UNIVERSITY OF INSUBRIA, VARESE

Department of Biotechnology and Life Sciences

(DBSV)

Ph.D. School:

Biological and Medical Sciences

Ph.D. Program in:

"ANALYSIS, PROTECTION AND MANAGEMENT OF BIODIVERSITY"

- XXVI Course –

Biodiversity for the protein sources utilized in feeds for intensive fish culture

Dr. Enrico Ingle

Guide Professor:

Marco Saroglia

December, 2013

Abstract

Biodiversity for the protein sources utilized in feed for intensive fish culture

The reduction of dependence on fishmeal (FM) in is compulsory for a sustainable growth of aquaculture. Concerning the protein component, feed companies turned towards the use of alternative protein sources, as vegetable meal (VM), that today vary in the range 48-68% in commercial aquafeeds.

Nevertheless, elevate VM in the diet may not always be tolerated by fish, resulting in gut inflammations and histological damages, with reduced digestive functionalities and integrity. Microbiota composition is influenced by the diet. The digestive apparatus adjusts to changing of diet composition, with the mucosal defence system providing the gastro intestinal tract (GIT) with mucopolysaccarides that protect the intestine walls from injurious agents; moreover, a damaged fish gut may become an important infection route for microbial pathogens in fish.

The GIT contributes to body health in many ways: of particular interest in this context is the emerging concept of "gut health". It covers multiple positive aspects of the gastrointestinal tract, such as the absence of gastrointestinal illness, normal and stable intestinal microbiota, and effective immune status, that are also able to determine a state of well-being. Among protective nutraceuticals that are produced with anaerobic bacteria fermentation and may be add to feed as well, the short chain fatty acids play a relevant role. Among them, butyrate could have a pivotal role in the context of "nutritional welfare": in particular, acting as an anti-inflammatory agent, providing an enterocytes trophic action, acting as promoter of oligopeptides transport, promoting production of antinflammatory cytokines, thus positively acting on fish growth.

Also the molecular biomarkers, that may early describe the progression of any intestine damages, could be useful tools to tune an appropriate nutritional strategy.

In feeding challenges, a scoring system has been set up to assess the diet-related histological changes in different gut tracts; such system showed that histological changes occur early, within the first 10 days after the new diet administration. The intestine conditions shown a sort of adaptation trend after 40-50 days trial, after which the score trends tend to recover toward the initial level. We speculate that, as long as such changes are reversible, healthy fish is able to respond quickly at diet variation returning toward the initial condition within 1-2 months. However, we have to take in account several parameters (growth performances, histological changes, molecular biomarker levels, gut microflora biodiversity, rearing condition etc.) to compose a comprehensive picture on the animal nutritional welfare.

The present work has enabled to evaluate the zootechnical performances of four diets at different level fishmeal substitution with protein vegetable sources. The result showed that seabass may be fed with aquafeed containing around 20% of fishmeal without relevant difference in growth performance from feed richer in FM.

The great importance of biodiversity concept, in fishing resources for feed as well as in maintaining the fish gut microbiota in good condition, is evident with this study.

INDEX:

1.0 Introcuction	Pag.	1
2.0 Materials and Methods	Pag.	18
2.1 Growth Performance		19
2.2 Histological analysis		20
2.3 Microbial culture from fish gut		23
2.4 DNA isolation and PCR-denaturing gradient gel electrophoresis (DGGE)		24
2.5 Molecular Biomarkers		25
2.5.1 Preparation of total RNA		26
2.5.2 Quantitative real-time RT-PCR		26
2.6 Statistic		30
3.0 Results	Pag.	31
3.1 Growth performance and biometrics parameters		31
3.2 Scoring: evaluation of histological changes		41
3.3 Measure of histological changes		57
3.4 Count of cell in gut and liver		61
3.5 Microbial culture from fish gut		65
3.6 DNA isolation and PCR-denaturing gradient gel electrophoresis (DGGE)		69
3.7 Molecular Biomarkers (mRNA levels with Real-Time PCR method)		70
4.0 Discussion	Pag.	78
5.0 Conclusion	Pag.	90
6.0 Acknowledgement	Pag.	92
7.0 References	Pag.	93

8.0 Appendix Pag. 107

9.0 Publications Pag. 112

1.0 Introduction

Aquaculture takes a variety of forms: extensive or intensive, in natural settings or tanks, in fresh water or seawater, in flow-through or recirculation systems, traditional or modern, and so on. Traditional, extensive fish farming consists in sustaining ponds (natural or artificial) and lagoons that promote the development of aquatic fauna, particularly encouraging the growth of juveniles/fry until they reach a desirable market size at a higher yield than that of the natural ecosystem. Combined forms of fish farming in ponds and lagoons have increasingly evolved more into managed production modes described as semi-extensive aquaculture. This assists nature by introducing fry from hatcheries into the sites and providing supplemental feed. For a yield much greater than semi- and extensive aquaculture provide, an intensive fish farming site is used, generally composed of several tanks of different sizes and depths suited to the different stages of growth of the fish, either floating at sea or in permanent structures on land. In such installations, water recirculation system are applied which offer the advantages of isolation from the external environment, meaning that all the parameters of the water can be monitored and controlled: temperature, pH, salinity, disinfection, etc. Moreover, to achieve a greater production it is necessary to introduce from outside all that the natural system is unable to supply for supporting a greater biomass: food, oxygen and removing catabolites and carbon dioxide.

Among the fish farming systems, saltwater aquaculture farming marine fish represents one important source of animal protein for human health.

In the 1960s, a floating cage technology started it's development as a major innovation in sea farming and its availability at a reasonable price proved to be an unprecedented commercial success and turned sea farming into an up-and-coming sector in Europe. Mediterranean countries studied and developed the rearing of sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) and, during the 1990s, farming of these species spread throughout the Mediterranean.

Salmon, sea bass, and seabream remain the flagship products of European sea farming, offering a diversification in quality that appeals to different market segments; the 1990s and the first decade of the new century saw the development of another form of intensive sea farming, this time of flatfishes.

Although the recirculation technology progress offered new prospects for land-based aquaculture, the start of the 21st century nevertheless has brought a sizeable new challenge for the industring. European coastal zones are saturated with activity and have

no more land to offer for expanding aquaculture. Sea farming is obliged to move further away from the coast. It must move off shore, far from sheltered coastal areas.

Actually off-shore technology is the new field of research in European aquaculture and it will be in the future. Therefore it remains a growing, vibrant and important production sector for high protein food.

Capture fisheries and aquaculture supplied the world with about 148 million tonnes of fish in 2010 (with a total value of US$ 217.5 billion), of which about 128 million tonnes was utilized as food for people, and preliminary data for 2011 indicate increased production of 154 million tonnes, of which 131 million tonnes were destined as food (FAO 2012 http://www.fao.org/docrep/016/i2727e/i2727e.pdf). With sustained growth in fish production and improved distribution channels, world fish food supply has grown dramatically in the last five decades, with an average growth rate of 3.2% per year in the period 1961–2009, outpacing the increase of 1.7% per year in the world's population. World per capita food fish supply increased from an average of 9.9 kg (live weight equivalent) in the 1960s to 18.4 kg in 2009, and preliminary estimates for 2010 point to a further increase in fish consumption to 18.6 kg per capita.

Of the 126 million tonnes available for human consumption in 2009, fish consumption was lowest in Africa (9.1 million tonnes, with 9.1 kg per capita), while Asia accounted for two-thirds of total consumption, with 85.4 million tonnes (20.7 kg per capita), of which 42.8 million tonnes was consumed outside China (15.4 kg per capita). The corresponding per capita fish consumption for Oceania, North America, Europe, and Latin America and the Caribbean were 24.6 kg, 24.1 kg, 22.0 kg and 9.9 kg, respectively. China has been responsible for most of the increase in world per capita fish consumption, owing to the substantial increase in its fish production, particularly from aquaculture. China's share in world fish production grew from 7% in 1961 to 35% in 2010.

In 2009, fish accounted for 16.6% of the world population's intake of animal protein and 6.5% of all protein consumed. Overall global capture fisheries production continues to remain stable at about 90 million tonnes.

Farmed food fish include finfishes, crustaceans, molluscs, amphibians (frogs), aquatic reptiles (except crocodiles) and other aquatic animals (such as sea cucumbers, sea urchins, sea squirts and jellyfishes). In 2010, global production of farmed food fish was 59.9 million tonnes, up by 7.5% from 55.7 million tonnes in 2009 (32.4 million tonnes in 2000) [See appendix:Tab.1]. The reported grow-out production from aquaculture is almost entirely destined for human consumption. The total farm gate value of food fish production

from aquaculture is estimated at US$119.4 billion for 2010.

In 2010, the top ten producing countries accounted for 87.6% by quantity and 81.9% by value of the world's farmed food fish. Asia accounted for 89% of world aquaculture production by volume in 2010, and this was dominated by the contribution of China, which accounted for more than 60% of global aquaculture production volume in 2010.

Other major producers in Asia are India, Viet Nam, Indonesia, Bangladesh, Thailand, Myanmar, the Philippines and Japan. In Asia, the share of freshwater aquaculture has been gradually increasing, from around 60% in the 1990s up to 65.6% in 2010. In terms of volume, Asian aquaculture is dominated by finfishes (64.6%), followed by molluscs (24.2%), crustaceans (9.7%) and miscellaneous species (1.5%). The share of non-fed species (they only use by environmental trophic resources) farmed in Asia was 35% (18.6 million tonnes) in 2010 compared with 50% in 1980. In North America, aquaculture has ceased expanding in recent years, but in South America it has shown strong and continuous growth, particularly in Brazil and Peru.

In terms of volume, aquaculture in North and South America is dominated by finfishes (57.9%), crustaceans (21.7%) and molluscs (20.4%).

In Europe, the share of production from brackish and marine waters increased from 55.6% in 1990 to 81.5% in 2010, driven by marine cage culture of Atlantic salmon and other species. In 2010, finfishes accounted for three-quarters of all European aquaculture production, and molluscs one-quarter.

Africa has increased its contribution to global production from 1.2% to 2.2% in the past ten years, mainly as a result of rapid development in freshwater fish farming in sub-Saharan Africa. Finfishes overwhelmingly dominate African aquaculture production, with only a small fraction from marine shrimps and marine molluscs.

Oceania accounts for a minor share of global aquaculture production and this consists mainly of marine molluscs and finfishes, with the latter increasing owing mainly to the development of farming of Atlantic salmon in Australia and Chinook salmon in New Zealand.

The least-developed countries (LDCs), mostly in sub-Saharan Africa and in Asia, remain minor in terms of their share of world aquaculture production (4.1% by quantity and 3.6% by value) with the main producers including Bangladesh, Myanmar, Uganda, the Lao People's Democratic Republic and Cambodia. However, some developing countries in Asia and the Pacific (Myanmar and Papua New Guinea), sub-Saharan Africa (Nigeria, Uganda, Kenya, Zambia and Ghana) and South America (Ecuador, Peru and Brazil) have

made rapid progress to become significant or major aquaculture producers in their regions. In contrast, in 2010, developed industrialized countries produced collectively 6.9% (4.1 million tonnes) by quantity and 14% (US$16.6 billion) by value of the world's farmed food fish production, compared with 21.9% and 32.4%, respectively, in 1990.

Aquaculture production has contracted or stagnated in Japan, in the United States of America and in several European countries. An exception is Norway, where, thanks to the farming of Atlantic salmon in marine cages, aquaculture production grew from 151 000 tonnes in 1990 to more than one million tonnes in 2010.

Freshwater fishes dominate global aquaculture production (56.4%, 33.7 million tonnes), followed by molluscs (23.6%, 14.2 million tonnes), crustaceans (9.6%, 5.7 million tonnes), diadromous fishes with species migrating between fresh and salt waters (6.0%, 3.6 million tonnes), marine fishes (3.1%, 1.8 million tonnes) and other aquatic animals (1.4%, 814 300 tonnes).

In the last three decades (1980–2010), world food fish production of aquaculture has expanded by almost 12 times, at an average annual rate of 8.8%, while the world population grew at an average of 1.6% per year. Aquaculture enjoyed very high average annual growth rates of 10.8% and 9.5% in the 1980s and 1990s, respectively, but has since slowed and today the annual average is around of 6.3%. In contrast, world capture fishery production has almost stopped growing since the mid-1980s.

The combined result of development in aquaculture worldwide and the expansion in global population is that the average annual per capita supply of food fish from aquaculture for human consumption has increased by almost twelve times, from 0.7 kg in 1970 to 8,3 kg in 2010.

Despite the long tradition of aquaculture practices in a few countries over many centuries, aquaculture in the global context is a young food production sector that has grown rapidly in the last 50 years.

European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) are among the most important marine finfish species reared in Europe, particularly in the Mediterranean region, and production of both species is still experiencing a rapid expansion (Basurco and Abellán, 1999). Mediterranean trend from 2003 to 2007 (Fao 2010 http://www.fao.org/docrep/013/i1696e/i1696e00.htm) showed a global increase of 50% (Seabass from 85 601 to 124 029 tons ; Seabream from 103 214 to 157 577 tons) [See appendix: Tab 2 and 3].

To sustain high rates of increased aquaculture production a matching increase in the

volume of fish feeds is required; also taking in account expected growth of the world population (food fish reached 62.7 million tonnes in 2011, up by 6.2% from 59 million tonnes in 2010 [See appendix: Fig.1].

We will need more than 60 million tons of compound aquafeed by 2020 (FAO 2012; Tacon, 2013) and such feed must have adequate characteristics. With such a figure it becomes evident the importance of a biodiversity of available protein source, not only based on the fishing to have success in fish feeding.

Fishmeal is the most costly components of milled feeds used in the production of carnivorous aquacultured species. Therefore, dietary protein is the main focus of the feeding trials in aquaculture production.

It 'should be noted that worldwide annual production of fish meal (FM) is about 6 million tons and fish oil (FO) is less than 1 million tons, being remained fairly stable for the last 20 years although the introduction of precautionary quotas and increased use for direct human consumption has resulted in reduced volumes of whole fish going for fishmeal and oil [See appendix: Fig.2]. Such data suggest that productive level could remain sustainable also in the future, however the fisheries will do not be able to follow the demand increasing of further FM and FO production, mainly from the Atlantic, Chile and Peru fishing areas.

FM and FO are also produced from fish processing factory (trimmings, offal etc.) and/or by-catch, although to a limited extent. However, the capacity of the fisheries to cope with the increasing demand for fish oil has reached the limit of sustainability, owing to problems such as overfishing, climate alterations, and increasing demand from other sectors (Rana, 2009; Shepherd and Jackson, 2012).

Concerning the use of FM and FO: the International Fishmeal and Fish Oil Organisation (IFFO) estimate that more than 60% of global fishmeal production goes to aquaculture and that 79% is splitted almost equally among salmonids, marine fish and crustacean, whereas remaining 21% is used for others species [See appendix: Fig.3]; regarding fish oil, IFFO estimate that 81% of production goes to aquaculture, 13% to direct human consumption and 6% to other usage including fat hydrogenation and industrial use. The 68% of aquaculture amount, is used for samonids (See appendix : Fig.4].

Global aquaculture production has continued to grow while usage of fishmeal & fish oil is static [See appendix : Fig.5].

Fishmeal is an excellent high protein feed ingredient used at least at some stage in almost all intensive aquaculture systems, Fish oil is the best source of the omega-3 fatty acids EPA & DHA and its use in feeds ensures a healthy product for the final consumer.

To ensure and increase protein fish source provision, we have to find sustainable alternatives commodities suitable for aquafeed and some plant seeds such as linseed, rapeseed, or soybean oils represent good candidates (Hashim 2006). At the some time well known seafood benefits, in regards to human health, should be ensured (Criteria for safe use of plant ingredients in diets for aquacultured fish 2009: http://www.vkm.no/dav/1232b28f3b.pdf).

The evidence favouring health benefits of dietary fish intake is overwhelming. Examples include a 16-year study demonstrating reduced risk of coronary heart disease in human subjects in response to increased fish ingestion (Hu and Willet, 2002). Many of the benefits of fish consumption have been attributed to the relatively low amounts of saturated fats and the high proportion of omega-3 fatty acids in fish and fish oils.

It's widely reported that dietary omega-3 fatty acids reduce the rates of all-cause mortality, cardiac and sudden death, and possibly stroke (Wang et al., 2006). Positive evidence of the health benefits of eating fish contrasts with some inherent risks of seafood contaminants.

Among the teleosts, freshwater species differ from marine species in their fatty acid requirements. The former have a recognized capacity to desaturate and elongate the fatty acids with 18 carbon atoms, linoleic (18:2 n-6) and linolenic (18:3 n-3) acids, into highly unsatured fatty acids (HUFA) with 20 and 22 carbon atoms such as arachidonic (AA, 20:4 n-6), eicosapentaenoic (EPA, 20:5 n-3), and docosahexaenoic (DHA, 22:6 n-3) acids (Sargent et al., 2002). The HUFA play a major role in ecosanoid production (AA and EPA), vision (DHA), brain development (DHA) and regulation of expression of several genes involved in lipid metabolism (Forman et al., 1997).

In contrast to freshwater fish, marine species are assumed to have a deficient capacity to bioconvert 18 carbon precursors into HUFA and, hence require preformed HUFA in their diet. Although long recognized, the reasons for this deficiency in marine fish remain unclear. Firstly, it could be because of a gene loss corresponding to an adaptation to the n-3 HUFA-rich marine food web (Sargent et al., 1995). Secondly, marine fish show a deficiency in desaturase enzymes, probably because of repressed desaturase enzyme activity associated with high levels of HUFA usually present in the diet, as previously seen in mammals. By determining the essential fatty acid requirements in marine fish and understanding the molecular basis of HUFA biosynthesis and how it is regulated in fish, the use of vegetable oils can be manipulated and optimized in aquaculture.

