. 4.20A, 4.20D). Dietary butyrate caused an upregulation of TNFα gene transcription; indeed, fish belonging to group B showed higher levels of TNFα messenger than the others ($p < 0.05$) (fig. 4.20B). Only IL-6, among the interleukins measured in this study, was not influenced by diet (fig. 4.20E).

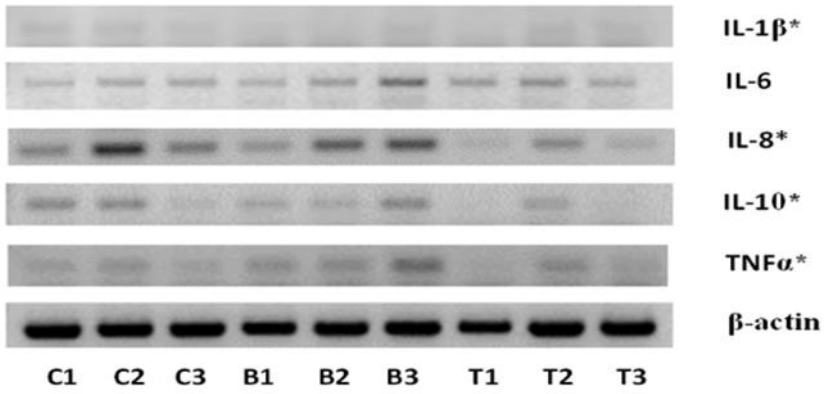

Fig.4.19 Agarose gel electrophoresis of the PCR products corrisponding to genes IL-8 (A), TNF (B), IL-1β, IL-10 and IL-6 and for the gene β-actin.

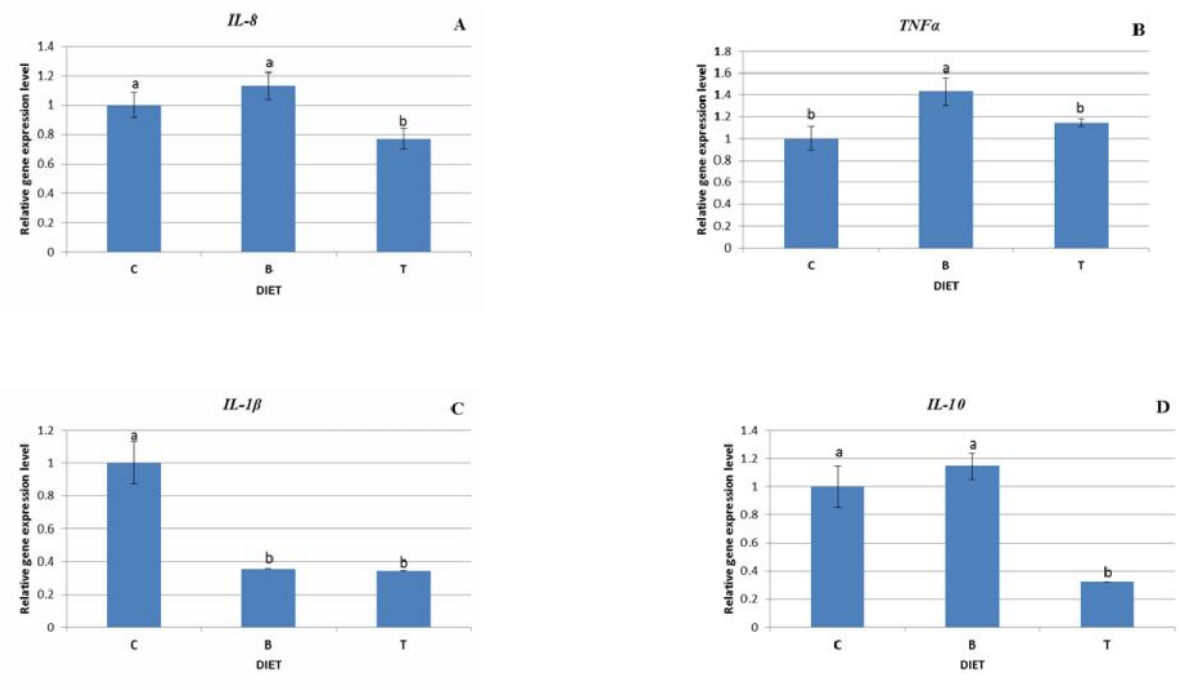

Figure 4.20Relative expression levels of IL-8 (A), TNFα (B), IL-1β (C), IL-10 (D) and IL-6 (E) genes.*

*Values are reported as average values (n = 3) ± SD. The group C represents the reference sample whose values of expression for each target genes were placed by convention as equal to 1. Differences between the letters indicate significant differences between groups ($p < 0.05$), ANOVA followed by Duncan test .

4.4 Commercial feeds and product quality

4.4a Lipid profile of feeds and fillets

In table 4.8 was reported the content for the six experimental feeds produced by three important feed industries. The raw materials of each feeds were not available due to the patent presence on the product. By our analytical analysis can formulate hypothesis on the main ingredients.

Among experimental diets, EPA was presented in a high concentrations respect DHA. Particularly, F4 and F5 diets reported 1203.56 mg/100g and 400.55 mg/ 100g, respectively.

The content of *n3*-PUFA and *n6*-PUFA of the following diets suggested that F4 and F2 feeds were rich in fish oil, whereas F1, F3 F5 and F6 showed high content in vegetable oils. The feed F4 reported high amounts for both *n*-PUFA.

Table 4.8 Fatty acid composition (mg/100g of feed) of the diets (6 treatments) of rainbow trout. Data represent mean of three replicates for each sample.

	F1	F2	F3	F4	F5	F6
C14:0	373.79	142.3	37.92	259.27	202.43	69.62
C16:0	1059.15	347.92	390.41	683.15	896.54	470.58
C16:1	308.23	126.68	34.95	254.30	171.20	68.68
C18:0	500.76	241.84	349.90	560.82	394.99	249.66
C18:1n-9	704.24	1478.99	762.03	1768.31	6535.71	2764.02
C18:2n-6	1854.26	551.78	824.65	1399.75	1666.49	868.29
C20:0	80.61	37.73	39.53	116.95	56.60	86.79
C18:3n-6	n.d	n.d	5.65	n.d	n.d	1.61
C20:1	590.24	27.75	73.72	623.42	287.82	183.66
C18:3n-3	9.27	9.46	7.94	19.4	87.46	41.42
C20:2n-6	15.04	n.d	8.06	58.26	23.96	12.49
C20:3n-6	n.d	n.d	n.d	n.d	n.d	n.d
C22:1n-9	342.65	n.d	68.53	1104.21	243.92	108.62
C20:4n-6	n.d	n.d	n.d	n.d	n.d	n.d
C20:5n-3	218.79	692.3	76.47	1206.56	400.55	165.36
C22:6n-3	103.21	37.57	34.50	525.98	103.67	50.29
C18:4n-3	43.03	19.99	n.d	86.11	30.80	1.75
C22:5n-3	12.8	25.71	9.35	108.51	38.18	13.82
C20:4n-3	8.45	n.d	n.d	47.41	n.d	n.d
n3-PUFA	395.55	785.03	128.26	1993.97	660.66	272.64
n6-PUFA	1869.3	551.78	830.3	1458.01	1690.45	882.39
n3-LC-PUFA	52.30	29.45	7.94	105.51	118.26	43.17
n6-LC-PUFA	n.d	n.d	5.65	n.d	n.d	1.61
EPA/DHA	2.12	18.4	2.21	2.3	3.86	3.28