Improving zootechnical performance like: FCR (Feed Conversion Rate) reducing and SGR

(Specific Growth Rate) increasing; we could be able to increase the aquaculture sustainability, reducing FM and FO, usage. Especially we have to improve the "Fish-In" and "Fish-Out" (FIFO) ratio; FIFO is transfer coefficient between pelagic forage fish equivalent and aquaculture production (Jackson 2009).

Tacon and Matian (2008) reported an overall fish-in fish-out ratio of 0.70, considering a wet fish to fish meal processing yield of 22.5% and wet fish to fish oil processing yield of 5%, but extraction a aquaculture is accounted on it, as algal and molluscs production.

At a species-group level, calculation of small pelagic forage fish input per unit of farmed fish output showed steadily decreasing fish-in fish-out ratios for all cultivated species from 2000 to 2010 (source IFFO 2012 http://www.iffo.net/downloads/Position%20Papers/Demonstrating%20responsible%20marine%20feed%20IFFO.pdf), with decreases being most dramatic for carnivorous fish species such as salmonids (decreasing from 2.6 to 1.8), eel (decreasing from 3,0 to 1,8) and marine fish (decreasing from 1,5 to 0,9) /.

Today only strictly carnivorous finfishes have FIFO higher than 1 while herbivorous and omnivorous finfish are below 0,6 [See appendix : Tab.4]

Even though the wide variation observed concerning dietary fishmeal and fish oil use within and between countries for the same species (Tacon et al. 2006); it will be possible reduce the FIFO ratio (value close to 1) also for carnivorous species. Taking in account also FM and FO produced from fish processing factory and proteins and oils supply from plants.

Currently in marine fish diets (marketable feed) the percentage of proteic meal from plant source varies from 48% to 68%.

When using high dietary levels of plant derived materials, particularly those derived from soybean, it is important to consider the impacts on gut microbiota as well as gut inflammation and histology as the gastrointestinal (GI) tract can be one of the important infection routes for some pathogens in fish. In some cases, diets containing up to 20% inclusion level of high-quality plant protein sources have resulted in better nutrient digestibility and growth parameters than the fishmeal-based control diets.

Ideally, the potential and eventual use of a new ingredient depends on several factors (Allan, 2004):

- in general, the higher the protein content and the closer the amino acid profile is to fish meal, the better.
- availability and price (ingredients that are easily or commercially available and cheap

are preferable).

- presence and concentration of anti-nutritive factors (anti-nutritive factors are common in plant proteins and can cause reduced feed intake, feed efficiency and growth).
- presence of contaminants (toxins from fungus and pesticides etc).
- digestibility and utilization of energy and nutrients.
- effects on attractiveness and palatability (while aquatic products such as fish meal and meat meal tend to make feeds more attractive and palatable, plant based ingredients may have an opposite effect).

Today various plant-derived ingredients are used in aqua feed. Wheat is the most widely used ingredient, and is used as a carbohydrate source with the currently most utilised feed technology (extrusion). As a lipid source, both rapeseed oil and some palm oil, among others, are used. As a protein ingredient, among others, the following have been used: soybean (with varying degrees of processing), maize, peas, lupine, and broad beans.

Despite the possible presence of anti-nutritional factors (ANFs), soybean meal remains one of main protein sources for aquafeed. The causal agent(s) in soybeans that lead to reduced nutrient digestibility and the inflammatory response in the distal intestine are still largely unknown, but affect many specific digestive processes, from feed intake to enzyme activities to nutrient transport to gut histology (Uràn, 2008).

However, such details are often lacking in studies focusing on other alternative protein sources that are already in use in formulated feeds for various fish species.

Losses due to disease represent a major cost in the aquaculture industry internationally, and diet composition is among several factors that may influence disease susceptibility. Optimal health and disease resistance is dependent not only on an optimal balance of nutrients available for all systemic needs, but also on optimal function of the gastrointestinal tract (GIT) and associated organs. The GIT is constantly exposed to a conglomeration of nutrients, ANFs and non-nutrients also comprising food antigens and micro-organisms. The digestive apparatus adjusts to changing diet composition, and the mucosal defence system provided by the GIT must protect the body from injurious agents and at the same time develop oral tolerance to antigens from the diet and commensal microbiota (Chehade and Mayer, 2005; Sansonetti, 2004). The microbiota's species composition, which may be influenced by various dietary nutrients, non-nutrients and anti-nutrients, is also of importance for the host's gut and general health (Ringo & Olsen, 1999; Ringo et al., 2006; Bauer et al., 2006; De Schryver et al., 2010; 2011).

In our work the attention was focused on histological changes in the gut of sea bass fed at

different levels replacement of fishmeal by plant protein sources. Such diets were related with marketable aquafeed for intensive aquaculture.

The aim was to evaluate the fish welfare by scoring system focused on four histological parameters (Uràn et al., 2004; Knudsen et al., 2007; 2008).

While histological scoring have been approached for some salmonid species (Gibson-Corley, 2013; Mitchell, 2012) with our study a new arbitrary scoring system has been approached for sea-bass gut histology, to evaluate the cellular and tissue changes in the GI tract.

The applied scoring protocol takes in account four parameter, empirically rated:

- supranuclear vacuolization of the absorptive cells
- lamina propria of the single intestinal fold
- connective tissue between base of folds and stratum compactum (or lamina propria at basal level)
- number and or shortening of folds (simple and complex) heights of mucosal foldings

For each parameter, the score rate ranges from 1 to 5: a rates 1-2 representing normal morphology, while a rate up to 5 being assumed as a morphological descriptor of severe enteritis (Crissman, 2004) .

Histological changes were not specific pathological lesions, but we have to take in account such changes as indicator of reversible gut modifications diet related.

Furthermore we assessed the productive performances of single fish groups fed different diets. We have to underlining that all diets had marketable-like aquafeed formulation and were able to supply the sea bass nutrient requirements. Each diet could be suitable for aquaculture commercial farms, therefore the histological changes and the variations concerning other parameters, should be expected not dramatic and in the most case reversible.

Because the gut represents the functional link between energy intake and metabolisable energy, it could be expected that gut size or its features would change under selection pressures diets related (D'Anatro at al., 2013; Gawlicka and Horn, 2005; German et al., 2010; Gaucher et al., 2012). In this sense, reversible adjustments in the size of the digestive organs is a widespread phenomenon among vertebrates, and the congruence between empirical data and optimal digestion models strongly supports the idea that it has evolved by natural selection (Olsson et al., 2007).

The histological score was tentatively compared to the transcriptomic activity of genes describing intestine walls functionality (molecular biomarkers). A biomarker is defined as

any biological response to a stress factor measured inside the organism indicating a deviation from the normal state. This response can range from molecular through cellular and physiological to behavioural changes. Molecular biomarkers directly indicate a gene activity and for this reason they have the characteristics for being useful early indicator.

Monitoring gene expression profile for many genes can help us to paint a global picture of gene expression under a specific developmental stage, environmental condition, or treatment. In practice, gene expression profile experiments often involve measuring the relative amount of mRNA expressed in two or more experimental conditions ("treatment"). The correlation of "treatment" and gene expression profile could provide inference on gene functions. In this work we are looking for new insights on the effect of fishmeal replacement with vegetable origin source monitoring several gene expression profiles included SLC6A19, cloned from sea bass in Insubria University laboratories.

In the course of the past few years, the study of intestinal peptide transport has rapidly evolved into a field of nutritional and biomedical applications. In particular, the effect of dietary protein on oligopeptide transporter 1 (PepT1) expression and activity has been an area of active research in recent years (Sangaletti et al., 2009: Terova et al., 2009).

Mammalian members of the SLC15 family are electrogenic transporters that utilize the proton-motive force for uphill transport of short chain peptides and peptido-mimetics into a variety of cells. The prototype transporters of this family are PEPT1 (SLC15A1) and PEPT2 (SLC15A2), which mediate the uptake of peptide substrates into intestinal and renal epithelial cells.

The intestinal oligopeptide transporter 1 (PepT1), responsible for the uptake of dietary di- and tripeptides in cells, is an integral plasma membrane protein. It transports peptides against a concentration gradient by coupling the movement of substrate across the membrane with the movement of protons down an inwardly directed electrochemical proton gradient. Recent studies on dietary protein and PepT1 expression, demonstrated that expression levels and function of PepT1 are very responsive to dietary treatments.

In general, peptide transporter expression and activity increase in conjunction with dietary protein and peptide levels, suggesting that up-regulation via a high-protein diet may represent a mechanism for taking advantage of the abundant resource, whereas up-regulation shown in response to a lack of substrate appears to be a compensatory mechanism to scavenge amino acids in the lumen.

In teleost fish, PEPT1 plays a significant role in animal growth by operating, at the gastrointestinal level, as part of an integrated response network to food availability that

directly supports body weight. Notably, PEPT1 responds to both fasting and refeeding and is involved in a phenomenon known as compensatory growth (a phase of accelerated growth when food levels are restored after a period of growth depression). In particular, PEPT1 expression decreases during fasting and increases during refeeding, which is the opposite of what observed so far in mammals and birds (Verri et al., 2011). This information would be of great importance, in particular for the farmed species raised in feed-based aquaculture systems, where any improvement of aquafeed utilization can lead to a decrease in production costs.

Also the Intestinal Neutral Amino Acids Transporter (SLC6A19), is reported as biomarker of intestinal oligopeptide and aminoacid transporters.

The SLC6A19, is an integral plasma membrane protein, responsible for the uptake of a broad range of neutral amino acids across the apical membrane of enterocytes and renal cells. It actively transports non-polar, bulky, branched amino acids against a concentration gradient from lumen into the epithelial cells of small intestine and kidney by coupling the movement of substrates across the membrane with the Na^+-electrochemical gradient.

In mammals, SLC6A19 is prominently expressed throughout the epithelium of the small intestine, where it is responsible for the transport of a significant fraction of dietary neutral amino acids across the brush border membrane. The corresponding information for fish is completely unknown. In particular, we know nothing about the response of SLC6A19 at the mRNA level to different feeding regimens in fish, although this knowledge could provide an accurate estimate of gastrointestinal function in fish and is relevant to understanding the mechanism by which the expression of aminoacid transporters is regulated.

It is a matter of fact that at the moment very limited information is available on the transport events that mediate the uptake of amino acids and oligopeptides, in spite of the physiological relevance of such nutrients intestinal absorption in fish growth (mostly in cultured species).

Today, a functional approach to sequences of genes, mRNAs, and proteins involved in solute transport functions across teleost fish cell membranes is needed to elucidate the role of transporters in teleost fish physiology, in the perspective of confirming old and establishing new concepts, and applying the most relevant information to health, nutrition, and welfare of cultured species (Verri et al., 2012).

When dietary amino acids are provided in the form of protein, these proteins must be degraded in the digestive tract for the cellular anabolic and catabolic processes in the

body tissues. Digested dietary proteins in both teleosts and mammals are subject to hydrolysis by a range of proteases and peptidases that generate a mixture of free amino acids and small peptides, which are absorbed across the apical membranes of enterocytes.

For monogastric mammals and stomach-possessing fish, protein digestion begins in the stomach lumen. Hydrochloric acid secreted by the stomach parietal cells denatures the dietary proteins and decreases stomach lumen pH to approximately 2.0-2.5.

The major gastric proteases, autocatalytically activated from zymogens (pepsinogen) at acidic pH, degrade proteins into polypeptides of different size, and some free amino acids (Clements and Raubenheimer, 2006). These hydrolytic products and some intact proteins will be passed into the small intestine for further digestion. For most marine fish larvae, gastric pepsin activity and hydrochloric acid secretion seems to be very low or absent (Tanaka et al., 1996, Cahu et al., 2001). Whether the lack of HCl, and pepsin will hamper enzymatic protein digestion in fish larvae needs to be further addressed (Rönnestad et al., 2003).

Among molecular biomarkers intestine walls functionality affected to the diets we have to take in account also growth indicator as "myosin" (muscular protein) and "IGF1" (Insulin-like Growth Factor 1).

One of the most important factors in deciding the formulated diet's effectiveness is what effect diet has on growth, in particular on muscle growth, being muscle (fillet) weight gain vs. visceral fat deposition, one of the most important criteria of fish growth, pertaining to commercial production.

The myotomal muscle comprises more than half of the body mass in most fish (Bone, 1978). This relevant mass is not only a specific mechanical adaptation to the aquatic life (due to the density of medium, rapid swimming requires a large amount of muscle without concurrent problems of economy of weight), but also serves as a major protein storage to be used for a number of energy-demanding activities (Weatherley and Gills, 1987) and has an important auxiliary function as a store of metabolites and amino acids. Muscle has the highest growth rate efficiency of any tissue, making the muscle protein synthesis per se an excellent overall measure of fish growth (Houlihan et al., 1989; 1995).

Different techniques has been used to estimate fish growth, such measuring the morphological parameters (weight and length), incorporation of labelled aminoacids (Cowey and Walton, 1988; Carter et al, 1994), nitrogen retention (Kim et al., 1987) proteasome activity (Dobly et al., 2004), growth hormone and IGF-1 levels (Farbridge and

Leatherland, 1991; Fuentes et al., 2011) and DNA/RNA ratios (McNamara et al. 1999; Peragòn et al. 2000). A promising alternative to these laborious techniques seems to be the molecular approach by quantitatively monitoring the expression of genes associated with muscle metabolism (Johansen and Overturf, 2006; Martins et al., 2010; Weber and Bosworth, 2005), or genes coding for the major muscular proteins such as myosin and actin (Overturf and Hardy, 2001; Hevroy et al., 2006).

Myosin is the most abundant protein accounting for about 50% of the total myofibrilar proteins, followed by actin with about 20% (Brechtel, 1986). Unlike mammals and birds, fish muscle continues to grow, albeit with possible variations in the growth rate, throughout a much greater period of the life cycle (Johnston, 1999). A typical teleost feature is the indeterminate nature of individual growth, due to both muscle hyperplasia and hypertrophy (Johnston, 2001), and, likely related to this, the continued presence of transcripts of myosin heavy chains (MyHC) in adult individuals (Gauvry and Fauconneau, 1996; Elworthy et al., 2008). Therefore, MyHC gene, which is expressed throughout muscle development in fish, represents an ideal candidate gene linking growth and protein accretion as well as validating the use of molecular techniques to monitor fish growth.

Since the initial characterization of MyHC in mouse, dozens of additional cDNAs have been isolated in different vertebrate species, whereas only few orthologues from commercially important fish species have been identified to date. Furthermore, only little is known about the role of this gene in modulating muscle growth in response to different feeding regimens. In this study we try to describe, by real-time RT-PCR, the MyHC levels in muscle of sea bass (*Dicentrarchus labrax*) in response to different planes of nutrition with the aim to relate its level to fish specific growth rate (SGR).

Although the mechanisms implicated in growth control are complex and not fully understood in fish, it is obvious that the brain processes and responds to nutritional stimuli with appropriate modifications in growth through hormonally mediated pathways (Duan, 1998). The majority of the growth promoting effects of the growth hormone (GH) are believed to be mediated by insulin-like growth factor-I (Leroith et al., 2001; Moriyama et al., 2000), which is structurally related to IGF-II (Shamblott and Chen, 1992). Both substances were termed “insulin-like” because of their ability to stimulate glucose uptake into muscle and fat cells (Randle, 1954). IGF-I and IGF-II are ubiquitous small peptides, well known for stimulating many anabolic responses on a variety of target cells in both endocrine and autocrine/paracrine fashions (Jones and Clemmons, 1995). IGFs (also known as somatomedins) are unique among the growth factors, having the property of

stimulating both proliferation and differentiation of muscle precursor cells (myoblasts or satellite cells) as well as myocyte hypertrophy during muscle regeneration (Florini et al., 1996; Musaro et al., 1999).

In most teleosts, the addition of new fibers continues to be an important contribution to normal muscle growth well into adulthood (Weatherley et al., 1988) and it also appears that the importance of the IGF system in muscle is maintained at a higher level for much longer throughout adult life (Méndez et al., 2001), suggesting a special role of these growth factors in fish physiology.

IGF-I and II transcripts have been detected in many fish tissues, with liver containing the greatest amount (Shamblott & Chen, 1993). The evidence that IGF-I and IGF-II mRNAs are also expressed in other tissues (Duan *et al*., 1993) and the presence of receptors in these tissues suggests that, in fish, paracrine and autocrine actions of IGFs are also involved in organ-specific functions.

Furthermore, in various fish species, it has been shown that hepatic (Duan & Plisetskaya, 1993) and muscular (Chauvigné *et al*., 2003) IGF-I and IGF-II mRNA levels depend on feeding status, but, to our knowledge, there is no information regarding a possible role of IGF-I and -II in sea bass (*Dicentrarchus labrax*) growth.

Also for these biomarkers we describe the quantification, by real-time PCR, of IGF 1 and 2 expression levels during feeding of sea bass with different diets with the aim to determine a possible role during growth.

Concerning molecular markers at regard to innate immunity we have to considered the antimicrobial polypeptides (AMPPs) as "dicentracin".

Several AMPPs have been isolated from fish, including commercially important species, such as misgurin in loach (*Misgurnus anguillicaudatus*) (Park et al., 1998) , epinecidin in grouper (*Epinephelus coioides*) (Yin et al., 2005), pleurocidin in winter flounder (Pleuronectes americanus) (Cole et al., 1997), moronecidin in hybrid striped bass (Lauth et al., 2002), pardaxin in sole (*Pardachirus marmoratus*) (Adermann et al. 1998), hepcidin in winter flounder (*Pseudopleuronectes americanus*), Atlantic salmon (Salmo salar), and perch (Perca fluviatilis) (Douglas et al., 2003; Rossi et al., 2007), parasin in catfish (Parasilurus asotus) (Park et al.,1998), and dicentracin in sea bass (*Dicentrarchus labrax*) (Salerno et al., 2007). AMPPs are abundantly and typically expressed in tissues that constitute the surfaces of access for pathogen entry such as skin, gills, and the intestinal tract, (Noga and Silphaduang, 2003) but they are also found in peripheral blood leukocytes and head kidney, which represents the primary organ involved in immune function

(Hancock and Chapple, 1999; Smith et al., 2000; Sarmento et al., 2004).

AMPPs have broad spectrum activity, directly killing pathogens such as bacteria, fungi, parasites, and viruses. Thus, these molecules have the potential to increase resistance to infectious and infestive diseases and to contribute in a preventive manner to fish health and welfare (Noga et al.,2011).

The mechanism of action of antimicrobial peptides is strictly related to their structure: most molecules are relatively small (less than 100 aminoacid residues), and contain both hydrophilic and hydrophobic residues, enabling insertion into biological membranes (Douglas et al., 2003). Microbial killing is a consequence of the interaction of the AMPP with the microbial outer membrane. The membrane is destabilizes in a disruptive or non-disruptive manner (Giuliani et al., 2007): in the first case, channel formation alone promotes leakage of cytoplasmic contents, resulting in the death of the micro organism; in the second situation, AMPPs enter the cytoplasm, where interaction with cellular components induces the microbial killing (Lauth et al., 2002).

In fish, the structure of AMPPs makes it possible for them to divide into different groups such as piscidins, histone-like proteins (HLPs), and hemoglobin-like proteins (Hb-LPs).

Piscidins, first isolated from mast cells of the commercially cultured hybrid striped bass [21], comprise a large number of AMPPs widely distributed in higher teleost families, such as Moronidae, Serranidae, Sciaenidae, Cichlidae, Siganidae, and Belontidae (Noga and Silphaduang, 2003; Silphaduang et al., 2006); they are linear, amphipathic, α-helical peptides, with a highly conserved N-terminus characterized by the consensus motif I-X_5-H-X_4-I-H (Fernandes et al., 2010).

It has been demonstrated that the expression levels of piscidin 4 in gill tissue of healthy hybrid striped bass are within concentrations that are lethal to important fish pathogens (Corrales et al., 2010). Moreover, they are expressed to a significantly greater degree in healthy fish than in those nutritionally stressed or in diseased ones (Corrales et al., 2009).

Gene expression of piscidins can be upregulated by integrating immunomodulators such as Bio-Mos® in the food (Dawson and Pirvulescu, 1999); this promotes the nonspecific immune system in sea bass (*Dicentrarchus labrax*), increasing the mRNA copy number of the dicentracin in the head kidney; the latter seems to have a positive relationship by fishmeal in the diet. (Terova et al., 2009).

Another group of broad spectrum antimicrobial molecules highly represented in fish are the histone-related AMPPs, proteins (HLP) highly homologous to histones (for a review see Noga et al., 2011).

HLPs were first isolated from the skin of channel catfish (Ictalurus punctatus) (Robinette et al., 1998) and then identified in the skin, gills, and spleen of hybrid striped bass and rainbow trout (*Oncorhynchus mykiss*) (Noga and Silphaduang, 2001; 2002) HLP-1 is the most common and most potent HLP and it is highly homologous to histone H2B. Robinette and Noga (2001) measured HLP-1 in channel catfish skin through an enzyme-linked immunosorbent assay (ELISA), detecting that chronic stress has a significant suppressive effect on this molecule as compared to levels in unstressed fish.

Other HLPs, histones or fragments of them, are related to the innate immune response: HLP-2, for example, which is highly homologous to histone H1, oncorhyncin II, a histone H1-derived protein (Fernandes et al., 2004), and parasin I, a peptide homologous to the N-terminal of human histone H2A (Park 1998), from which hipposin is also derived, isolated from skin of Atlantic halibut (*Hippoglossus hippoglossus*) (Birkemo et al., 2003).

Like histones, whose main functions concern nuclear regulation and chromatin structure in nucleosome, the hemoglobins, which bind respiratory gasses, have also been found to be a source of peptides with potent antimicrobial activity (Liepke et al., 2003). Both intact hemoglobin tetramers and single alpha and beta subunits from various species, including human, exhibited considerable activity against gram-positive and gram-negative bacteria and fungi (Parish et al., 2001). A variant of the β-chain of hemoglobin, Hbβ peptide 1, is expressed in skin and gill epithelium of channel catfish (*I.punctatus*) in response to the ciliate parasite (*Ichthyophthirius multifillis*) infestations (Ullal et al., 2008).

As a result of chronic stress, including unbalanced diets, AMPP expression can be downregulated (Mock and Petrs, 1990; Robinette and Noga, 2001) and at the same time increase disease susceptibility. In contrast, upregulation of AMPP expression can clearly protect against disease, as reported for mammals (Bals et al., 1999; Salzman et al., 2003; Sawamura et al., 2005) and in a number of studies with transgenic fish (Sarmasik et al., 2002; Dunham et al., 2002; Yazawa et al. ,2006; Weifeng et al., 2004).

Therefore, antimicrobial peptides could be valuable in aquaculture, functioning as indicators of chronic stress and to prevent disease moreover could be considered a new promising therapeutics.

Finally the present study attempted to evaluate the effect of the partial substitution of fishmeal by plant source protein, on the diversity of gut mucosal bacterial populations (Merrifield, 2011).

Hosted microorganisms may provide exogenous enzymes and fermentation products to fish with various feeding habits, especially when their diet is rich in recalcitrant substrates

such as fibre (Clements, 1997). Interest in the possible role of gut microbiota in fish digestion emerged with the increasing proportion of plant protein sources introduced into fish feed to compensate for the shortage of fish meal (Gatlin et al., 2007; Barrows et al., 2008), but there are still few reports on the effect of plant protein sources on gastrointestinal microbiota in fish.

A wide range of microbes derived from the surrounding aquatic environment, soil/sediment and feed are found to colonize in the GI tract of fish.

Considering the fact that a large population of GI bacteria in fish is uncultivable (Pond 2006; Navarrete et al., 2009), the total bacterial load is higher as recorded from the total cultivable heterotrophic bacteria.

Indeed conventional methods often lack in accuracy and sensitivity (Asfie et al. 2003); for instance in characterizing certain fastidious and obligate anaerobes that require special culture conditions. Therefore, culture-based study of microbiota often leads to a very uncertain picture of the total microbial (Ringo et al., 2003; 2006;).

The Application of a recent molecular technology has provided a major breakthrough in the detection and identification of the microbial composition in gut ecosystem (Romero & Navarrete, 2006; De Paula Silva et al., 2009; Navarrete, 2010).

Among the recent technologies, DGGE, a "genetic fingerprint" method based on PCR amplification of 16S rDNA, has been successfully used to study the dynamic behaviour of the dominant microbes in fish gut. Such method provides an accurate picture of the complexity of the GI microbiota of fish (Nayak, 2010).

Although the presence of native GI microbiota in fish has been recognized, little is known about the bacterial communities and their establishment, diversity and most importantly their role in fish nutrition and health, including innate immunity. There still remain doubts about the complete microbial composition and load in the GI of majority fish species, and in the near future, culture-independent molecular tools may be able to provide a more detailed picture of the true complexity in the GI tract of different fish species.

2.0 Materials and Methods

The trial was conducted at “Agroittica Toscana”, a marine fish farm located in Piombino (Italy). European sea-bass (*Dicentrarchus labrax*) with an initial average weight of 23.59±1.09 g were fed four different diet for 90 days. It was used marketable like aquafeed at different level of fish meal replacement with plant product. After the trials, all the fish ware fed by high fish meal level diet (Diet A), for 50 days (Recovering).

Fish were randomly distributed to 8 circular 900 L fiberglass tanks at a stocking density of 150 fish per tank. The tanks were continuously supplied with marine water (complete refill every 4-5 hours per tank). During the experiment: Temperature was 24.7±0.6 °C; Oxygen was maintained sharply over 100% saturation with pure oxygen supply; pH was 7.9±0.4 unit

The four different diets were fed to duplicate tanks (two tanks per treatment) twice a day until apparent satiety:

Diet A = 48% vegetable meals and 35% Fish meal

Diet B = 57% vegetable meals and 27% Fish meal

Diet C = 52% vegetable meals and 21% Fish meal

Diet D = 68% vegetable meals and 15% Fish meal

All diets was iso-proteic, iso-lipidic, iso-energetic (≈45.0% Protein and ≈18.5% Fat inclusion) and supply the Sea bass nutrient requirements; each one was a commercial-like diet formulation by extrusion processes. In tab.2.1 and 2.2 is reported each diet composition and ingredients.

Tab.2.1 Experimental extruded diets compsition

Composition	Experimental Extruded Diets			
	A$_{48\%Veg-}$	**B**$_{57\%Veg-}$	**C**$_{52\%Veg-}$	**D**$_{68\%Veg-}$
Crude Protein (%)	45.0	44.0	45.0	44.0
Crude Fat (%)	18.5	18.5	19.0	18.0
Ash (%)	8.0	7.5	7.0	6.0
Crude Fiber (%)	2.0	2.0	2.0	2.6
NFE (%)	20.5	20.5	21.0	23.3
Moisture (%)	6.0	8.0	6.0	6.5
Digestible Energy	19.9	19.7	20.1	19.4
DP/DE (mg/kJ)	21.5	21.7	21.2	20.5

Tab.2.2 Experimental extruded diets ingredients

Raw Materials	Experimental Extruded Diets			
	A48%Veg-	**B**57%Veg-	**C**52%Veg-	**D**68%Veg-
Fish meal (%)	35.00	27.00	21.00	15.00
Vegetable meals (%): *Soybean, Wheat, Corn gluten,*	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	4.05	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

2.1 Growth Performance

The growth performance was recorded: biometrics parameters as weight, total length, standard length, hepatosomatic index, viscerosomatic index. Such measures were carried out from every fish collected for histological and microbiological analysis at 0, 10, 20, 27, 40, 90 and 140 days (see bellow)

The mean weigh was obtained by global biometry in each tank. All fish in every tank were counted e weighed together (either three or four weightings, according to the total weight, to reduce the stress and ensure sufficient animal welfare). Global biometries have been made at 0; 20; 40; 60; 90 and 140 days.

Daily food administration was recorded to calculate FCR (Feed Conversion Ratio). The FCR is simply the amount of feed it takes to grow a kilogram of fish it is calculated by following formula:

FCR = feed administered (Kg) / Fish weight (Kg)

We used biometric measurements to calculate also the Specific Growth Rate (SGR) of fish, during a specific time frame, by following formula:

SGR = [Final Weight (g) - Initial Weight (g)] x100 / Time between weightings (days)

2.2 Histological analysis

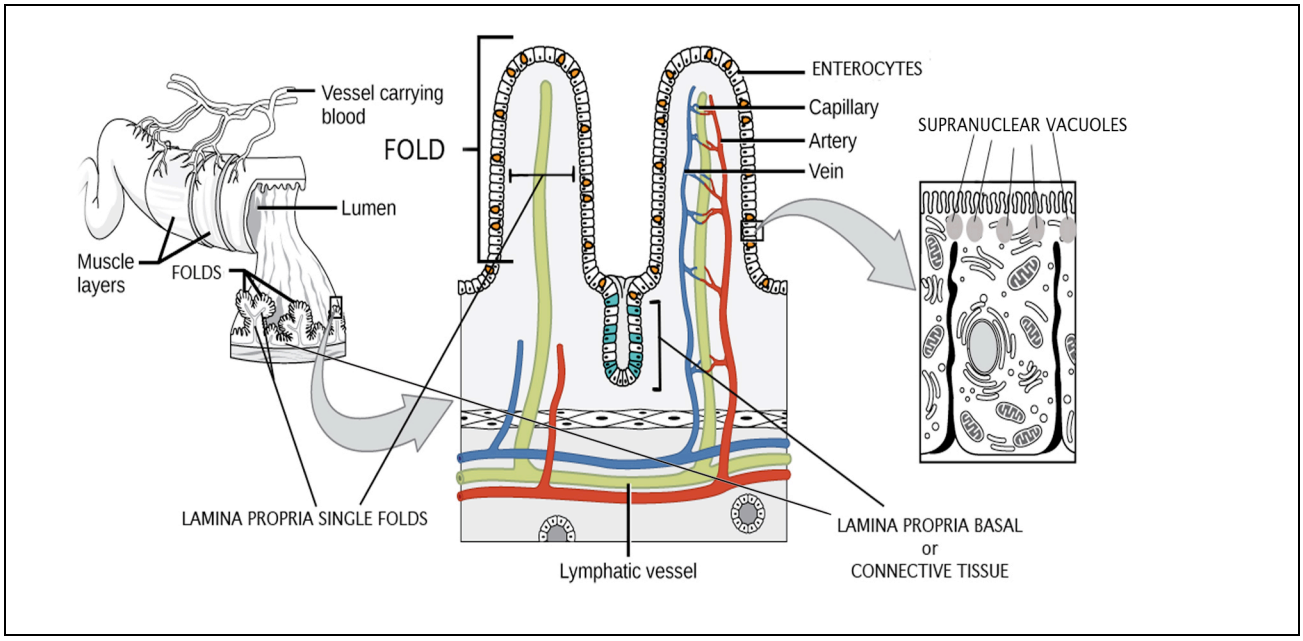
At the beginning, after 10, 20, 27, 40, 90 day and at end of the recovering period (140 days), five fish from each tank (10 fish in total per treatment) were sacrificed with an overdose of anaesthetic (tricaine methanesulfonate, Finquel MS-222, Argent Chemical Laboratories, United States), for histological examination. Histological analysis were focused on intestine and liver.

A section (around 2 cm) of foregut (anterior part of the intestine), midgut and hindgut (distal part of the intestine) of the digestive tract were excised carefully, rinsed in saline water, and fixed in phosphate buffered formaldehyde (4%, pH 7.2).

Samples were then dehydrated, embedded in paraffin, and cut according to standard histological procedures. Slides were then stained with a combination of hematoxylin and eosin.

Four different morphological parameters were evaluated using light microscopy according to semi quantitative scoring system adapted from Uràn et al. (2004) and Knudsen et al. (2007). In table 2.3 the scoring criteria applied to each parameters are detailed: supranuclear vacuoles; lamina propria of single folds; connective tissue (or lamina propria basal); mucosal folds. The considered parameters are reported in a schematic view in figure 2.1.

Fig.2.1 Histologic analysis: schematic representation of parameters considered (Folds, Lamina Propria single fold, Lamina propria basal or Connective Tissue, Supranuclear Vacuoles) for the scoring.



For each parameter a score of “1-2” represented normal morphology, while a score of “5” was given to morphological symptoms of severe enteritis.

The scoring system was tested for intra-observer agreement. As recommended by several authors (Desmet, 2003; Mitchell et al., 2012), the different criteria were discussed and defined in detail by observers prior to assigning scores to the slides to improve agreement and reduce inter-observer variability. Histological samples were randomized and blindly evaluated.

To better point out the histological changes regarding a global evaluation the summation of the mean of each single parameter considered was done. The latter with global score "4 - 8" represented normal morphology or slight alteration, "9 -14” value, show more evident alteration, while "15-20" score, represent serious injury until to severe enteritis.

In order to support the results obtained using arbitrary scoring scale, we used "Image J" free software to measure tissues and to count cells on pictures taken from histological slides. We measured: the connective tissue thickness and lamina propria of single fold width at three levels (Up - Middle and Down). We olsocounted: granulocytes concentration at the base of single folds and hepatocytes concentration in the liver.

Tab.2.3 Histological scoring system for morphological changes induced by diet in the intestine of european sea-bass (D.labrax). (Adapted from Knudesen et al. 2007)

Score Appearance	Summation (∑ medium value of single score)
Supranuclear vacuoles 1 Large vacuoles occupy almost the entire apical part of the enterocytes. 2 Medium-sized vacuoles, which occupy less than half of the enterocytes, are present. 3 Small-sized vacuoles are near the apical membrane in most enterocytes. 4 Scattered small vacuoles are still present in some enterocytes. 5 No supranuclear vacuoles are present.	Supranuclear Vacuoles +
Lamina propria of simple folds 1 There is a very thin and delicate core of connective 2 The lamina propria appears slightly more distinct and robust in some of the folds. 3 There is a clear increase of lamina propria in most of the simple folds. 4 There is a thick lamina propria in many folds. 5 There is a very thick lamina propria in many folds.	Lamina Propria +
Connective tissue (between base of folds and stratum compactum) 1 There is a very thin layer of connective tissue between the base of folds and the stratum compactum. 2 There is a lightly increased amount of connective tissue beneath some of the mucusal folds. 3 There is a clear increase of connective tissue beneath most of the mucosal folds. 4 A thick layer of connective tissue is beneath many folds. 5 An extremely thick layer of connective tissue is beneath some folds.	Connective Tissue +
Mucosal folds 1 Simple and complex folds (CFs) appear long and thin. Thin side branches on the CF. 2 Simple mucosal folds have medium length. CFs are still long but appear thicker. 3 Simple folds have short to medium length. Side branches on CF are stubby. 4 Thick CFs are prevalent. Simple folds are short. Almost no side branches are on the CF. 5 Both complex and simple folds appear very stubby.	Mucosal Folds =
For each parameter a score of “1 - 2” represented normal morphology, while a score of “5” was given to morphological symptoms of severe enteritis.	∑ score "4-8" = normal morphology or slight alteration, ∑ score "9-14" = evident alteration ∑ score "15-20" = serious injury until to severe enteritie

2.3 Microbial culture from fish gut

At the beginning, after 30 and 90 day, five fish from each tank (10 fish per dietary treatment) were sacrificed with an overdose of anaesthetics (tricaine methanesulfonate, Finquel MS-222, Argent Chemical Laboratories, United States), for microbiological analysis.