In table 4.9, the fatty acid fillets composition (mg/100g) was reported. Here, the fatty acid EPA was lower than in diets, only in F2 group was detected an inversion between EPA (315.03 mg/100g of fillet) and DHA (39.18 mg/100g of fillet).

No significant differences were detected for C16:1, C18:3n-6 and C20:4n-3.

The highest EPA value was recorded in F5 fillets group (982.93 mg/ 100 g) with a significance $P \leq 0.01$ and $P \leq 0.05$. The lowest EPA value was reported for F3 fillets group (113.50 mg/ 100 g).

The highest DHA content was showed in F3 fillets group with a significant difference of $P \leq 0.05$ from F5 fillets and $P \leq 0.01$ from the others fillets groups. The lowest DHA amount was detected in F2 fillets group (39.81 mg/100g). Comparing the fatty acids profile of diets and fillets, it is worth noting the higher DHA amount in fillets than in diets. We also evaluated the grams of fillet per week to cover the requirement 500 mg/die EPA and DHA. 130-144 grams of fillet per week covered the daily requirements of EPA and DHA recorded in F5 and F3 fillets groups, while it would be needed 989 grams of fillet of F2 fillets group per week to cover the same requirements.

All fillet groups showed high content in oleic acid (C18:1*n*-9) and linoleic acid (C18:2*n*-6), representing the precursors of biosynthesis of *n*-9 and *n*-6, respectively.

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Table 4.9 Fatty acid composition (mg/100g of fillet) of the fillets of rainbow trout. Data represent mean ± standard deviation (n=4, N=24)

Fillets	F1	F2	F3	F4	F5	F6	S.D.
C14:0	157.23A Ca	32.63Bb	42.81A Bb	51.26A Bb	252.95Cb	59.07A Bb	31.75
C16:0	195.15A	154.2A	469.87A C	460.61A C	1853.66B	969.92A C	177.97
C16:1	165.20	85.37	55.42	61.0	165.51	67.56	43.34
C18:0	1216.53a	425.44a	676.5a	343.95b	797.04a	647.05a	272.09
C18:1n9	13751.04A Ba	3096.05A b	7472.87A Bab	3276.68A Bb	17556.53C	10079.26A Bab	3471.88
C18:2n6	3513.15A a	1202.27A Bb	895.87Bab	964.10Bab	1693.28A Bab	151.61Bab	638.64
C20:0	8.04A ab	7.68A ab	16.88A a	9.66A ab	33.49B	4.87A b	3.78
C18:3n6	15.57	5.28	17.31	9.81	9.61	8.08	5.01
C20:1	321.49A	178.18A	172.70A	174.3A	747.65B	38.52A	106.75
C18:3n3	41.51A a	11.08B	7.45Bb	13.48A Bb	28.16A Babc	32.58A Bc	7.18
C20:2n6	147.91a	53.15b	46.18b	59.87b	150.61a	56.32b	26.23
C20:3n6	115.29a	41.51b	57.06ab	49.94b	55.80b	55.50b	20.04
C22:1n9	403.12abc	243.96abc	83.63a	204.64abc	633.71b	38.16a	149.91
C20:4n6	6.79A	1.54A	28.26B	4.18A	1.84A	7.36A B	5.36
C20:5n3	590.86A Babc	315.03A Ba	113.50A a	204.68A Ba	982.93Bbc	286.76A Bbc	178.09
C22:6n3	605.22A D	39.81CD	2311.03Ba	409.34D	1716.39Bb	483.11A CD	196.16
C18:4n3	50.52A BCa	57.54A BCa	16.48A Cab	31.72A Cab	117.01Bb	19.38A cab	18.05
C22:5n3	10.93A	9.75A	10.24B	50.84B	40.11BCa	20.26A Cb	5.74
C20:4n3	41.79	32.42	29.06	18.42	63.64	47.61	19.32
EPA+DHA	1196.08	354.84	2424.53	614.02	2699.32	769.87	
EPA/DHA	0.97	7.91	0.05	0.5	0.57	0.6	
Need (*)	293	989	144	570	130	455	

*Grams of fillet per week, to cover the EPA plus DHA of 500mg/die.

Different capital and small letters in the same line (for each main effect) indicate significant differences at P≤0.01 and P≤0.05, respectively.

4.4b Amino acid profile of feeds.

In Table 4.10, amino acid diets profiles were reported in % on dry matter. Unfortunately, taurine was not detectable as alone, but quantified with arginine peak. Since that the digestibility coefficient (ADC) was not calculated, we had to consider only a 30% for each detected amino acid.

Moreover, due to the impossibility to know the raw materials of diets, we could conclude that amino acids were overestimated. For example, the amount of methionine corresponded to the totally methionine and did not allow to distinguish in supplemented methionine (e.s. DL-methionine) or one derived from fishmeal.

Table 4.10 Amino acids profile of 6 diets. Each amino acid is reported in dry matter (%).

AMINOACID	F1	F2	F3	F4	F5	F6
	%	%	%	%	%	%
ASP	1.15	1.29	1.33	1.09	0.97	1.06
SER	11.72	12.61	12.44	12.01	12.36	13.12
GLU	7.11	7.28	7.79	7.25	7.65	8.25
GLY	8.60	8.26	5.70	5.43	6.64	6.00
HIS	3.99	3.73	4.86	4.24	3.44	3.51
ARG+TAU	6.82	6.76	7.29	6.59	6.48	6.37
THR	4.59	4.55	4.27	5.04	5.11	4.95
ALA	7.30	7.37	6.89	7.27	6.63	6.33
PRO	6.62	6.63	5.06	5.42	6.21	6.29
TYR	3.31	3.25	3.20	3.86	3.41	3.27
VAL	6.38	6.10	6.80	6.17	5.89	6.18
LYS	7.08	8.20	9.41	9.65	9.07	8.51
ILE	4.65	4.45	3.71	3.29	5.03	5.19
LEU	9.35	9.04	10.46	11.15	8.63	9.00
PHE	5.65	5.34	6.02	6.05	5.27	5.61
CYA	3.16	2.87	3.14	3.38	4.13	3.84
MET	2.53	2.27	1.62	2.11	3.09	2.51

In table 4.11, the fatty acids composition of fillets mirrored the lipid profile of diets and this was confirmed by increasing positive correlations. The F5 and F6 groups showed high correlation values 0.98 and 0.85, respectively. The group F1 showed the lowest value of correlation (0.31

Table 4.11 Pearson's correlation of diets and fillets fatty acids composition. The level of significance was established at P<0.0001.

	FILLETS (1)	FILLETS (2)	FILLETS (3)	FILLETS (4)	FILLETS (5)	FILLETS (6)	TOTAL
F1	0.31 (0.0065)						
F2		0.58 (<0.0001)					
F3			0.56 (<0.0001)				
F4				0.6 (<0.0001)			
F5					0.98 (<0.0001)		
F6						0.85 (<0.0001)	
TOTAL							0.67 (<0.0001)

In table 4.12, Spearman's correlation reported the comparison between two feeds' ranks: one based on correlation values and the other based on increasing amount of each fatty acid.

If a fatty acid showed a positive correlation meant that its presence on the feed promoted the assimilation of the other fatty acids. On the other hand, a negative correlation meant for its presence decreased the assimilation of others fatty acids. Therefore, if we evaluated high positive correlations, docosapentaenoic acid and palmitic acid recorded 0.77 and 0.71 values, respectively. High negative correlations were showed for erucic acid (-0.66) and linoleic acid (-0.43).

Table 4.12 Spearman's correlation was reported as comparison between two feeds' ranks: one based on correlation values and the other based on increasing amount of each fatty acid.

	r
C14:0	0,03
C16:0	0.71
C16:1	-0,42
C18:0	-0,25
C18:1n9	0,08
C18:2n6	-0,43
C20:0	-0,03
C18:3n6	-0,37
C20:1	-0,65
C18:3n3	0,25
C20:2n6	-0,31
C20:3n6	-0,25
C22:1n9	-0,66
C20:4n6	0,37
C20:5n3	-0,42
C22:6n3	0,08
C18:4n3	-0,2
C22:5n3	0,77
C20:4n3	0,48

5. DISCUSSION

Separately we will discuss the effect of butyrate and taurine, following with a comparative analysis of the effects due to both the nutraceuticals.
A final tentative assessment of the effects of different commercial diets on the quality of fillet and trout is reported.

5.1 Diet integrations with prebiotics

Butyric acid is one of the three major short-chain fatty acids (SCFAs) produced within intestinal lumen by fermentation of polysaccharides, the other being acetic and propionic acid. Butyrate in particular is known for a trophic action at level of intestine, particularly important when the vegetable meals in the diet promote local flogistic reactions. The assessment of butyric acid in the fecal matters is of great importance, not only with the goal of monitoring the effects of the butyrate addition in the diet, but even more when the the intent is to tune intestinal microbiota toward a butyric fermentation. The two methods that have been implemented within the study, in GC-mass and in HPLC, modifying the protocol reported by De Baere *et al.* (2013), were both suitable to assess butyrate residual in fecal pellets, as well as in bacterial cultivations. Nevertheless, neither acetic nor propionic acid were quantified due to matrix effect at the beginning of elution.

In spite the large knowledge existing on ruminal butyrate fermentation, a paucity of study exists related to fish, with the exception of Fidopiatis et al. (2006). We detected butyric acid-producing bacteria in seabass fed with different vegetable meals percentages (48, 57, 52 and 68%). The two bacterial species detected being butyrate producers, belonged to the family of Vibrionaceae. That differs from ruminants and humans where genus Clostridium are designated to this kind of fermentation, apparently due to the lower temperature characterizing the fish gut environment. The family Vibrionaceae is composed by a genetically and metabolically diverse group of heterotrophic bacteria that are routinely found in all ocean environments (Takemura *et al.,* 2014). A few Vibrio species have extended their range beyond the marine environment, occurring predominantly in brackish and even freshwater environments (Thompson *et al*., 2004).

In our tentative to induce butyrate fermentation in fish gut, were followed by feeding fish with additional fibers in the diet, as chitosan, novelose, inulin, ß-glucan. Fecal products were then cultivated under anaerobiosis and the so produced short chain fatty acids monitored. After the anaerobic growth of seabass feces, chitosan and novelose330 resulted the best prebiotics promoting

butyric acid production. Novelose330 is defined a resistant starch (RS) of 3 type, as the sum of starch produced from gelatinised debranched starches by retrogradation with butyrogenic properties (Jacobasch et al., 2006). Chitosan is obtained by deacetylated product of chitin prepared from the shells of shrimps, prawn and crabs. Both resulted complex polysaccharides not adsorbed by intestine so not degraded by pancreatic enzymes (Englyst et al., 1992). In vitro butyric acid production confirmed the potential properties of chitosan, that compared with others substrates, increased the amount of short chain fatty acid.

According to Chen *et al.* (2014) that reported the potential effect of chitosan on stimulating immunity system of gibel carp, in our study the respiratory burst activity (ROS production) decreased with increasing of phagocytosis and chitosan administration; after 75 days of experiment, there was a significant reduction of ROS level in diet containing 7500, 10000 and 20000 mg kg-1 chitosan. We did not test different percentages of chitosan but only 2000 mg kg-1 in seabass, comparing the same amount of novelose330 and inulin. In 30 days, chitosan reduced significantly the ROS level in contrast with novelose330 and inulin. An on-farm longer period treatment with chitosan, could eventually further confirm the data.

In conclusion, the different trials confirmed the potential butyrogenic property of chitosan and its capacity on stimulating the seabass immunity system.

Future studies may be focalized on the composition of gut microbiota linked to chitosan administration and/or other probiotics. So, our results support the possibility to tune the seabass microbiota by diet, promoting a butyrate fermentation. Nevertheless, a strong gap in our study was due to the limitations imposed by the DGGE approach, that did not allow to completely assess the microbiota composition. Further study are envisaged, that will utilize the next generation sequencing (NGS) with an Illumina facilities.