After accurately disinfection by alcohol *70%*, ventral belly surface of the fish was opened to expose the peritoneal cavity.

In aseptic manner, spleen, gallbladder, liver and fat deposits surrounding the gastrointestinal tract were removed. The hindgut (distal intestine) section of the digestive tract were excised. Adherent and non-adherent (associated to digesta) bacteria of the gut section were isolated as described by Ringo (1993). Briefly, digesta from gut sections were squeezed (however the fishes were sampled after fasting) out in order to isolate the non-adherent bacteria. Thereafter, the intestinal segments were thoroughly rinsed three times in 3 ml sterile 0.9% saline in order to isolate adherent bacteria. The intestinal segments were transferred to sterile plastic bags and homogenised in a Stomacher (Seward Laboratory, London,UK).

Homogenates of the hindgut were diluted (1:9) in sterile BHI (Brain Heart Infusion) broth and then were spread, immediately and after 24 hours incubation in BHI at 20°C, onto other different bacterial growth mediums (Oxoid, Italia):

- TSA (*Tryptone soy agar*) plates with added 5% glucose. TSA is a general purpose medium , providing enough nutrients to allow for a wide variety of micro organisms to grow.
- MRS agar (is so-named by its inventors: de Man, Rogosa and Sharpe); this medium was designed to favour the luxuriant growth of Lactobacilli although some other Lactobacillaes, like Leuconostoc and Pediococcus, may grow.
- ADBS (Azide Dextrose Broth Simple) The presence of sodium azide for its bacteriostatic action inhibits the growth of Gram-negative favouring the growth of faecal streptococci
- VBBGA (Violet Red Bile Agar with Glucose, containing Bile and Violet Red dye): is based on the MacConkey Medium for the detection and enumeration of bile-tolerant Gram-negative Enterobacteriaceae.
- PSA (Pseudomonas Selective Agar) the *supplement CFC (Cetrimide-Fucidina-Cefaloridina) is added for the isolation of the various species of Pseudomonas*
- Blood agar: a culture medium consisting of blood (usually sheep's blood) and nutrient

agar, to isolate Aereomonas spp.

- TCBS Agar (Thiosulfate-Citrate-Bile-Sucrose Agar); TCBS Agar is also called Vibrio Selective Agar (VSA). TCBS plus CHROM agar it was used for the selective isolation of Vibrionaceae.

Plates were incubated at 22 °C and inspected regularly for up to 72 hours. Bacteria isolates associated with the hindgut wall were subcultured until purity was achieved.

The isolates (randomly chosen) were classified on the basis of cell morphology, Gram staining, motility, catalase and oxidase reactions. Samples of bacteria were characterized with API 20E (Enterobacteriaceae) and API 20 NE, (Not Enterobacteriaceae); by Biomerieux, (France). API 20E is one of the most commonly used kits for the rapid diagnosis of bacteria from fish (Popovic et al., 2004). The strips were used according to the manufacturer's instructions.

Moreover an automated system for identification and susceptibility tests for bacterial identification (*VITEK® 2 System Biomérieux France*) was utilized.

The aim was to identify and quantify the principal strains of cultivable bacteria: *Aereomonas spp*; Enterobacteriaceae; faecal Streptococci; Lactobacillaceae; *Pseudomonas spp*; *Vibrio spp*.. The results were expressed, for single bacterial strain as: presence/absence or Colony Forming Unit (CFU) or Most Probable Number per gram (MPN/g). For each bacterial genus, when isolated, it was reported also the specie.

2.4 DNA isolation and PCR-denaturing gradient gel electrophoresis (DGGE)

In order to ensure the detection of non-cultivable bacteria (Paula Silva 2011), DNA was purified directly from the hindgut contents after 40 days of feeding the respective diets. Total genomic DNA was extracted by glass bead beating (Sigma G4649) as described by Godon et al. (1997), with some modifications.

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'; Nubel et al., 1996). The PCR was performed in a final volume containing 50 ng mL^{-1} of genomic DNA, 0.025U ng mL^{-1} Taq DNA polymerase (MP Biomedicals), 0.2mM of each dNTP (Eurogentec premix), 0.4 mM of each primer and 2mM $MgCl_2$. The PCR conditions in the thermocycler TC-512 (Techne) were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles including denaturation at 94° C for 1 min, annealing for 2 min – with

'touch down' from 65 to 52 °C at the 10th cycle and subsequently – and a 2-min elongation at 72 °C. A final extension cycle was performed at 72 °C for 30 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 mL of PCR products was loaded onto an 8% polyacrylamide gel (16 cm 16 cm 1.5 mm) prepared from 40% w/v acryl - amide - N,N' - methylenebisacrylamide stock, 37.5 : 1 (Bio-Rad). The denaturing gradient was prepared by mixing 35% and 60% solutions of 7M urea and 40% formamide.

The electrophoresis was run in 1 x Tris/Acetic acid/EDTA (TAE) buffer diluted from 50 x TAE buffer stock solution (Bio-Rad). The migration was performed at 80 V (constant voltage) for 18 h, with a temperature gradient of 60°C. After electrophoresis, the gel was stained for 30 min by 200mL of TAE solution with SYBR Green (0.1 mL $^{-1}$; Sigma). After rinsing, the gel was scanned on Typhoon 9400 (Amersham Biosciences), and the image was analysed using the GELCOMPAR software (version 6.1, Applied Maths).

Some well-separated bands with a high peak intensity were excised from the gels, and left overnight soaking in 200-mL 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 25 cycles including denaturation at 95 °C for 30 sec, annealing at 52 °C for 30 sec and a 1-min elongation at 72 °C; a final extension cycle was performed at 72 °C for 30 min). The products were purified using a Gene Elute PCR clean-up kit (Sigma) and sent for sequencing.

2.5 Molecular Biomarkers

Five fish from each of the experimental groups (diets A - B - C - D) were sampled, after one day fasting, at the following time points: at the beginning (day 0), after 40 and 90 days (end of feeding trials). The fish were sacrificed with an overdose of anaesthetic (tricaine methanesulfonate, Finquel MS-222, Argent Chemical Laboratories, United States) and body weight and length (standard + total) were measured and used to calculate the condition factor: (K=body weight x 100/standard body length^3).

For the molecular biology analysis, proximal and distal intestine, muscle, anterior kidney and liver were dissected out, frozen immediately in liquid N_2, and then stored at −80 °C until the analysis.

2.5.1 Preparation of total RNA

Total RNA was extracted from seabass tissues using Pure Yield RNA Midiprep System (Promega, Italy), following the manufacturer's protocol. Briefly, 2ml of ice-cold Lysis Solution containing β-mercaptoethanol were transferred to a 10 ml tube. Target tissues were excised, placed in the tube and then homogenized until no visible tissue fragments remained. Two ml of the lysate prepared above were transferred to a 15ml centrifuge tube, and 4ml of RNA Dilution Buffer were added. The tube was sealed, mixed thoroughly by inverting it 3–4 times, and vortex. One ml of thoroughly mixed Clearing Agent was added to the diluted lysate mixture which was then mixed inverting 2–3 times, and vortex until homogeneous. Samples were incubated at 70°C for 5 minutes to denature Tubes were then removed, and cool at room temperature for at least 5 minutes. One blue PureYield™ Clearing Column for each sample was placed in a 50ml collection tube. Each sample was mixed by vortexing or vigorously shaking until homogeneous and the mixture was immediately poured into the assembled PureYield™ Clearing Column/collection tube. The PureYield™ Clearing Column assembly was then centrifuged in a swinging bucket rotor at 2,000 × g at 22–25°C for 10 minutes to clear the lysate. The blue Clearing Column was discarded, whereas the cleared lysate was saved in the collection tube.

The quantity of the extracted RNA was calculated using the absorbance at 260 nm, whereas the integrity of RNA was assessed by agarose gel electrophoresis. Crisp 18S and 28S bands, detected by ethidium bromide staining were indicator of the intact RNA.

2.5.2 Quantitative real-time RT-PCR

Generation of in vitro-transcribed mRNAs:

The approach used for the real-time quantification of the expression of five target genes relied on the standard curve method for target mRNA quantification. The target genes were PepT1, SLC6A19, Myosin heavy chain (MyHC), IGF1 (insulin-like growth factor 1) and dicentracine. Following this method, the number of each target gene transcript copies could be quantified by comparing them with a standard graph constructed using the known copy number of mRNAs of each target gene. The first step in this direction is the generation of standards of mRNAs by *in vitro*-transcription. As an example, in the case of PepT1, a forward and a reverse primer were designed based on the mRNA sequences of *D. labrax* PepT1 (accession n° FJ237043). These primer pairs were used to create templates for the *in vitro* transcription of mRNAs for this gene. The forward primer (*gtaatacgactcactataggg*GGAATGTGGCATTCACACC) was engineered to contain a T7/T3

phage polymerase promoter gene sequence to its 5' end and used together with the reverse specific primer (GTCCATCTTGAGCCCTGCT) in a conventional RT-PCR of total sea bass head kidney RNA. RT-PCR products were then checked on a 2.5 % agarose gel stained with ethidium bromide, cloned using pGEM®-T cloning vector system (Promega, Italy) and subsequently sequenced in the SP6 direction.

In vitro transcription was performed using T7/T3 RNA polymerase and other reagents supplied in the Promega RiboProbe *In Vitro* Transcription System kit according to the manufacturer's protocol.

The molecular weight (MW) of the *in vitro*-transcribed RNA for cFos was calculated according to the following formula:

PepT1-MW = [129 (n° of A bases) x 329.2) + 69 (n° of U bases) x 306.2) + 66 (n° of C bases) x 305.2) + 98 (n° of G bases) x 345.2)] + 159. The result was 114920.4.

Spectrophotometry at 260 nm gave a concentration of 550 ng/μl for PepT1. Therefore, the concentration of the final working solution was 2.88×10^{12} molecules/μl.

The same aforementioned approach was used for the *in vitro* transcription of the other target genes such as SLC6A19, MyHC, IGF1, and Dicentracin.

The primers used were the followings:

SLC6A19 (Genebank accession nr. **KC812315**):
forward (5'- *gtaatacgactcactataggg*GGCCCAAATGGGACAACAA - 3');
reverse (5'- CCAGACAGAAGAGCATGATGAA -3').

Dicentracin (Genebank accession nr. **AY303949**):
forward (5'- *gtaatacgactcactataggg*AGTGCGCCACGCTCTTTC - 3');
reverse (5'- CTAGTCAAAAGCTGCGCGCT -3')

IGF1 (Genebank accession nr. **AY800248**):
forward (5'- *caattaaccctcactaaaggg*ATGTCTAGCGCTCTCTCCTTTC - 3');
reverse (5'- GGGGCAGAAATTACAGAATGTAG -3')

MyHC (Genebank accession nr. **DQ317302**):
forward (5'- *gtaatacgactcactataggg*CTCAAGAACGGTGAGGAGTGG - 3');
reverse (5'- AGGAGCTGAAGAGACAGGTTG -3')

The molecular weight (MW) of the *in vitro*-transcribed RNAs calculated according to the aforementioned formula were of 253362.8 for SLC6A19; 77090.8 for dicentracin, 182371.8 for IGF1, and 112325 for MyHC.

Spectrophotometry at 260 nm gave a concentration of 112.6 ng/μl for SLC6A19, 571 ng/μl for dicentracin, 278 for IGF1, and 544 for MyHC. Therefore, the concentration of the final working solutions were of 2.68 x 10^{11} molecules/μl for SLC6A19, 4.46 x 10^{12} for Dicentracin, 9.18 x 10^{11} for IGF1 and 2.92 x 10^{12} molecules/μl for MyHC.

Generation of standard curves for PepT1, SLC6A19, IGF1, MyHc, and Dicentracin

The mRNAs of five target genes produced by *in vitro* transcription were used as quantitative standards in the analysis of experimental samples. Defined amounts of mRNAs of each gene, at 10-fold dilutions, were subjected to real-time PCR using One-Step TaqMan EZ RT-PCR Core Reagents (Life Technologies, Italy), including 1x Taqman buffer, 3 mM MnOAc, 0.3 mM dNTP except dTTP, 0.6 mM dUTP, 0.3 μM forward primer, 0.3 μM reverse primer, 0.2 μM FAM-6 (6-carboxyfluorescein-labeled probe), 5 units *rTH* DNA polymerase, and 0.5 units AmpErase UNG enzyme in a 30 μl reaction. AmpErase® uracil-N-glycosylase (UNG) is a 26-kDa recombinant enzyme encoded by the *Escherichia coli* uracil-N-glycosylase gene. UNG acts on single-and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA. For Taqman® assays, AmpErase® UNG treatment can prevent the reamplification of carry over PCR products from previous PCR reactions. When dUTP replaces dTTP in PCR amplification, AmpErase UNG treatment can remove up to 200,000 copies of amplicon per 50 μl reaction. RT- PCR conditions were: 2 min at 50°C, 30 min at 60°C, and 5 min at 95°C, followed by 40 cycles consisting of 20 s at 92°C, 1 min at 62°C. The Ct values obtained by amplification were used to create standard curves for target genes.

Quantification of PepT1, SLC6A19, IGF1, MyHc, and Dicentracin transcripts by One-Step TaqMan real time RT-PCR

A hundred nanograms of total RNA extracted from the experimental samples was subjected, in parallel to 10-fold-diluted, defined amounts of standard mRNA, to real-time PCR under the same experimental conditions as for the establishment of the standard curves. Real-time Assays-by-

Design[SM] PCR primers and gene-specific fluorogenic probes were designed by Life Technologies. Primer sequences and Taqman® probe of the four target genes were the followings:

PepT1:
Forward primer: 5'– GCTACCCTCTGGCCTTTGG –3'
Reverse primer: 5'- GAACACAATCAGAGCTACCACCAT-3'
Taqman® probe: 5'- TCCCCGCTGCTCTC-3'

SLC6A19:
Forward primer: 5'- TCACCTGTGTGGGCTTTTGT - 3'
Reverse primer: 5'- CATGGCTTTGACACAGGTAAGG - 3'
Taqman® probe. 5'- ACGTTGCCGAGTCCCAC - 3'

Dicentracin:
Forward primer: 5'- TGCGCCACGCTCTTTCTT - 3'
Reverse primer: 5'- CCCCAGGTTCAGCCATGAG - 3'
Taqman® probe. 5'- ACGACCATCGACAGCAC - 3'

IGF1:
Forward primer: 5'- GCAGTTTGTGTGTGGAGAGAGA - 3'
Reverse primer: 5'- GACCGCCGTGCATTGG - 3'
Taqman® probe. 5'- CTGTAGGTTTACTGAAATAAAA - 3'

MyHC:
Forward primer: 5'- TGGAGAAGATGTGCCGTACTCT - 3'
Reverse primer: 5'- CGTGTCATTGATTTGACGGACATTT - 3'
Taqman® probe. 5'- AACTGAGTGAACTGAAGACC - 3'

TaqMan® PCR was performed on a StepOne Real Time PCR System (Life Technologies). To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 30 µl) for each sample.

Sample quantification

Data from Taqman® PCR runs were collected with StepOne Real Time Sequence Detector Program. C_T (cycle threshold) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA.

2.6 Statistic

The data were statistically compared using: one and two way analysis of variance (ANOVA) and multiple comparisons post-hoc. All the analysis performed by "R software" version 2.15.2 (2012-10-26) - "Trick or Treat".The level of statistical significance was set at $p<0.05$.

3.0 Results

3.1 Growth performance and biometrics parameters

The growth performance of sea-bass (*D.labrax*) during the feeding test (90 days fed with four different diets: A-B-C-D), and following the 50 days recovering period, when all fish were fed by diet A (highest level of fish meal), is represented in table 3.1 and figure 3.1.

The initial fish weight was respectively: 23.29±1.39 for diet A; 23.57±0.72 for diet B; 24.45±0.84for diet C and 23.02±0.95 for diet D. After the first 40 days of feeding, the higher mean weight was reached with diet A (62.03±2.59 g); diets B and C, quite similar to each other, reached respectively 57.35±2.13 g and 55.50±2.29 g; whereas the weight off with diet D was much lower (41.10±3.20 g).

Diets B and C were also similar in FCR (respectively 0.90 and 0.93), and in SRG (respectively 2.17 and 2.00). The better SRG was obtained by diet A (2.39) while the worst one by diet D (1.41), that gave also the worst FCR (1.28). The best FCR were achieved with diets B and C.

The daily feeding ratio is the amount of feed fed to the fish daily; it is expressed as a percentage of the raised biomass and calculated by multiplying FCR x SGR.

Until 40 days, daily ratio is in agreement with SGR decreasing trend from diet A to diet D. We have to underline that increasing substitution of fishmeal with protein plant source implies a worsening of palatability. Consequently SGR decreases and then decreases feed daily ratio.

At the end of the experimental trials (90 days), the highest final weight (Fig.3.2) was achieved by diets A and B (respectively 128,41±3,36 and 124,52±4,54 grams), then diet C (118,28±3,78 grams) and diet D (100,71±7,87 grams).

Diet D is still the worst one, but its gap with other diets decreases: the weight gain in the first 40 days was less than 50% of the gain obtained by diet A; at 90 day the difference in total weight gain between diets D and A was reduced at 25%. Considering the partial weight gain from 40 to 90 days, the performances of A and B diets were nearly the same (respectively 66,38±3,35 and 67,17±2,78 grams) while a little worse for diets C (62,78±2,35 grams) and D (59,61±6,54 grams); in this case the D diet's gap, towards diet A it was less then 10%.

At the end the comparison between the four diets showed the better SGR by diet A (1.90) followed by diet B (1.85), diet C (1.75) and diet D (1.64). Whereas, the better FCR was by

both B and C diets (1.07), while diet A and D gave a worse conversion rate (respectively 1.16 and 1.14).