Several studies have addressed the effects of organic acids and their salts on growth performance and health of fish. These include Arctic charr (Ringø, 1991), Atlantic salmon (Ringø *et al*., 1994; Lückstädt, 2008b), rainbow trout (Rungruangsak and Utne, 1981; deWet, 2005; Pandey and Satoh, 2008; Gao *et al.*, 2011), tilapia (Ramli *et al.*, 2005; Ng *et al.*, 2009; Zhou *et al.*, 2009), catfish (Owen et al., 2006), red sea bream (Sarker et al., 2005, 2007; Hossain et al., 2007), gilthead sea bream (Robles et al., 2013) and rohu (Baruah et al., 2007a,b). Results indicate that growth performance, nutrient digestibility and gut health, can be improved by some organic acids and their salts in some fish species (reviewed by Lückstädt, 2008a). In our study on seabass, sodium butyrate

did not promote any effects on growth performances, but on gut health the beneficial effects were evident. In Scollo *et al.* (2012) was reported the expression of neutral amino acid transporter (SLC6A19) and peptides transporter (PEPT1) at intestinal level of seabass fed with sodium butyrate against a negative control. Sodium butyrate increased the gene expression of PEPT1 at proximal and distal intestine respect to control. While, SLC6A19 transporter resulted more expressed at distal intestine in seabass feeding with sodium butyrate. Therefore, sodium butyrate increased the intestinal absorption of peptides and amino acids carrying out its trophic and anti-inflammatory effect in intestinal structure. There is a high energy requirement for renewal of the intestinal epithelium. Butyric acid and butyrates are efficient at providing energy for epithelial growth (Topping and Clifton, 2001). Moreover, butyric acid and butyrates influence a wide array of cellular functions relevant to gut health (Hamer *et al.,* 2008). Thus, butyric acid and butyrates may exert a positive effect on gut health.

Actually, there is a 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 (Hu and Guo, 2007; Øverland *et al*., 2000; 2008), but some questions remain regarding their efficacy in fish farming. Indeed, following the experiments in pig and poultry feeding, a wide variety of organic acids and their salts were tested in aquaculture diets for different fish species. These included carnivore species such as rainbow trout (Oncorhynchus mykiss), Atlantic salmon (*Salmo salar*), and Arctic charr (*Salvelinus alpinus*), herbivorous tropical warm-water species such as tilapia (*Oreochromis niloticus*), and omnivore fish such as carp (*Cyprinus carpio*), and catfish (Ictalurus punctatus) (reviewed by Lückstädt, 2008). However, conflicting reports exist on the subject. Growth was significantly enhanced in some species, such as rainbow trout (O. mykiss), when fed an organic acid blend supplement mainly consisting of formate and sorbate (de Wet, 2005), but not in trout fed other commercial aquaculture supplements such as lactic acid (Pandey and Satoh, 2008) or citric acid (Vielma *et al.,* 1999; Pandey and Satoh, 2008). In other species, neither hybrid tilapia (Oreochromis niloticus × O. aureus) fed potassium diformate (Zhou *et al.,* 2009) nor Atlantic salmon (*S. salar*) fed sodium salts of acetic, propionic, and butyric acid (5:5:2 w/w/w) showed any growth enhancement (Gislason *et al.,* 1994; Bjerkeng *et al.,* 1999). The results of our study are in line with those of Gislason *et al.,* (1994) and Bjerkeng *et al.,* (1999), as we did not find differences in the growth of European sea bass fed a diet supplemented with Na-butyrate. Indeed, the mean body weight and SGR of sea bass in our study did not reveal significant differences between fish fed the sodium butyrate-supplemented diet and the control fish.

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 (Owen et *al.*, 2006; Gao *et al.*, 2011; Robles *et al.*, 2013; Liu *et al.*, 2014). However, only few reports have described butyrate-induced epigenetic and transcriptional changes in intestinal and hepatic genes of farmed fish (Scollo *et al*., 2012; Liu *et al.,* 2014). In view of this scarcity of information, the present work contributes to our current understanding of the epigenetic regulatory effects of butyrate in marine 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) (Canani et al., 2012). Histone deacetylases (HDACs) are critical enzymes involved in epigenetic transcriptional regulation, i.e., histone acetylation associated with chromatin structure and function (Dokmanovic and Marks, 2005). Acetylation of core histones at specific lysine residues in the NH2-terminal tails results in a decrease in the overall positive charge of histone tails, which diminishes their strength of binding to the negatively charged DNA. The effect is an opened chromatin structure, which makes DNA more accessible to transcriptional factors (Waterborg, 2002). Therefore, as a result, HDACs act as transcriptional repressors, whereas HDACi upregulate gene expression. There are very compelling data showing that sodium butyrate increases the quantities of acetylated H3 and H4 histone proteins in certain cells and tissues. In 1978 Candido et al. found that butyrate exposure caused hyperacetylation of histones H3 and H4 in vertebrate cell lines (Candido et al., 1978) and the same result was obtained in several in vitro studies using cultured mammalian cells (Mohana et al., 2007; Koprinarova et al., 2010; Paskova et al., 2013). 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 (Mátis et al., 2013; Shimazu et al., 2013; Gundersen and Blendy, 2009; Kien et al., 2008); hence, the present study represents the first in fish. Our results on sea bass hepatic histones clearly confirmed the capability of butyrate to induce histone hyperacetylation even in vivo. In agreement with what Mátis and colleagues (2013) observed in liver of chicken 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 (Gundersen et al., 2009). This indicates 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/100g feed), which was perhaps not sufficient to induce histone H3 hyperacetylation. Moreover, in sea bass and likewise in chicken, two

isoforms of histone H3 were separated on the immunoblots; in mammals, in contrast, three H3 variants were well characterized (H3.1, H3.2, H3.3) (Hake et al., 2006). 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 (Mátis et al., 2013). Similarly, acetylation of histone H4 in mammals (Gundersen et al., 2009) 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; on the other hand and likewise in chicken, acetylation of H3 histone was improved only at a higher dose (Gundersen et al., 2009). 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 (Kurdistani et al., 2004; Agricola et al., 2006). In a recent in vitro study using microplate-scanning FRET (Fluorescence Resonance Energy Transfer) analysis, Gansen et al. (2015) showed that acetylation of histone H3 promoted nucleosome opening and disassembly, whereas acetylation of H4 alone increased unwrapping of the DNA ends, but did not enhance disassembly. The same study also revealed that H4 acetylation significantly counteracted H3 acetylation in nucleosome disassembly. Therefore, it would not be surprising if histone H3 and H4 differ from each other in response to dietary butyrate.

Among all core histones, H2A has the largest number of variants. In mammalian Jurkat cells, at least thirteen H2A variants were identified (Bonenfant et al., 2006). According to Brower-Toland et al., (2004), and Ishibashi et al., (2009) acetylation of H2A is involved in conformational changes of nucleosomes, which influence some strong, specific, and key histone-DNA interactions, whereas Gansen et al. (2015) suggested that acetylation of H2A and H2B histones did not influence nucleosome stability, but could affect the nucleosome entry-exit region. However, multiple studies revealed that butyrate caused hyperacetylation of H2A both in vivo (Mátis et al., 2013) and in cell culture (Mohana Kumar et al., 2007; Ishibashi et al., 2009; Tobisawa et al., 2010). Unfortunately, we could not verify in sea bass whether butyrate induced H2A hyperacetylation since the antibody we used did not work in our species. 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; the expression was in general, higher in the liver than in the intestine. As previously found in European sea bass reared in different temperatures (Piferrer, 2013; Díaz and Piferrer, 2015), three of these 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 (Aliyari & Ding, 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, also known as pattern recognition receptors (PRRs). The double-stranded (dsRNA)-specific Dicer nucleases represent a distinct family of PRRs. Upon viral infection, one domain of PRRs interacts directly with microbial signatures shared by major classes of microbes, whereas the second protein-protein interaction domain activates the downstream signaling events, leading to transcription of immunity effector genes with broad-spectrum anti-microbial activities (Aliyari and Ding, 2009). Moreover, dicer1 knockdown experiments showed an increase in the interferon response against pathogens (Chiappinelli et al., 2012). Although our results showed a slightly increase in the expression of irf1, a higher expression of dicer1 was also observed in the liver in the intestine, 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 (Takahashi *et al*., 2012). A similar effect is possible in our experiment with butyrate treatment since il6 expression was downregulated 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 (Villagra *et al.,* 2009). 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 (Rubtsov *et al.,* 2008) and the scenario fits nicely with the known anti-inflammatory effect of butyrate in the fish that received the supplemented diet.

In conclusion, results of the 8-week-long feeding trial showed no significant differences in weight gain and SGR (specific growth rate) of sea bass that received 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 threefold 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 2-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 were differences observed in the expression (P=0.003) due to the butyrate treatment.

Beside the reduced availability of some essential aminoacids as lysine and methionine, taurine is either poor or absent in vegetable protein sources utilized to substitute fishmeal. The essentiality of this sulphonic aminoacid has been reported by several authors being variable with the fish species and ontogenic development, as well as environmental conditions as water salinity. Reduced growth rates and feed efficiency was attributed to imbalance in amino acidic profile of soybean meal, by Jirsa *et al.* (2010), in which the authors reported a significant decrease on growth performance in white seabass (*Atractoscion nobilis*) fed with high level of plant protein. In a successive paper, they reported as taurine promoted the growth in juvenile stages of the same species fed soy-protein added 0.99% taurine (Jirsa *et al*., 2014). In juvenile common dentex (*Dentex dentex*) sizing 39.1 ±0.7g, a 40% of fishmeal replacement with soybean meal have compromised the growth rates and feed efficiency, but a taurine addition of 2 g kg-1 improved the growth rates (Chatzifotis *et al.,* 2008). In seabass (*Dicentrarchus labrax*) fry, taurine brought to a higher specific growth rate when fed an addition as 0.2% and 0.3% of taurine, compared to 0% and 0.1% (Brotons Martinez et al., 2004). This suggests that different requirements of taurine could be related to different size (body weight) of marine fish (El-Sayed, 2014) and to the fish ontogenesis (Wang *et al*., 2015). Particularly, the larval and early developmental stages of marine fish were most influenced by the consumption of taurine (El-Sayed, 2014) with the exception for gilthead seabream larvae where supplementation with taurine did not enhance the growth performance, maybe due to the capacity of this fish species to synthetize taurine at larval stage (Pinto *et al.,* 2013).

The latter conclusions reviewed in El-Sayed (2014), suggest that beside possible different responses among species, the juvenile stages may better show benefits from taurine addition in feed than older stages. Accordingly, in spite our experimental design was not focused on a growth study and the fish were affording forced swimming challenges possibly disturbing growth, the observed growth trend in sub-adult seabass sizing 86.4 – 98.71 g fed with 1.5% of taurine, indicated an improved SGR among fish fed 70 days complexively with taurine additions. Similar trend was found from a second experiment with seabass initially sizing 514 g, sustaining a clear positive effect of taurine on growth performances effects in body weight (see lather in this Discussion).

Furthermore, taurine has been known for its protection against the main toxicity cellular causes; that is the production of free radicals and overload calcium (Jong et al., 2012). Concerning the role as antioxidant effect, taurine was considered a scavenger of reactive oxygen species (ROS) (Ekremoğlu *et al.*, 2007).

In our experiment, the effect of taurine as antioxidant was studied testing the swim performance of fish, where the swimming activity was assumed to magnify the ROS production.

Seabass fed 1.5% taurine achieved a good swimming performance and U_{crit} resulted significantly higher in the first swim trial (t_2) with the difference even increased in the further trial (t_3). The addition of taurine indeed increased the fish swimming resistance and the U_{crit} was able to demonstrate not only the fish speed but the duration of the effort as well. Such enduro resistance capacity induced by taurine was previously observed by Yatabe et al. (2003) in rat fed 0.5g $kg^{-1}$of taurine. The authors also observed that an oral administration of taurine increased its concentration skeletal muscle. According to above, seabass treated with 1.5% of taurine in our experiment performed U_{crit} of 4.16± 0.078 BL s^{-1} compared to the control group performing a U_{crit} 3.97± 0.08 BL s^{-1}. The increased U_{crit} performance observed in the group supplemented with 1.5% taurine, could find on interpretation after Ripps & Shen (2012) who reported an increased ATP through activation of the respiratory chain by taurine, directing electrons to the production of ATP.

Obviously, the training also improved aerobic performance of red muscle due to the increased volume of mitochondria (Davison, 1997) in spite an increase of ROS production.

The link between swimming training and ROS production was studied for the first time by (Mortelette *et al*, 2010a) in trained and untrained eels (*A. anguilla L.*). The red muscle fibres collected along the lateral line in trained eels showed “in vitro” an increased HO• production, as well as an higher maximal oxygen consumption, compared to the muscle of untrained eel. That confirmed previous study by Palstra *et al.* (2008) on the same species, reporting an increased oxygen consumption and ROS production of red muscle, after forced swimming aerobic exercise. Our experiment didn’t fail to confirm the same results on ROS production associate to forced

swimming, in spite our observations on Seabass where carried out “in vivo”, immediately after the aerobic exercise.