Tab.3.1 Mean weights, feed conversion (FCR) and specific rate growth (SRG) of sea-bass (*D.labrax*) fed with four diets (A – B – C - D) different level of fishmeal replacement with proteic plant source.

		A48Veg-35Fish Weigth (g)	**B57Veg-27Fish** Weigth (g)	**C52Veg-21Fish** Weigth (g)	**D68Veg-15Fish** Weigth (g)
first 40 days	**(0-days) INITIAL WEIGTH**	**23.29±1.39**	**23.57±0.72**	**24.45±0.84**	**23.02±0.95**
	(40-days) weight	**62.03±2.59[a]**	**57.35±2.13[b]**	**55.50±2.29[b]**	**41.10±3.20[c]**
	Weight gain (40-day-0-days)	38.73±3.02[a]	33.79±2.13[b]	31.04±2.90[b]	18.08±2.67[c]
	FCR	0.96	0.90	0.93	1.28
	SRG	2.39	2.17	2.00	1.41
	Daily Ratio	2.30	1.95	1.85	1.82
last 50 days	**(90-days) FINAL WEIGTH**	**128.41±3.36**	**124.52±4.54**	**118.28±3.78**	**100.71±7.87**
	Partial Weight gain (90-day-40-days)	66.38±3.35	67.17±2.78	62.78±2.35	59.61±6.54
	TOTAL Weight gain (90-day-0-days)	105.12±1.38	100.96±4.27	93.83±3.41	77.69±4.55
	FCR	1.16	1.07	1.07	1.14
	SRG	1.90	1.85	1.75	1.64
	Daily Ratio	2.20	1.98	1.87	1.87
Recovering 50 days (all tanks Diet A)	**(140-days) FINAL WEIGTH**	**194.47±6.55**	**191.63±10.13**	**187.39±13.52**	**181.31±11.72**
	Partial Weight gain (140-day-90-days)	89.35	90.68	93.57	103.62
	TOTAL Weight gain (140-day-0-days)	171.17	168.07	162.94	158.29
	FCR	1.78	1.71	1.80	2.02
	SRG	1.51	1.49	1.44	1.46
	Daily Ratio	2.68	2.55	2.60	2.95

[a,b,c] Significant differences, P<0.05

Fig.3.1 Growth trend in fishes fed by four diets (A – B – C - D) at different level of fishmeal replacement with protein plant source until 90 days, plus recovering period (50 days), when fishes in each tank were fed only with diet A.

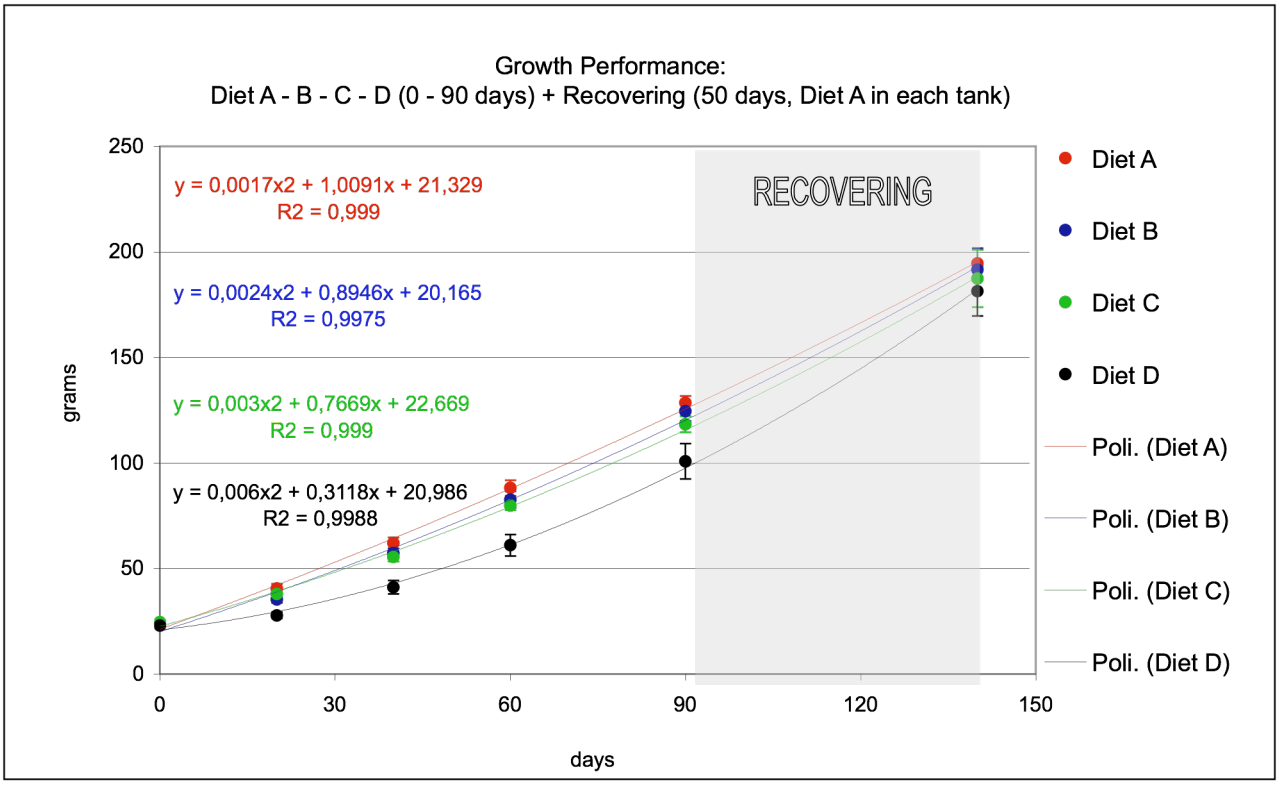
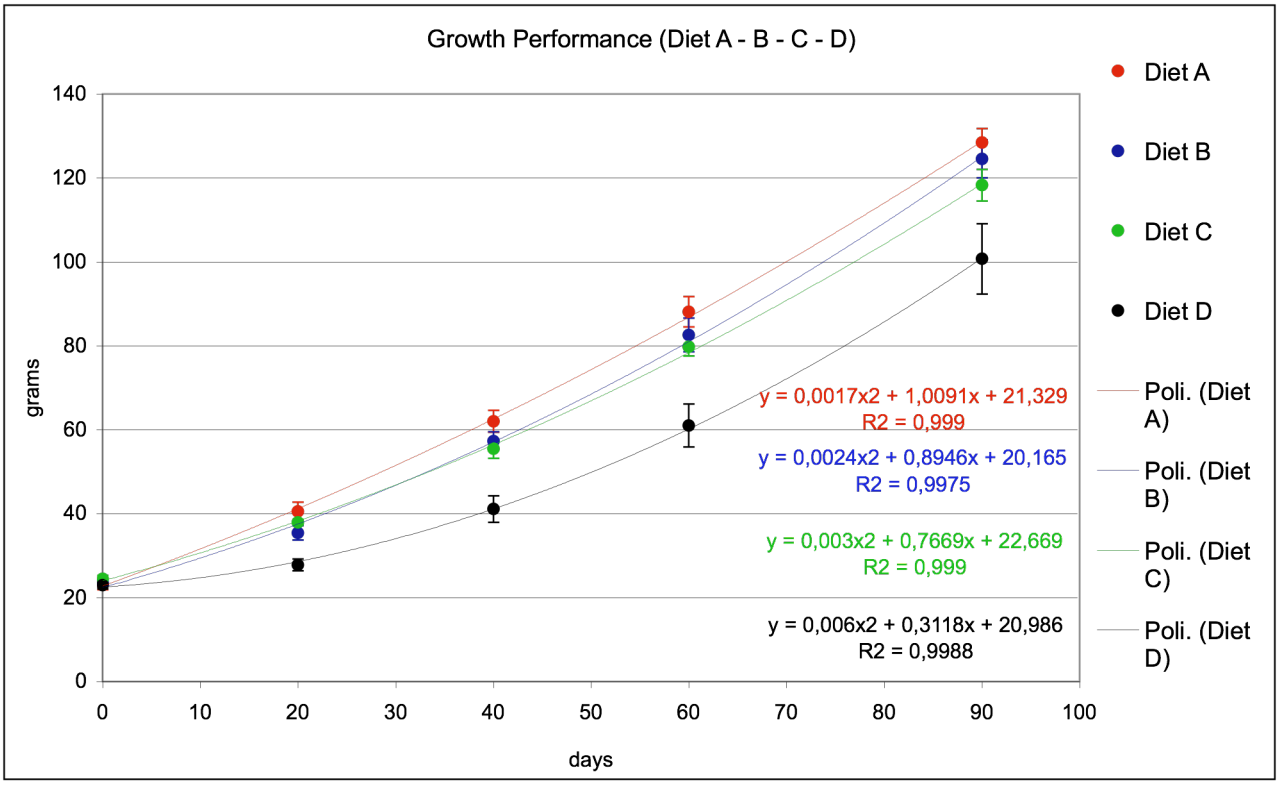


Fig.3.2 Growth trend in fishes fed by four diets (A – B – C - D) at different level of fishmeal replacement with protein plant source

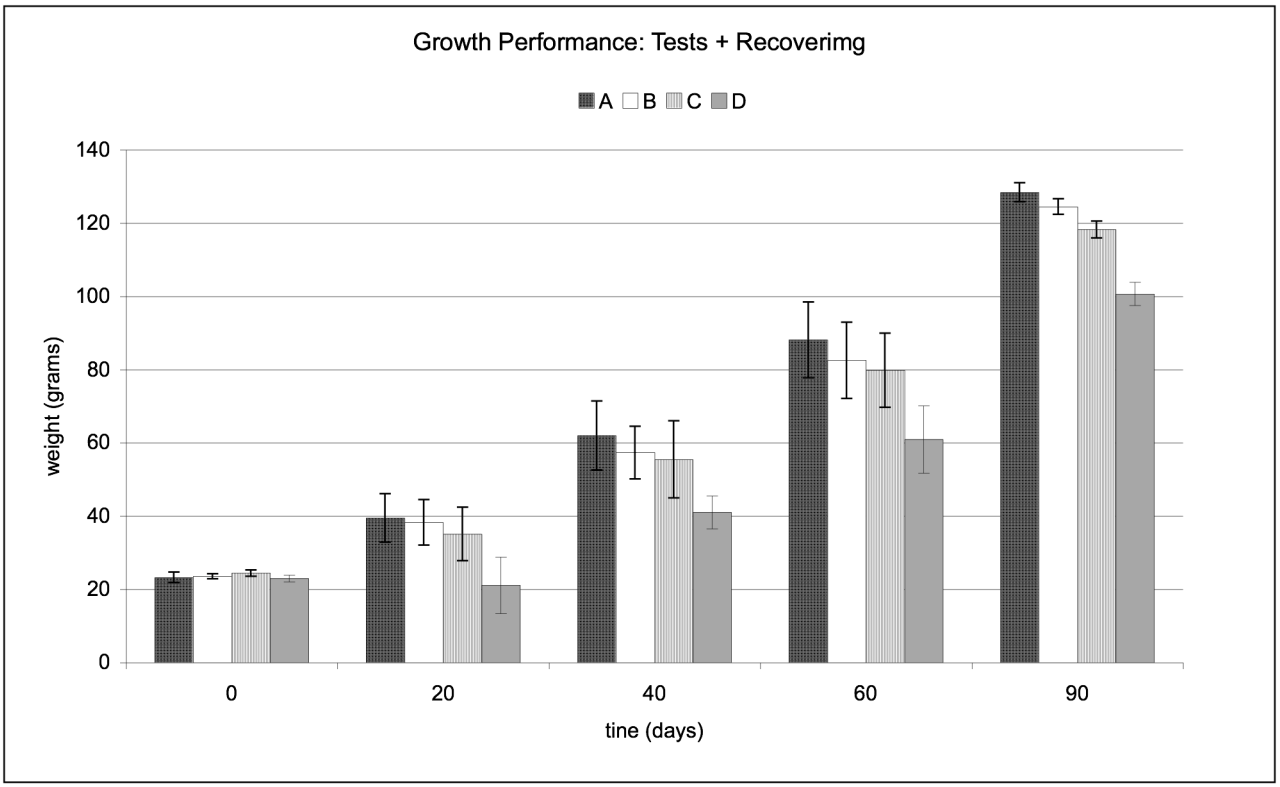


It is reasonable to think that in both case increase of FCR is related to the food palatability because the fishes were fed *ad libitum* until apparent satiety.

Therefore it is possible that feeding by diet A (high palatability because high level of fishmeal) led to a supercharging, with feed waste while feeding by diet D (low palatability because high level of plant source protein) could give under nourishment with lack growth.

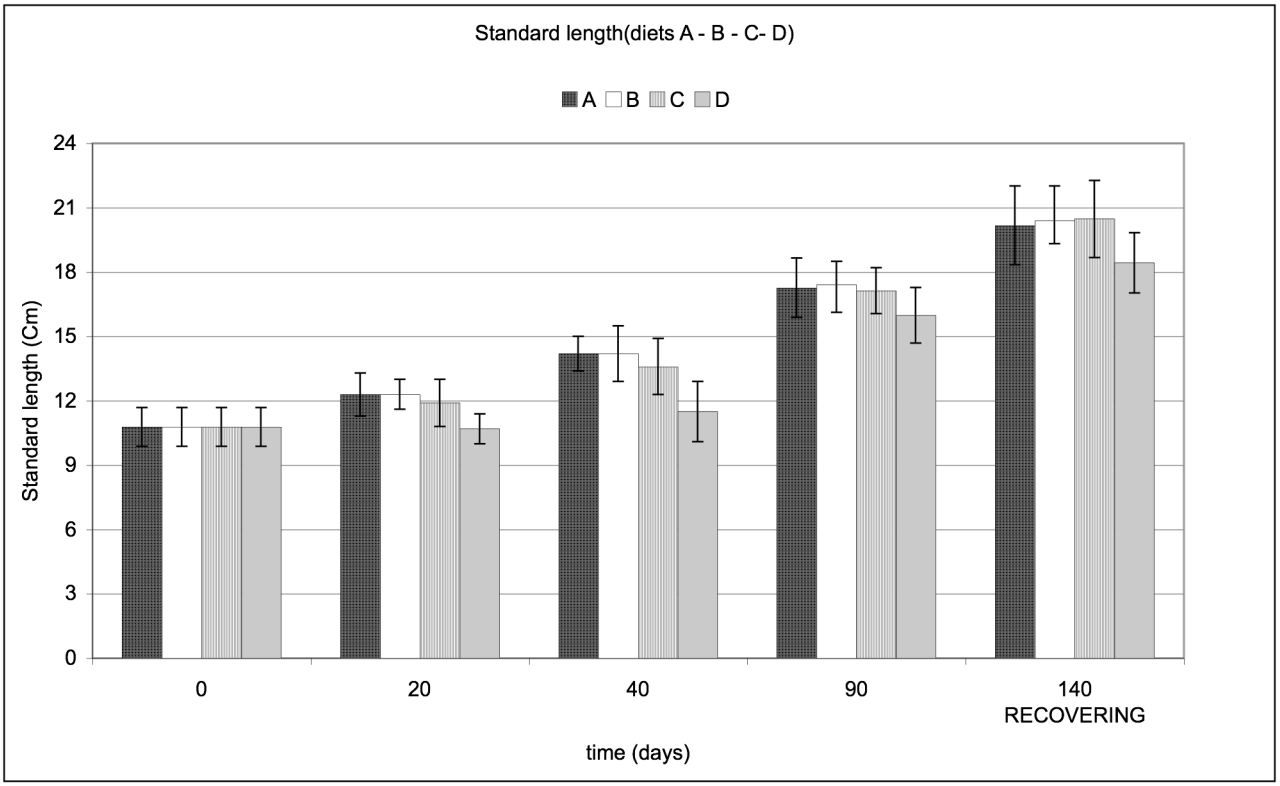
After the recovering period (Fig. 3.3), the fishes coming from A and B diet achieved the higher final weight (respectively 194.47±6.55 and 191.63±10.13 grams without significant differences), followed by those coming from diet C (187.39±13.52 grams) and at the last those from diet D (181.31±11.72 grams). The FCR didn't show significant difference for the fishes from diet A and C (respectively 1.78 and 1.80), while those from diet B showed the better value (1.71) and those from diet D (2.02) the worst one. The SRG didn't show significant difference for the fishes from every diet. Interestingly the inverse correlation between partial weight gain and total weight gain. The fishes earlier from diet D showed the higher weight gain during the recovering period (103,62 grams) those earlier from diet C (93,57 grams), then from diet B (90,68 grams) and diet A (89,35 grams).

Fig.3.3 Medium weights of fishes fed by four diets (A – B – C - D) at different level of fishmeal replacement with protein plant source until 90 days, plus recovering period (50 days), with diet A only



Standard length and total length were measured at beginning, at 10 - 20 - 40 - 60 - 90 day and after the recovering period (140 days). Diets A, B and C showed a similar trend, while diet D gave lower value in each measurement, although there aren't difference statistically significant (Fig.3.4 and 3.5) diets related.

Fig.3.4 Standard length of fishes fed by four diets (A – B – C - D) at different level of fishmeal replacement with protein plant source until 90 days, plus recovering period (50 days), with diet A only.



Also in this case the diet D, tended to reduce the gap during the test; the fishes seemed to be able to adapt to new food, improving the growth performance. Additional reduction of D diet gap, were observed during the recovering period.

During test and recovering, the viscerosomatic index showed no statistically significant differences neither between diets, nor time related. (Fig.3.6).

Fig.3.5 Total length of fishes fed by four diets (A – B – C - D) at different level of fishmeal replacement with protein plant source until 90 days, plus recovering period (50 days), with diet A only.

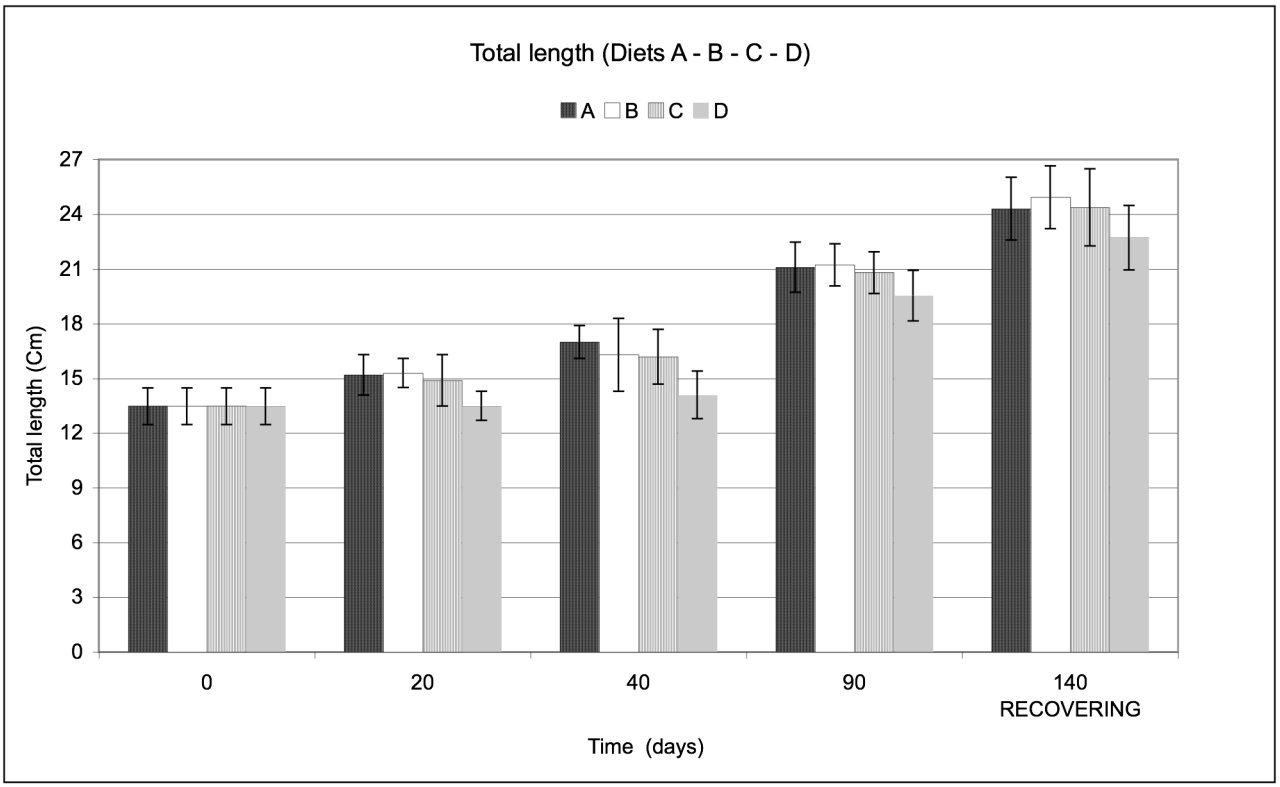


Fig.3.6 Viscerosomatic index (VSI) in fishes fed by four diets (A – B – C - D) at different level of fishmeal replacement with protein plant source until 90 days, plus recovering period (50 days), with diet A only.

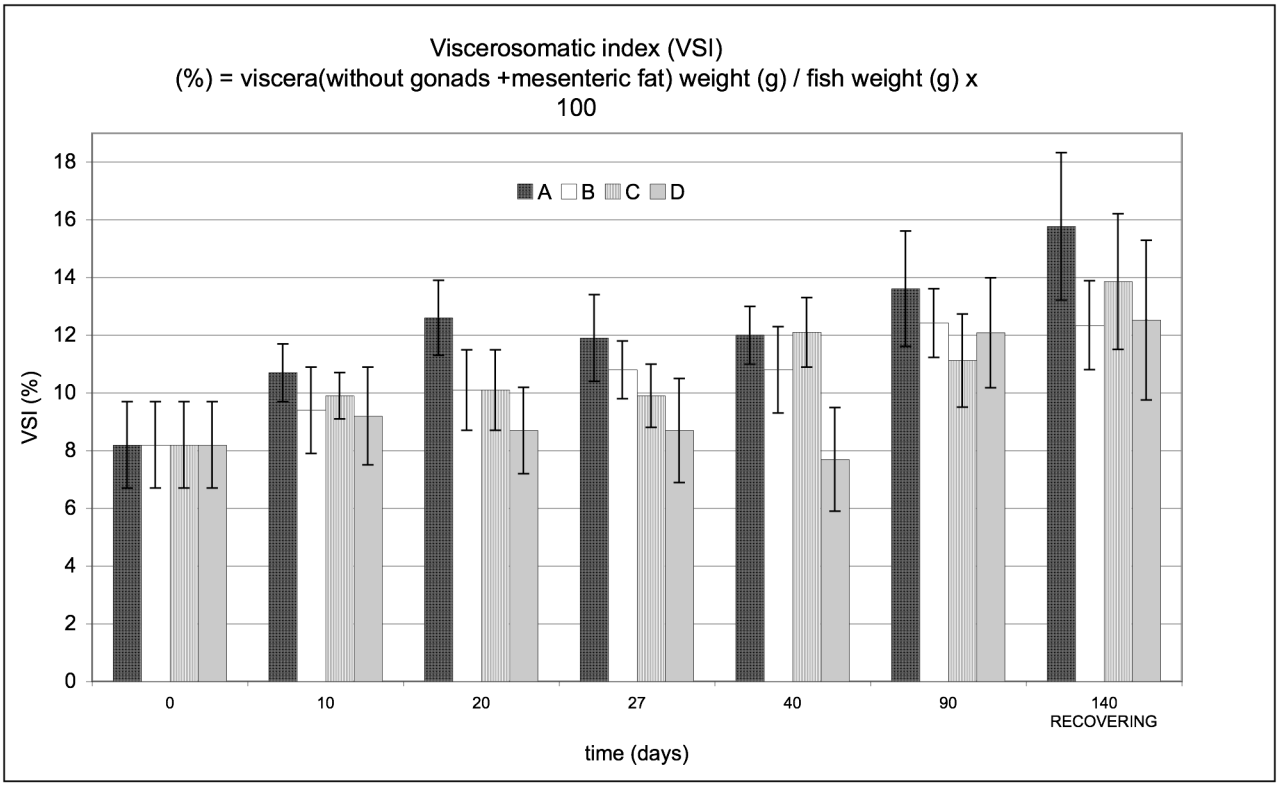
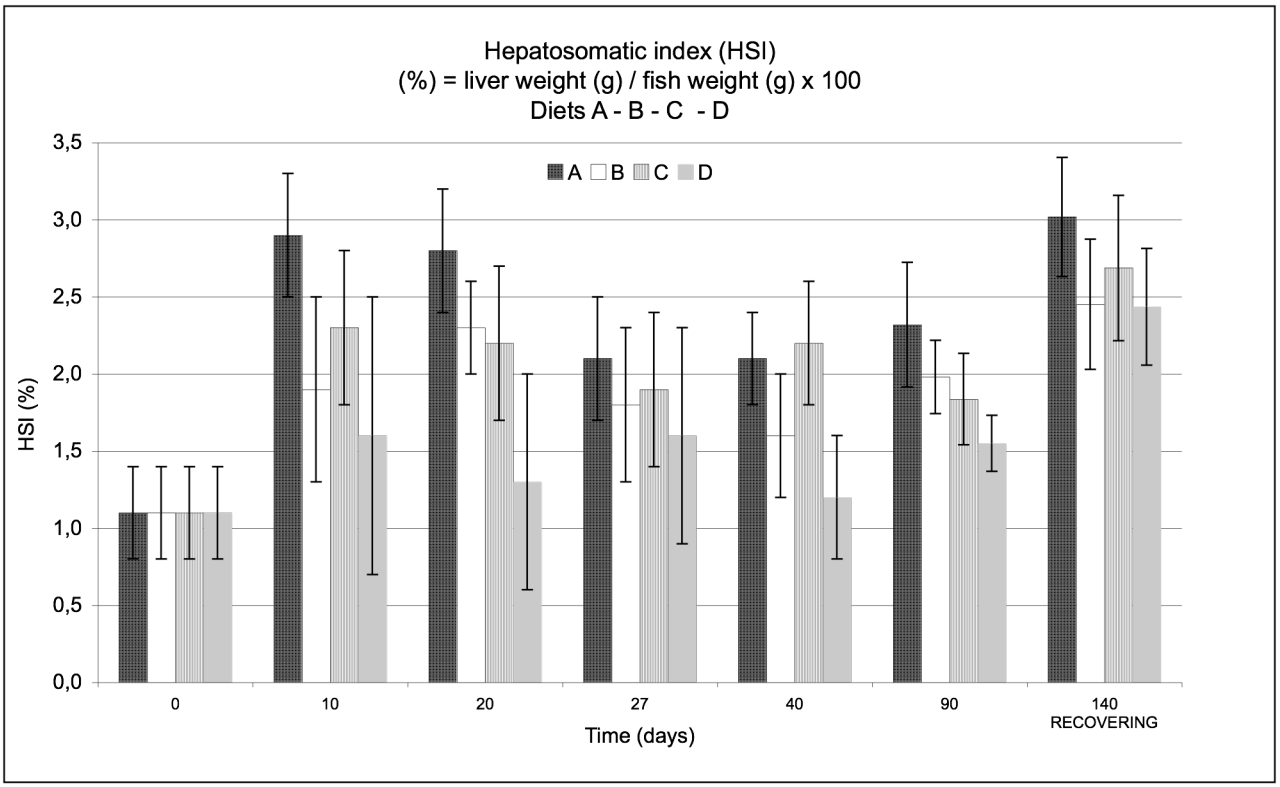


Fig.3.7 Hepatosomatic index (HSI) in fishes fed by four diets (A – B – C - D) at different level of fishmeal replacement with protein plant source until 90 days, plus recovering period (50 days), with diet A only.



The hepatosomatic Index values (Fig.3.7) recorded for diet A (almost ever higher) were significantly different from those observed for diet B (intermediate value) and D (lower value); there is no significant difference between diets A and C. Reminding that diet A, contained the highest fishmeal level and in diet B haemoglobin was added. Such compounds could increase hepatic volume and then increase the hepatosomatic index. Diet A high palatability can induce hyperphagia: the fishes can accumulate fats into the liver with hepatocytes hypertrophy. Although we did not find references in literature, we tentatively hypothesize a relationship also between hepatocytes hypertrophy and haemoglobin presence in diet C.

The hyperphagia can also increase perivisceral fat accumulation, on the other hand the relative undernourishment, due to low diet palatability, can lead to slimming without perivisceral fat accumulation. Figure 3.8 reports the anatomic views of abdominal cavity where fish fed diet A show higher perivisceral fat, compared to the one fed diet D that shows no perivisceral fat accumulation. Fish fed diet B shows intermediate condition.

At the end of the tests it was did a global statistical evaluation on biometric parameters: it was applied two-way analysis of variance crossing "time" effect with by four diets ("Treatments") effect due, using the following model:

$$Y = Time + Treatments + Time*Treatments + Error$$

Results were summarised in table 3.2 from which we can see:

- Hepatosomatic index is the only parameter that shows the existence of diet related effect (considered independently of the time). It is significant ($F(3,3) = 10:59$ $p = 0.04$).
- The others parameters show a significantly ($p < 0.05$) effect due by time (diet independent) except viscerosomatic index for which the ANOVA is not significant.

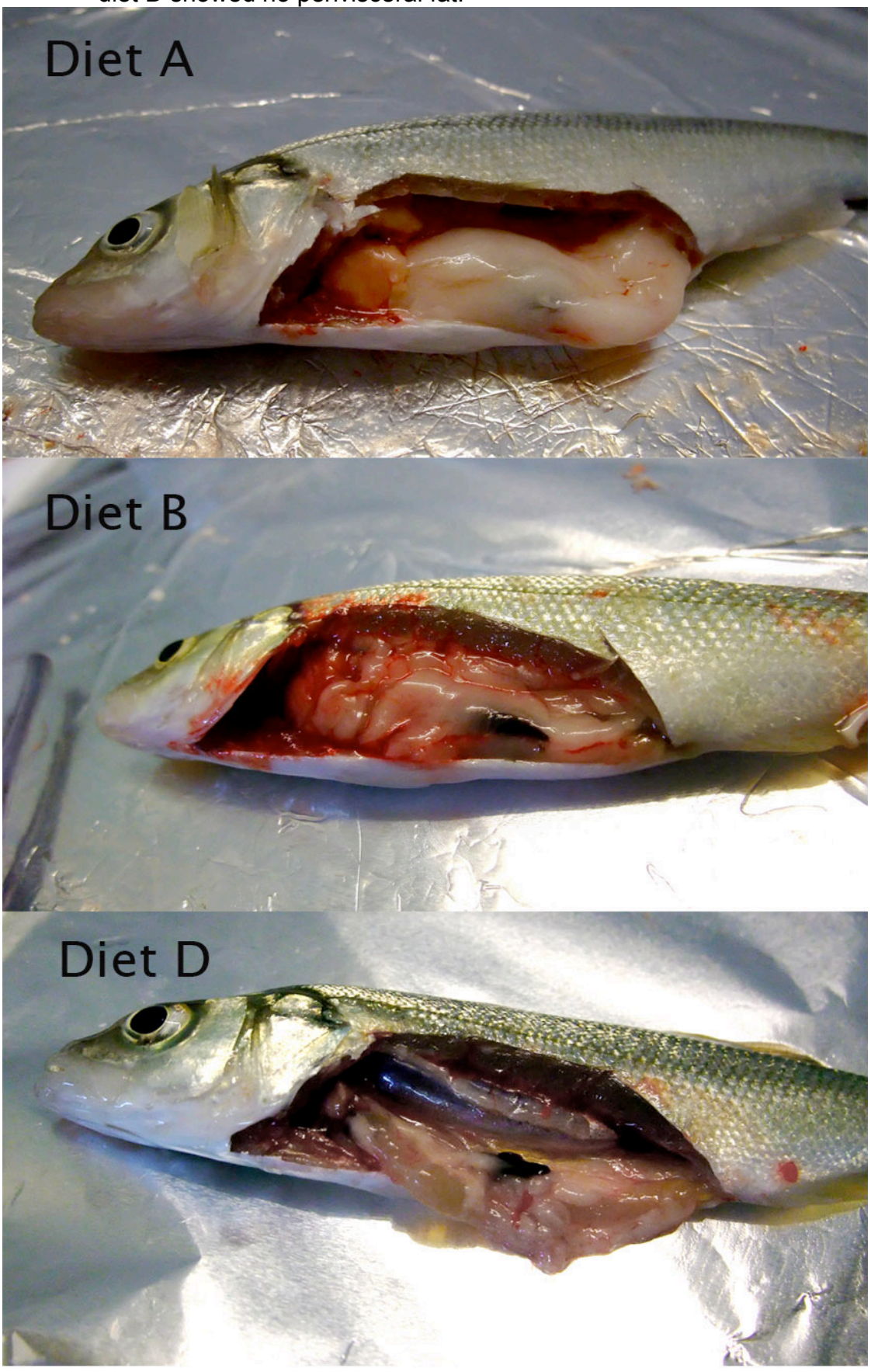
To Hepatosomatic Index was applied multiple comparisons post-hoc analysis of variance for the comparison between diets (regardless of the time), while on the significant parameters for the effect of time post-hoc multiple comparisons between times (independent from the diet). the results are shown below:

- hepatosomatic index: diet A values are significantly different than diet B and D, as well to initial detection.
- Total length: at day 90 (end of test) it was significantly higher than previous extent. It happened the same at day 140 after recovering period (50 days) when all fish were fed diet A.
- Standard length: at day 90 (end of test) it was significantly higher than previous extent. It happened the same at day 140 after recovering period (50 days).
- Total weight: at day 90 (end of test) it was significantly higher than previous extent. It happened the same at day 140 after recovering period (50 days).
- Liver weight: at day 90 (end of test) it was significantly higher than previous extent. It happened the same at day 140 after recovering period (50 days).
- Viscera weight: at day 90 (end of test) it was significantly higher than previous extent. It happened the same at day 140 after recovering period (50 days).

Tab 3.2 Two-way analysis of variance (significant values highlighted in grey) applied to biometric parameters

Parameter	Effects	Df	Sum Sq	Mean Sq	F value	p
Total Lenght	Time	6	353.2	58.87	64.76	0.0029
	Diet	3	10.8	3.61	3.97	0.143
	Time*Diet	12	2.7	0.22	0.24	0.968
	Residuals	3	2.7	0.91		
Standard Lenght	Time	6	255.93	42.65	43.41	0.0052
	Diet	3	7.96	2.65	2.70	0.218
	Time*Diet	12	3.57	0.30	0.30	0.942
	Residuals	3	2.95	0.98		
Total Weight	Time	6	80744	13457	27.64	0.010
	Diet	3	1215	405	0.832	0.558
	Time*Diet	12	317	26	0.054	0.999
	Residuals	3	1461	487		
Liver Weight	Time	6	54.67	9.11	12.37	0.032
	Diet	3	1.44	0.48	0.65	0.633
	Time*Diet	12	0.40	0.03	0.045	1.000
	Residuals	3	2.21	0.73		
Hepatosomatic Index	Time	6	2.799	0.466	6.665	0.074
	Diet	3	2.224	0.741	10.595	0.042
	Time*Diet	12	0.693	0.058	0.826	0.652
	Residuals	3	0.210	0.07		
Viscera Weight	Time	6	1516.0	252.66	12.32	0.032
	Diet	3	29.5	9.84	0.48	0.719
	Time*Diet	12	10.6	0.88	0.043	1.000
	Residuals	3	61.5	20.52		
Visceral Index	Time	6	49.79	8.229	3.181	0.185
	Diet	3	18.26	6.088	2.333	0.252
	Time*Diet	12	7.59	0.632	0.242	0.968
	Residuals	3	7.83	2.609		

Fig. 3.8 Perivisceral fat accumulation: fish fed diet A showed high perivisceral fat quantity, fish fed diet B showed medium accumulation and fish fed diet D showed no perivisceral fat.