We also considered the ROS production by the cellular mechanism of respiratory burst (RB), at time t_2 and t_3 after swim performance, in isolated phagocytes. Seabass fed with 1.5% taurine showed a decrease of RB activity compared to the control group performing an increase of RB activity. The stressful exercise of forced swim (t_2 and t_3) showed, in control group, an increase of ROS production; while the scavenger, taurine, decreased the level of ROS. Comparing to the control group, taurine addition controlled the ROS production during the time and in the second swimming performance (t_3), its effect was more amplified; supporting the definition of taurine as a scavenger of reactive oxygen species (Bañuelos-Vargas *et al.*, 2014) protecting from mitochondrial dysfunction. In other words, taurine has prevented the diversion of electrons into superoxide anion generation by improving the function of the electron transport chain (Jong *et al.*, 2012). It is widely accepted that slowing of electron flux through the respiratory chain can result in a diversion of electrons from complexes I-III to an alternate acceptor, such as oxygen (Turrens, 2007). Particularly, the complexes I-III of respiratory chain were considered the primary mitochondrial sources of superoxide generation and it has been documented that in cells lacking of taurine, their activities was compromised (Jong *et al.*, 2012).

Concerning oxygen consumption, no noticeable differences were detected between the two groups at the t2 challenge. Nevertheless, at t3 the group with the addition of taurine 1.5% consumed less oxygen and achieved a higher Ucrit than the control group, in spite MO2 data not resulting statistically significant. The recovery time between t_2 and t_3 allowed fish to adapt metabolism to swimming training when fed with 1.5% of taurine. This conclusion is in according to Mortelette et al., 2010b, that compared two fish species: rainbow trout (*Onchorynchus mykiss*), than yellow and silver eel (*Anguilla anguilla L.*), for the oxygen consumption and hydroxyl radical in mitochondria of red muscle fibers.

The authors found that trout consumed more oxygen and produced more hydroxyl radical than silver eels, while yellow eel placed in the middle. These oxygen consumption results were explained by the kind of species (Sébert & Theron 2001). In fact, the energetic demand is linked to the life conditions of species where silver eels are migratory under extreme conditions than non-migrating yellow eel and trout taking from a fishery (Mortelette *et al.*, 2010b). The same result has been obtained for ROS production leading to physiological conclusion: high consumers of oxygen, high mitochondrial activity in red muscle fibers, so high ROS production (Mortelette *et al.*, 2010b). Beside the parameter $HO^{\bullet}$ as a controller of oxidative status, the proton leak was considered as an antioxidant mechanism by Skulachev, (1998) through uncoupling proteins (UCPs) at the

mitochondrial level production (Mortelette *et al.,* 2010b). In the cardiac muscle of trout, it was estimated that 65% of oxygen consumption is due to proton leak (Mortensen & Gesser 1999). At mitochondrial level, proton leak through UCPs subtracts the energy available from the electrochemical gradient, lowering ATP synthesis and consuming oxygen substrates, as heat (Echtay 2007). The oxidative forms production may be even more attenuated by taurine, working as a buffer molecule. The buffering property of taurine is lent by the pKa value of the amino group (9.0-8.6) that allows contributing to mitochondrial pH in the range of 7.5-8.5. In addition, a proper taurine concentration is relevant to balance the mitochondrial pH with the regulation of proton pumping due to proton leak, electron transport chain, ATP-synthase (Hansen *et al.,* 2010). In fact, extracellular treatments with high taurine concentrations stabilized or buffered the mitochondria functions, as observed in the protection to oxidative burst detected in reperfusion (Tseng et al.,1990; Milei et al., 1992), that is the restoration of blood flow to ischemic tissue (Verma *et al.,* 2002).
Considering the latter statement, in our experiment the 1.5% taurine addition had the ability to mitigate the ROS production, even in forced swimming conditions, due to buffer molecule property of managing the proton flux, protecting the mitochondrial integrity and directing protons for ATP production.
In conclusion, others taurine concentrations in feed formulation might be trialed to monitor fish physiological effects on swimming performances and ROS production.

In our study where 514 g average weight seabass intestine was monitored, growth was observed after 60 days on fish receiving as control diet with 10% fish meal only, with a substitution with vegetable meal (diet C), than either a diet with 2% butyrate supplementation (diet B) or a supplementation with 2% taurine (diet T), the trend shows a similar SGR from diets C and B, while when compared to diet C, the SGR of diet T was 2.7 times higher ($P<0,05$). This result is in line with the findings of Conceicao *et al.* (1997) on larval stages of turbot and with Brotons-Martinez et al. (2004) on seabass fingerlings fed a diet containing soybean meal (SBM) and also taurine for 30 days.
In addition to the observed reduction in the number of goblet cells, intestinal samples from feed C examined by histology presented some aspects that are unusual in the literature (Beverfiord and Krogdal, 1996; Iwashita *et al.,* 2008), for example, elevated concentrations in the enterocyte cytoplasm of absorptive supranuclear vacuoles filled with PAS-positive material. We interpret this as the result of an interruption in the digestive processes within the enterocyte, with accumulation of a substance which, owing to its acidophilic properties and PAS positivity, could consist of glycoproteic materials. In contrast, other authors reported, in salmonids and in carps, a reduction of

the same supranuclear vesicles. However, other observations that lamina propria and epithelial lining were heavily infiltrated with inflammatory cells, including macrophages and polymorphonuclear leukocytes, shortening of the mucosal folding height, and widening of the lamina propria, are similar to those effects reported in the literature after a diet enriched with SBM (Beaverfiord and Krogdal, 1996; Uran *et al*., 2008a,b). The important signs of inflammation were confirmed in our samples by immunohistochemistry staining, showing a strong positivity for anti-CD45 that indicates a relevant diffusion of leukocytes in all the distal intestinal tissue. The TEM observations confirmed the presence of leukocytes and showed an apical tubulovescicular system, large vacuoles occupying the supranuclear cytoplasm containing a homogeneous electrodense content, and local intracellular degeneration, which may be due to a fragility of the vacuolar membrane, with consequent liberation of lytic enzymes and a recall of leukocytes.

According to Noaillac-Depeyre and Gas (1973), the supranuclear body is of complex origin. Indeed, in the adult carp, the apical cytoplasm of the enterocytes of the medium intestine shows the presence of a dense tubolovesicular network that form a voluminous supranuclear body. However, the supranuclear bodies are moreover independent of the presence of food material in the intestinal lumen since they are also present in fasting animals. Therefore, it seems that they do not result only from the running together of the food vacuoles but probably arise from the fusion of the Golgi vesicles. The supranuclear bodies would thus have a dual origin, endogenous for the hydrolytic enzymes that contain, and exogenous for the di- and tripeptides supplied by the food which are then further digested inside them (Noaillac-Depejre and Gas, 1973).

By observing samples from fish fed with diet B, the *muscularis externa* is thinner than that of samples associated with diets C and T, suggesting that butyrate facilitates the formation of softer fecal pellets. The complex structure of the folds is suboptimal, not perfectly organized, and includes a high number of goblet cells, a thick lamina propria, a significant number of leukocytes as confirmed by the positive staining with the CD45-antibody, although the inflammatory infiltrate is actually less dramatic than that found in samples of the C group, and more concentrated at the submucosa level. The supranuclear vacuoles are less concentrated in number than those of fish fed with diet C and are often heterogeneous. In enterocytes TEM showed an alteration at the microvilli level, corresponding to cells in some state of degradation, while other areas appeared definitively less compromised, with microvilli regularly displayed; the cells show an intense vesicular system and supranuclear vacuoles characterized by inhomogeneous content, partly dense, like that found in group C, partly clear. The fact that the acidophilic and PAS-positive content found in the vacuoles as already descibed for diet C was now reduced, might allow us to assume that the metabolization processes started after butyrate was added to the diet, allowing a sort of recovering process.

In fish fed with diet T, the *muscularis externa* was more developed, as in diet C. The folds were long and regular, with enterocytes showing a limited number of vacuoles and an increased number of goblet cells that were larger in size and mainly concentrated in the medioapical part of the fold. Leukocytes, immunoreactive to the CD45 antibody, are still present both in the mucosa and the submucosa, similar to diet B.

The TEM images of enterocytes are very different from the ones from diets C and B, with cells showing well-displayed microvilli, frequently covered by a protective mucus layer, no alterations in the structure, and supranuclear vacuoles filled with a heterogeneous content, including membranous bodies suggesting that an autophagic processes are in progress. To our knowledge no other example of taurine associated with a diet causing autophagic processes has been reported in the literature, and thus these findings should be further investigated. Furthermore, such a process seems to enable enterocytes of fish fed with diet T to tentatively cope with the stress induced by the SBM, helping to protect the intestinal mucosa.

Molecular markers for inflammatory cytokines substantially supported the optical and TEM microscopy observations. The transcription level of the IL-1β gene was highest in distal intestine of sea bass fed diet C, indicating a chronic inflammation that was less evident in diets B and T. Cytokine IL-8 was less expressed in the T than in C and B groups, which supports a protective action of taurine to oxidative stress that would induce a "call" for the inflammation agents that would generate a cascade inflammatory reaction. For IL-10, reported elsewhere as an anti-inflammatory cytokine, transcription activity was strongly reduced in diet T but still relatively high in diets C and B. Among the cytokines considered, only IL-6 gene expression was not affected by dietary butyrate or taurine supplementation. Proinflammatory IL-6 is of particular interest since it is expressed to a small extent on normal colonic epithelium but to a much greater degree in colonic carcinomas (Shirota *et al.*, 1990). In human colonic cells, increased expression of IL-6 and its receptor IL-6R have mainly an anti-apoptotic effect (Yuan *et al.*, 2004). IL-6, indeed, upregulates the expression of anti-apoptotic protein Bcl-xl (Fujio *et al*., 1997; Scwarze and Hawly, 1995). Yuan and colleagues (2004) clearly demonstrated that butyrate downregulates IL-6 signaling in human colonocytes by inhibiting IL-6R rather than IL-6 expression. Similarly, butyrate did not influence IL-6 expression in sea bass, but we cannot exclude that, even in fish, it could act principally on its receptor expression. It is well documented that taurine inhibits the overproduction of inflammatory mediators such as TNF-α (Kim and Cha 2014). TNF-α induces further production of other pro-inflammatory mediators, such as IL-6, IL-8 and TNF-α, too. In sea bass intestine, TNFα tends only to be expressed at higher levels in fish fed diet B, which was associated with upregulation of IL-8

but not IL-6 in the same group. Moreover, higher expression of TNF-α in fish receiving B diet supports the TEM observation of an effect of increased cellular turnover, promoted by butyrate.
Our findings on cytokine activity are mainly in agreement with the literature. Indeed, in the intestine of carps receiving soy as protein source, a peak of IL-1β at was observed at week 1, continuing to be above the control level for the entire experiment, as well as TNF-α1. A strong upregulation was observed for IL-10 after 1 week of SBM feeding, but at weeks 3 and 5 expression levels were downregulated again at values either lower or similar to the control (Uran *et al*., 2008a). In zebrafish intestine, after eating diets containing different amounts of SBM, Hedrera *et al.* (2013) reported a significant increase in transcription of the mRNA synthesizing proinflammatory cytokines such as IL-1β and IL-8. The IL-10 transcriptional activity, expected to have anti-inflammatory action, was increased in the group receiving feed with soy protein.
In conclusion, sea bass fed a diet containing a concentration of a soy protein source close to 30% (16.7% as SBM and 12.8 as full-fat soy) for two months developed a severe inflammatory status in the distal intestine. The symptoms of inflammation seem to be mitigated, albeit at different success rates by adding sodium butyrate or taurine at concentrations as low as 2%. However, the meccanisms underlying these effects should be further investigated along with any possible synergistic effect of the two substances combined in the feed in normalizing the intestinal abnormalities caused by soyabean meal.

5.2 Commercial feeds and product quality

The quality of farmed fish can be described by several physical parameters like product freshness and appearance and by nutritional and organoleptic properties. The substitution of fish oil with vegetable oils can affect the lipid composition of fish fillet, by increasing the ratio n-6/n-3. The study of fatty acids diet and fillet composition is a preliminary step to further investigations. Growth performances, together with physiological and physiopathological markers, may give a first figure on the effects due to dietary imbalances, but to have a complete view of the problems generated by FM and FO substitutions, an assessment of the fillet quality is paramount. A specific marker for fish quality is the content in *n-3* LCFAs and the ratio n-3/n-6. The fatty acids composition of fillets generally reflects the fatty acids content in the diet, as largely reported in literature (Rosenlund *et al.*, 2001; Torstensen *et al.*, 2004, 2005; Turchini *et al.*, 2009; 2011; 2013). Viceversa, as occurred in our case, the fillet may not be a direct projection of the diet, when the fatty acids were involved on metabolic processes, as β-oxidation of lipid to produce energy, in vivo bioconversion (capability on chain elongation and desaturation) and lipogenesis. The evaluated fatty

acids composition of fillets mirrored those of the experimental diets and this positive correlation was documented by several research (Rosenlund *et al.*, 2001; Torstensen *et al*., 2004, 2005; Turchini *et al.*, 2009; 2011; 2013). On the contrary, the fillet was not a copy of diets because fatty acids were involved on metabolic processes as, β-oxidation of lipid to produce energy, *in vivo* bioconversion (capability on chain elongation and desaturation) and lipogenesis.