3.2 Scoring: evaluation of histological changes

We report the results obtained according the histological scoring system adapted from Knudesen et al. (2007) for arbitrary evaluation of morphological changes induced by diet in the intestine of European sea-bass (*D.labrax*). The supranuclear vacuoles (easy to identify if present), are generally connected with gut absorption activity but their presence or absence is conditioned by many other factors, in addition to feed compound; for instance, by extended fasting, pathologic syndromes or specific diseases that could give enteritis.

Because all the fishes were sampled after fasting (minimum 24 hours) and in majority of case, the supranuclear vacuoles were absent, in the scoring we did not consider this parameter. In figure 3.9 we reported four histological slides representing the physiological condition with or without supranuclear vacuoles and some slight alteration of lamina propria and connective tissue.

To better point out the histological changes regarding a global evaluation it was done the summation of the mean of each single parameter considered.

When we take in account all parameters (Supranuclear Vacuoles, Lamina Propria of single folds, Connective Tissue, Mucosal Folds): global score rating "4 - 8" represents normal morphology or slight alteration, a rate "9 -14" show more evident alterations, while score "15-20" represents serious injury until to severe enteritis. Instead, don't taking in account the supranuclear vacuoles (only three parameters): global score "3 - 6" equals to normal morphology or slight alteration; "7 - 10" equals evident alterations; "11 - 15" equals serious injury. Clearly for each parameter the score (1-5) increases, increasing histological alteration.

In figures from 3.8 to 3.13 the global score is reported (either taking in account or not supranuclear vacuoles) for different gut tracts: proximal, medium and distal. In each case the global score is between normal condition and histological alterations slight or more evident.

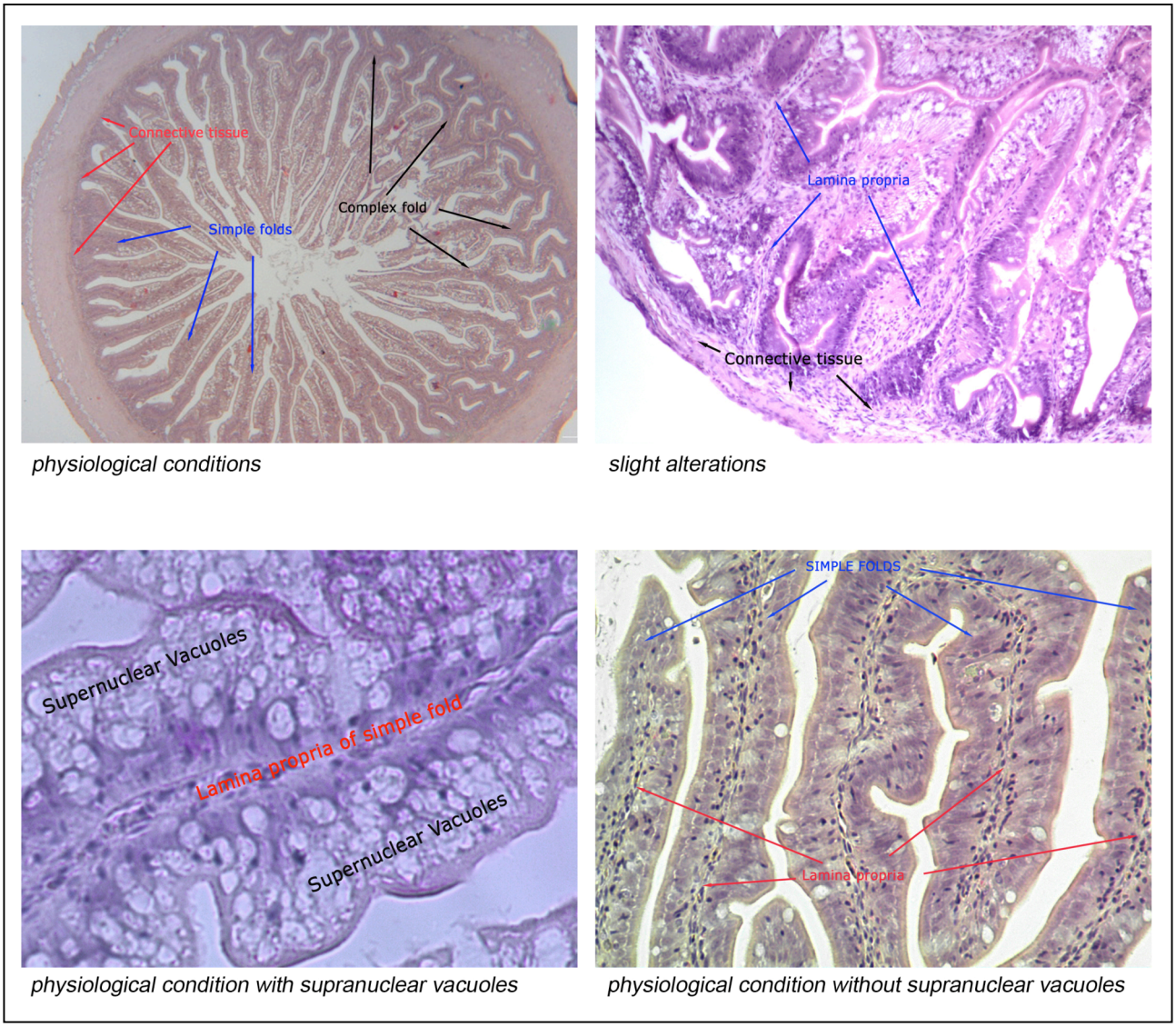
Vacuoles in global score play a decisive role because their value it is ever near to maximum, with some variation seems no diet related, although lower values were generally recorded for diet A.

Indeed when we take in account the scoring without vacuoles, the value remains below to 6 in normality range with few variations, this are below in detail. The score range for proximal intestine was "3 - 5" with highest values by diets C and D. Medium intestine showed a score range between 3,2 and 5,4; also in this case diets C and D gave highest

values but lower than to proximal tract. Distal intestine score range was "3 - 4,6" with diet D values generally higher.

Tables 3.3; 3.4 and 3.5 show, schematic view, the scoring values recorded during the test and at the end of recovering period, along three different gut tracts (proximal, mid and distal intestine).

Fig.3,9 Evaluation of histological changes, in gastrointestinal (GI) tract of sea bass (D.labrax), related to four parameters: supranuclear vacuoles; lamina propria of single folds; connective tissue (or lamina propria basal); mucosal folds.



physiological conditions

slight alterations

physiological condition with supranuclear vacuoles

physiological condition without supranuclear vacuoles

Fig.3.8 Proximal intestine scoring, considering four parameters (Supranuclear Vacuoles; Lamina Propria of single folds; Connective Tissue; Mucosal Folds) summation of the mean of each single parameter

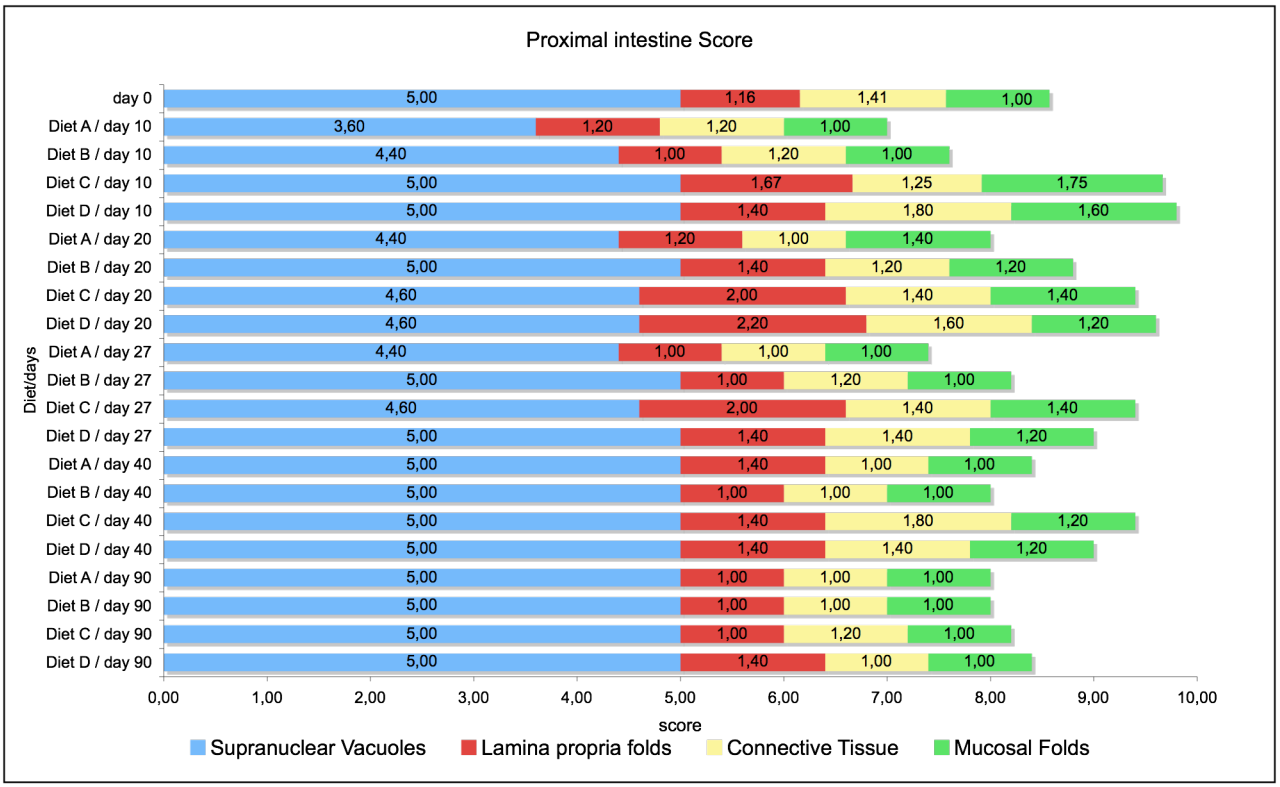


Fig.3.9 Proximal intestine scoring, considering three parameters (Lamina Propria of single folds; Connective Tissue; Mucosal Folds) summation of the mean of each single parameter

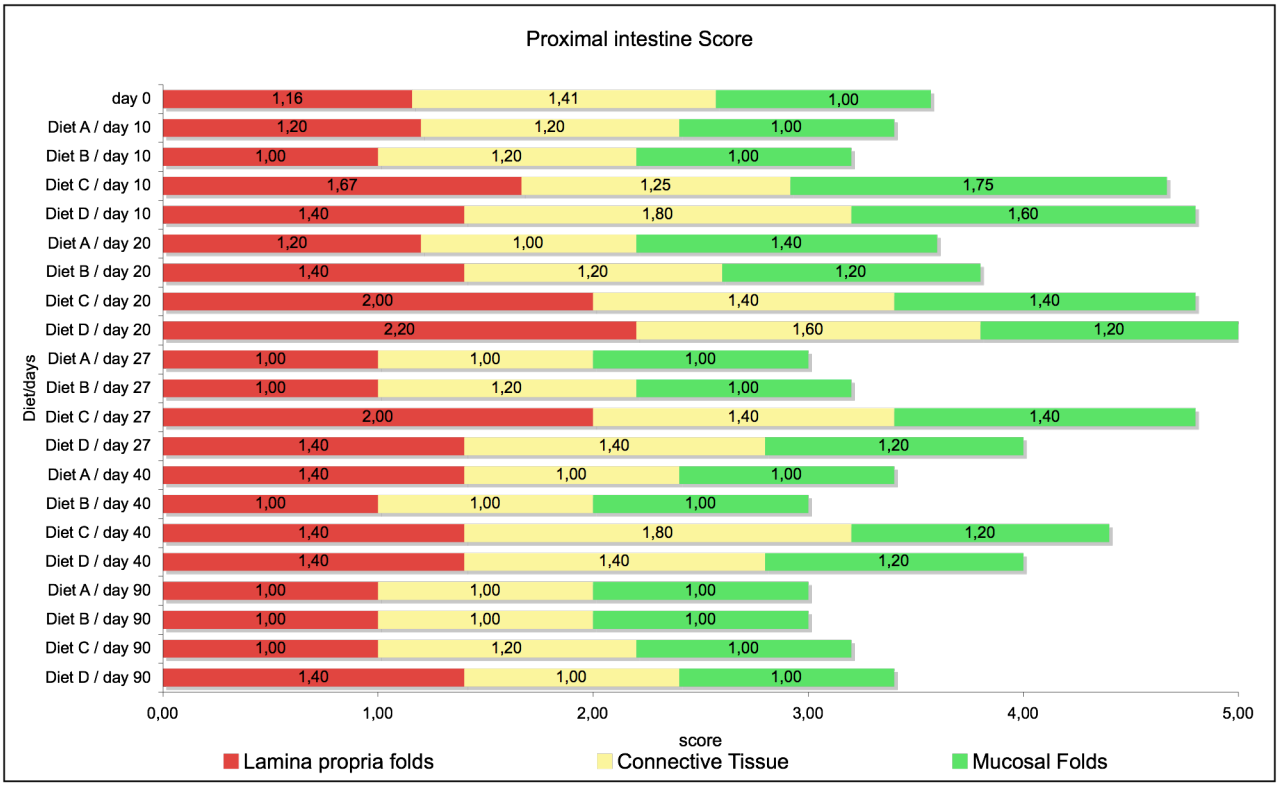


Fig.3.10 Medium intestine scoring, considering four parameters (Supranuclear Vacuoles; Lamina Propria of single folds; Connective Tissue; Mucosal Folds) summation of the mean of each single parameter

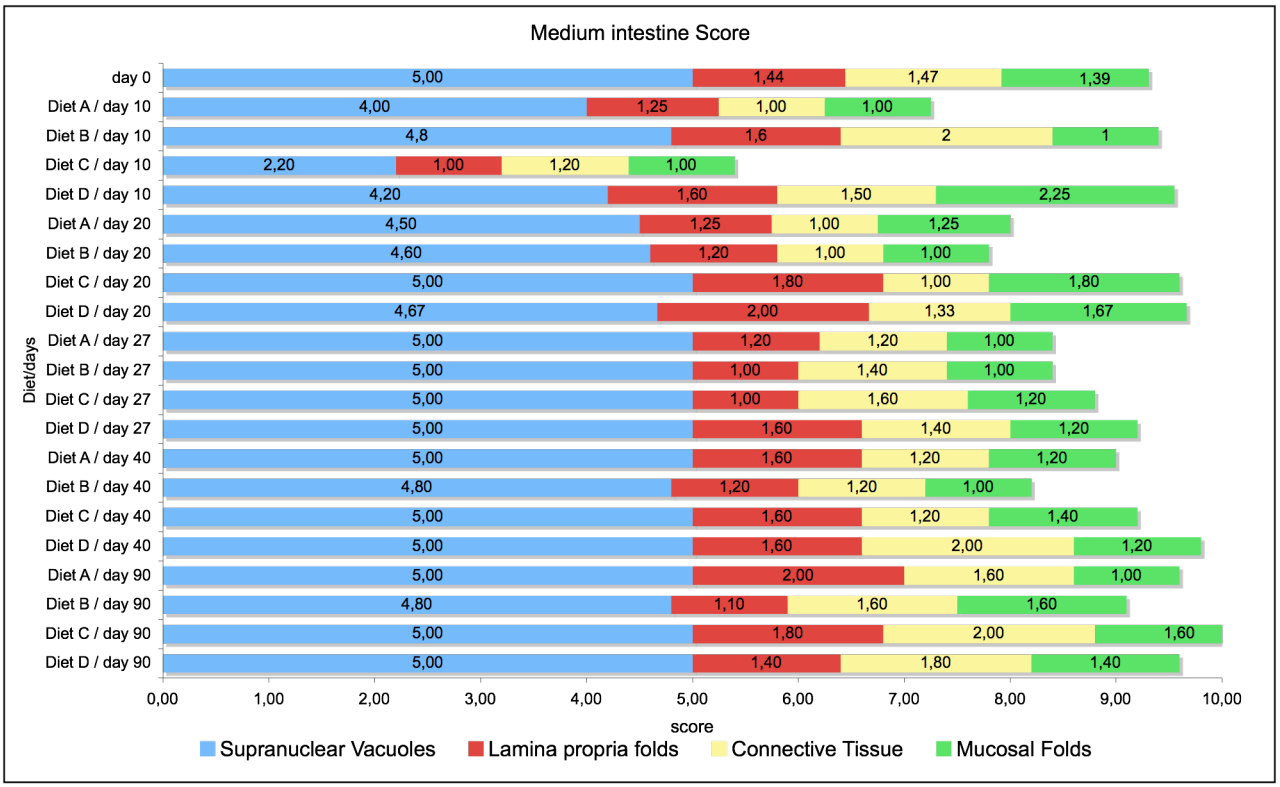


Fig.3.11 Medium intestine scoring, considering three parameters (Lamina Propria of single folds; Connective Tissue; Mucosal Folds) summation of the mean of each single parameter