Generally, the amino acid composition of feeds revealed the main dietary imbalances due to inclusion of plant feedstuffs in fish feed. Crystalline forms of lysine and methionine are generally added to the substituted diets, to recover the poor availability of such EAAs in the vegetable meals. Many studies reported reduced growth rates, poor feed utilization when using crystalline amino acids to replace protein-bound AA. In channel catfish was documented the low utilization of free lysine compared with a protein-bound lysine (Zarate *et al.*, 1999). The high rate of stomach evacuation and absorption makes the crystalline amino acids insufficiently available to protein synthesis and consequently on promoting the growth (Sveier *et al*., 2001). On the contrary, recent work reported by Rolland *et al.* (2015) in juvenile rainbow trout has claimed a positive correlation between increase level of crystalline methionine and growth, protein utilization and feed conversion. So, the external methionine supply was considered fundamental in light of feeds formulation.

In rainbow trout, the highest level of DHA in fish fillet than in the feed, showed the ability of this species to elongate and desaturate C20 *n-3* (EPA) in C22 *n-3* (DHA). In fact, EPA amount was higher than DHA in the feeds while this ratio resulted reversed in the fillets, except for F2 fillet group. EPA plus DHA fillets content highlighted the nutritional quality of fish food. In fact to cover the human daily requirement (500 mg/die), we should consume 130-140 grams of F3 and F5 fillets per week, while we should consume around 900-1000 grams of F2 fillets per week to cover the same daily-based requirements. Therefore, at nutritional level fish feed formulation remained a focal point of aquaculture in order to provide a quality product.

We tentatively explained, by a statistical approach (Spearman's correlation), how the amount of a fatty acid could influenced the correspondence between lipids feed and fillet composition. The results obtained showed that the negative or positive values of fatty acids correlation coefficients could decrease or increase the lipid fillet and diet correspondence, respectively. If we would hypothesize on the mentioned correlation, DHA (C22:6n3) reported a high and positive correlation (0.77) that could mean its presence increasing the assimilation of the others fatty acids and it could be linked to its involvement in n-3 biosynthetic pathway.

A negative Spearman’s correlation was reported for erucic acid (C22:1n-9) and its presence could decreased the lipid correspondence between fillets and feeds. It was known that erucic acid feed presence was linked to some vegetable oil supply and it was not advised to human diet because of promoting cardiac lesions. In fact, breeding programs have focused on lowering erucic acid in rapeseed oil with the production of canola oil (Turchini *at al*., 2010). Further analysis could deepen this statistical approach (Spearman’s correlation) whether it can be a real support to the feed formulation.

6. CONCLUSIONS

To protect oceanic resources and biodiversity, and to face the increasing costs of fish meal and oil on the international markets, the future of aquaculture is oriented toward a progressive reduction of oceanic resources in the feed. They are substituted by alternative sources of proteins and oils, vegetable sources being at the moment the most applied substitutions. Indeed, the problems arising with such substitutions represent a serious bottleneck for the expected growth of the industry, that from the actual almost 70 million tons produced worldwide, is expected growing up to 50% by the next 30 years. Adequate corrections are possible, in order to compensate any aminoacids missing in the substitutive diets, as well as mitigation of inflammatory damages due to undesired compounds associated to the vegetable sources of proteins. The nutritional quality of fish fillet, that is expected to supply the needed amount of *n-3* LCFAs to human consumer assuming 2 meals of fish per week, is another aspect to face with, when large amount of FO are substituted and when unbalanced diet aminoacids are supplied to the fish fed vegetable proteins.

With this study, we contribute to demonstrate the effectiveness of butyrate in protecting fish intestine and apparently liver as well, from the flogistic processes due to antinutritional and inflammatory compounds associated to the vegetable sources of proteins. In spite butyrate doesn't seem to directly improve fish growth, it has been demonstrate to sustain enterocytes functionality and to reduce intestine inflammatory focus, by inducing the hyperacetilation of histones core (H4) and increased the expression level of genes (*il-10*, *dicer1*, *ehmt2*, *hdac11*) related to immune response.

The limited amount of butyrate expected being available by the liver, was sufficient to induce a response that may be supposed having an anti-inflammatory and anti-viral protection of this organ. Nevertheless, because of the fast utilization of butyrate by the gut walls, a way to ensure a continuous availability is envisaged. So, the possibility to induce a butyric fermentation by the gut microbiota could help to solve the problem of availability and to avoid the cost of its introduction in the diet. Our results actually indicate this possibility, by addition of undigestible fibers in the diet, chitosan representing a promising solution. Nevertheless, further studies in this direction should be carried on and the newly available sequencing techniques as NGS Illumina could be the approach to follow, as long as the DGGE approach we followed did not provide enough information on the composition of the gut microbiota.

We also contribute to support the need of taurine supplementation in the diet for sea bass, when missing or not sufficient in the protein sources. This sulphonate aminoacid demonstrate a direct effect on fish growth, indicating its indispensability in the diet. Moreover, its role as antioxidant, supporting fish activity as forced swimming with a clear control of the produced ROS, has been demonstrated when a concentration as 1.5% is ensured in the diet. Its role in reducing intestine inflammations has been demonstrated, in a synergistic action with butyrate. Further studies on the physiological role of this aminoacid are envisaged, as its action may involve a number of delicate equilibriums in internal metabolism.

The amount and quality of oils in the feed is pivotal when the fillet quality is concerning, nevertheless, for the restitution of EPA and DHA from the feed to the fillet, an aminoacids balanced diet and antioxydants must be ensured in the fish diet.

Salmonids but not sparids show an active desaturation and elongation of fatty acids from the feed. In Rainbow trout, the feed EPA/DHA ratio shows generally an inversion when assessed in the fillet, as EPA is consumed by trout to produce DHA. This does not occur with diets being poor in essential aminoacids as lysin and methionine, as well as taurine, where DHA is consumed by the fish, instead. Far to be conclusive, our data show that depending from the fish diet, fillet contains such different amount of EPA and DHA that human consumer should obtain his weekly needs by consuming 1-6 meal of fish per week. From our data, the difference does not seem to be due to the feed EPA and DHA content, but to an unbalanced aminoacid and antioxydants content in the diet, instead.

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8 Submitted papers

- 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.
- Butyrate and taurine exert a mitigating effect on the inflamed distal intestine ofEuropean seabass fed with a high percentage of soybean meal.

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