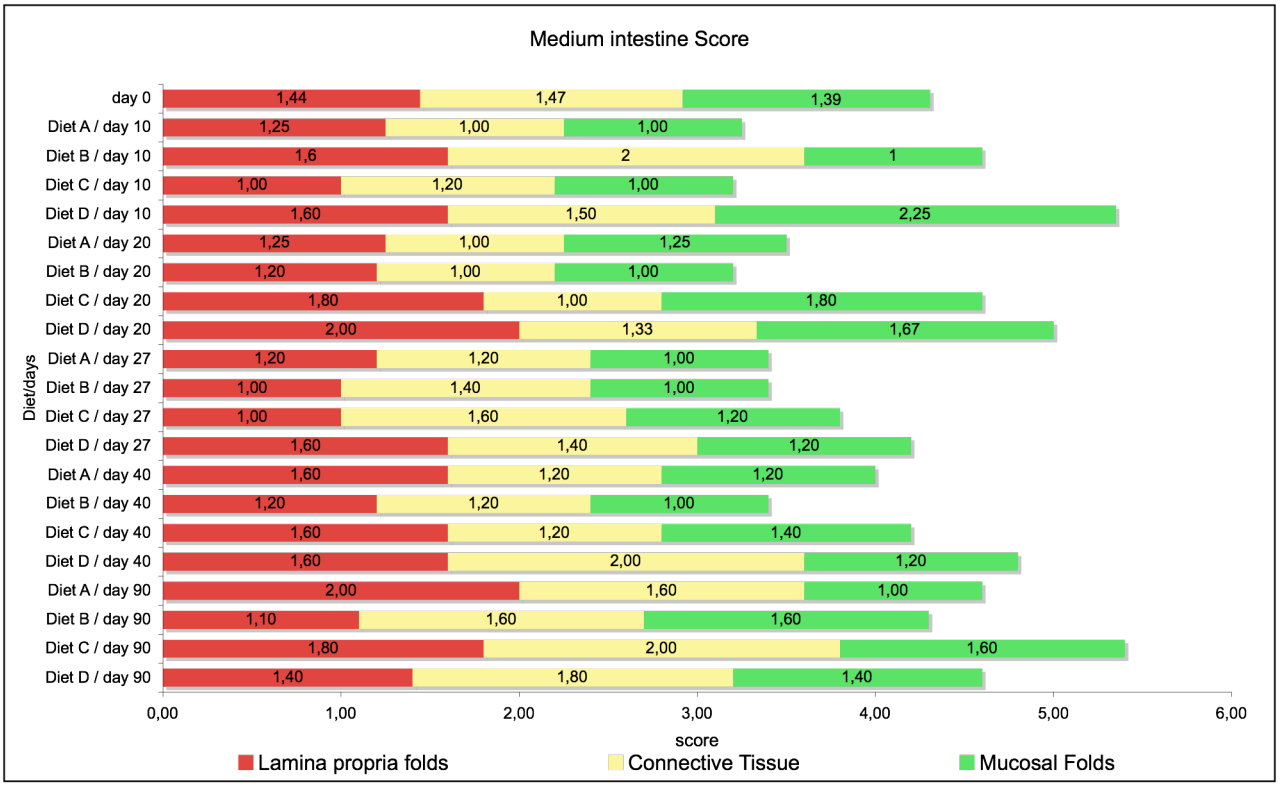


Fig.3.12 Distal intestine scoring, considering four parameters (Supranuclear Vacuoles; Lamina Propria of single folds; Connective Tissue; Mucosal Folds) summation of the mean of each single parameter

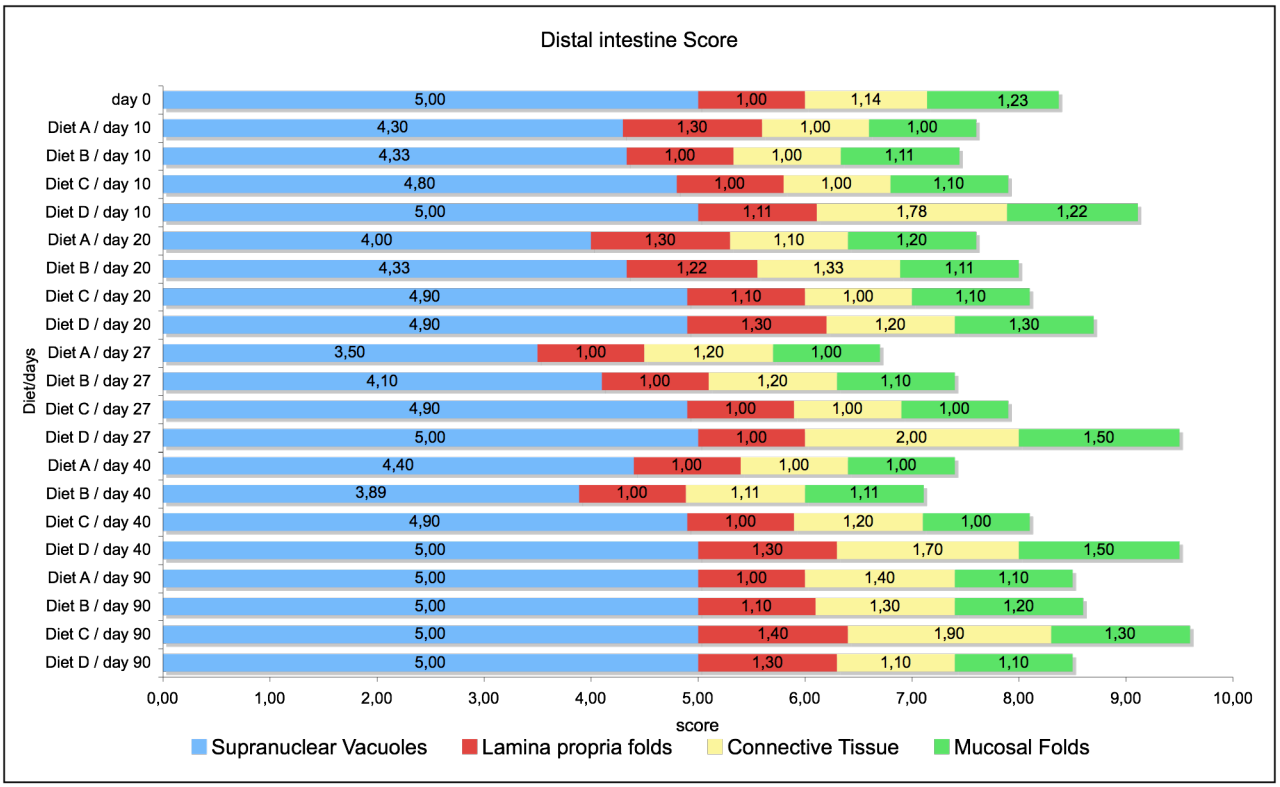
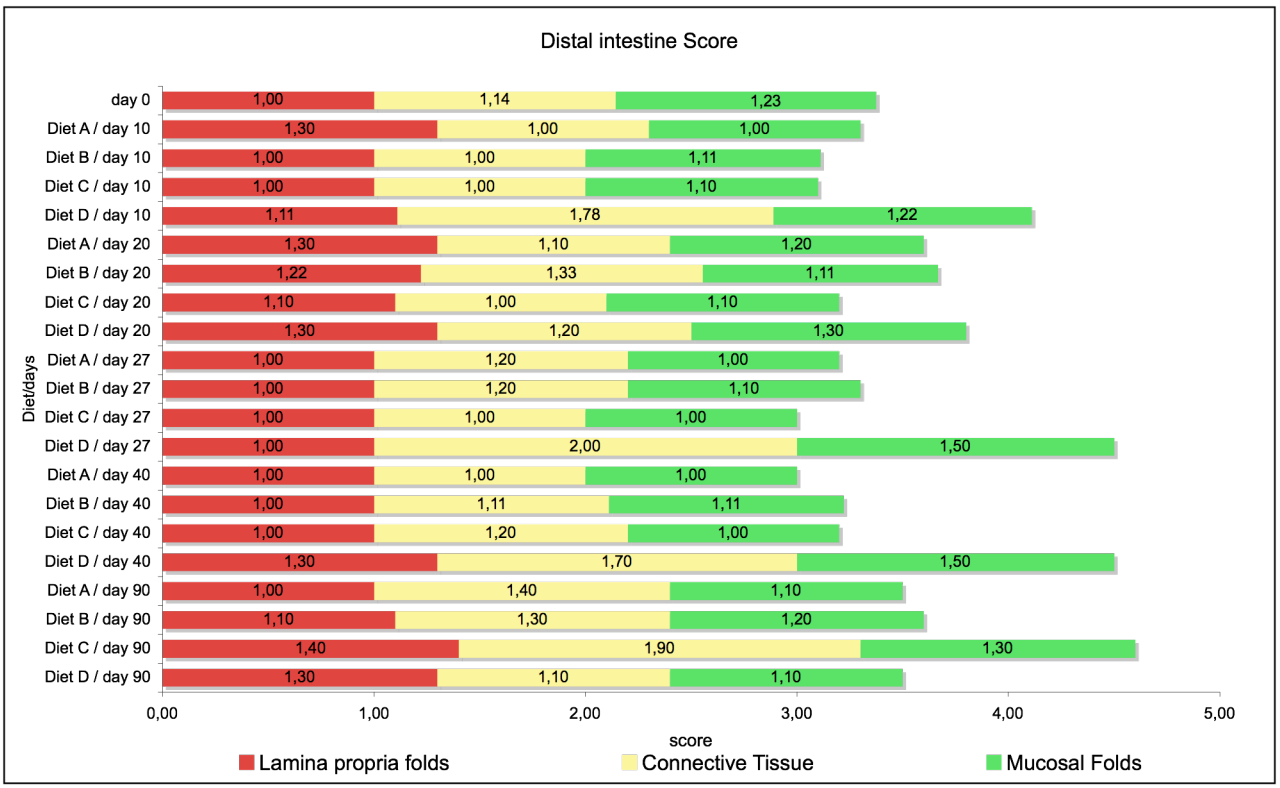


Fig.3.13 Distal intestine scoring, considering three parameters (Lamina Propria of single folds; Connective Tissue; Mucosal Folds) summation of the mean of each single parameter



Tab.3.3Proximal intestine scoring: mean and standard deviation for each considered parameter (Lamina Propria of single folds; Connective Tissue; Mucosal Folds) and their summation

		Med + S.d.			
Gut tract	Diet / time	**Lamina Propria Folds**	**Connective Tissue**	**Mucosal Fold**	**∑ (Summation)**
PROXIMAL INTESTINE	day 0	1.16 ± 0.37	1.41 ± 0.51	1.00 ± 0.00	3,59 ± 0,58
	Diet A / day 10	1.20 ± 0.45	1.20 ± 0.45	1.00 ± 0.00	3,40 ± 0,55
	Diet B / day 10	1.00 ± 0.00	1.20 ± 0.45	1.00 ± 0.00	3,20 ± 0,45
	Diet C / day 10	1.67 ± 0.58	1.25 ± 0.50	1.75 ± 0.50	4,67 ± 1,08
	Diet D / day 10	1.40 ± 0.55	1.80 ± 0.45	1.60 ± 0.55	4,80 ± 1,30
	Diet A / day 20	1.20 ± 0.45	1.00 ± 0.00	1.40 ± 0.55	3,60 ± 0,89
	Diet B / day 20	1.40 ± 0.89	1.20 ± 0.45	1.20 ± 0.45	3,80 ± 1,79
	Diet C / day 20	2.00 ± 0.71	1.40 ± 0.55	1.40 ± 0.55	4,80 ± 1,64
	Diet D / day 20	2.20 ± 0.45	1.60 ± 0.55	1.20 ± 0.45	5,00 ± 0,71
	Diet A / day 27	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3,00 ± 0,00
	Diet B / day 27	1.00 ± 0.00	1.20 ± 0.45	1.00 ± 0.00	3,20 ± 0,45
	Diet C / day 27	2.00 ± 0.00	1.40 ± 0.45	1.40 ± 0.00	3,20 ± 0,45
	Diet D / day 27	1.40 ± 0.55	1.40 ± 0.55	1.20 ± 0.45	4,00 ± 1,00
	Diet A / day 40	1.40 ± 0.55	1.00 ± 0.00	1.00 ± 0.00	3,40 ± 0,55
	Diet B / day 40	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3,00 ± 0,00
	Diet C / day 40	1.40 ± 0.55	1.80 ± 0.84	1.20 ± 0.45	4,40 ± 1,44
	Diet D / day 40	1.40 ± 0.55	1.40 ± 0.45	1.20 ± 0.45	4,00 ± 1,00
	Diet A / day 90	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3,00 ± 0,00
	Diet B / day 90	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3,00 ± 0,00
	Diet C / day 90	1.00 ± 0.00	1.20 ± 0.45	1.00 ± 0.00	3,20 ± 0,45
	Diet D / day 90	1.40 ± 0.55	1.00 ± 0.00	1.00 ± 0.00	3,40 ± 0,55
	Diet A / day 140	1.25 ± 0.46	1.25 ± 0.46	1.00 ± 0.00	3,50 ± 0,67
	Diet B / day 140	1.20 ± 0.42	1.30 ± 0.48	1.10 ± 0.32	3,60 ± 0,70
	Diet C / day 140	1.44 ± 0.53	1.22 ± 0.44	1.22 ± 0.44	3,89 ± 0,99
	Diet D / day 140	1.11 ± 0.33	1.11 ± 0.33	1.00 ± 0.00	3,22 ± 0,42

Tab.3.4 Medium intestine scoring: mean and standard deviation for each considered parameter (Lamina Propria of single folds; Connective Tissue; Mucosal Folds) and their summation

		Med + S.d.			
Gut tract	Diet / time	**Lamina Propria Folds**	**Connective Tissue**	**Mucosal Fold**	**∑ (Summation)**
MEDIUM INTESTINE	day 0	1.44 ± 0.62	1.47 ± 0.70	1.39 ± 0.70	4,44 ± 1,75
	Diet A / day 10	1.25 ± 0.50	1.00 ± 0.00	1.00 ± 0.00	3,25 ± 0,43
	Diet B / day 10	1.60 ± 0.55	2.00 ± 0.00	1.00 ± 0.00	4,60 ± 0,55
	Diet C / day 10	1.00 ± 0.00	1.20 ± 0.45	1.00 ± 0.00	3,20 ± 0,45
	Diet D / day 10	1.60 ± 0.55	1.50 ± 0.58	2.25 ± 0.96	5,35 ± 1,60
	Diet A / day 20	1.25 ± 0.50	1.00 ± 0.00	1.25 ± 0.50	3,50 ± 0,50
	Diet B / day 20	1.20 ± 0.45	1.00 ± 0.00	1.00 ± 0.00	3,20 ± 0,45
	Diet C / day 20	1.80 ± 0.45	1.00 ± 0.00	1.80 ± 0.84	4,60 ± 1,14
	Diet D / day 20	2.00 ± 1.00	1.33 ± 0.58	1.67 ± 1.15	5,00 ± 1,87
	Diet A / day 27	1.20 ± 0.45	1.20 ± 0.45	1.00 ± 0.00	3,40 ± 0,55
	Diet B / day 27	1.00 ± 0.00	1.40 ± 0.55	1.00 ± 0.00	3,40 ± 0,55
	Diet C / day 27	1.00 ± 0.00	1.60 ± 0.55	1.20 ± 0.45	3,80 ± 0,84
	Diet D / day 27	1.60 ± 0.55	1.40 ± 0.55	1.20 ± 0.45	4,20 ± 0,84
	Diet A / day 40	1.60 ± 0.55	1.20 ± 0.45	1.20 ± 0.45	4,00 ± 0,71
	Diet B / day 40	1.20 ± 0.45	1.20 ± 0.45	1.00 ± 0.00	3,40 ± 0,55
	Diet C / day 40	1.60 ± 0.55	1.20 ± 0.45	1.40 ± 0.55	4,20 ± 1,10
	Diet D / day 40	1.60 ± 0.55	2.00 ± 0.00	1.20 ± 0.45	4,80 ± 0,45
	Diet A / day 90	2.00 ± 0.00	1.60 ± 0.55	1.00 ± 0.00	4,60 ± 0,55
	Diet B / day 90	1.10 ± 0.55	1.60 ± 0.00	1.60 ± 0.00	4,40 ± 0,55
	Diet C / day 90	1.80 ± 0.84	2.00 ± 0.71	1.60 ± 0.55	5,40 ± 1,52
	Diet D / day 90	1.40 ± 0.55	1.50 ± 0.45	1.40 ± 0.55	4,60 ± 0,55
	Diet A / day 140	1.00 ± 0.00	1.29 ± 0.49	1.57 ± 0.79	3,86 ± 0,73
	Diet B / day 140	1.10 ± 0.32	1.60 ± 0.52	1.60 ± 0.84	4,30 ± 1,06
	Diet C / day 140	1.44 ± 0.53	1.22 ± 0.44	1.22 ± 0.44	3,89 ± 0,74
	Diet D / day 140	1.00 ± 0.00	1.00 ± 0.00	1.29 ± 0.49	3,28 ± 0,40

Tab.3.5 Distal intestine scoring: mean and standard deviation for each considered parameter (Lamina Propria of single folds; Connective Tissue; Mucosal Folds) and their summation

		Med + S.d			
Gut tract	Diet / time	**Lamina Propria Folds**	**Connective Tissue**	**Mucosal Fold**	**∑ (Summation)**
DISTAL INTESTINE	day 0	1.00 ± 0.00	1.14 ± 0.36	1.23 ± 0.44	3,38 ± 0,42
	Diet A / day 10	1.30 ± 0.48	1.00 ± 0.00	1.00 ± 0.00	3,30 ± 0,48
	Diet B / day 10	1.00 ± 0.00	1.00 ± 0.00	1.11 ± 0.33	3,10 ± 0,32
	Diet C / day 10	1.00 ± 0.00	1.00 ± 0.00	1.10 ± 0.32	3,10 ± 0,32
	Diet D / day 10	1.11 ± 0.33	1.78 ± 0.83	1.22 ± 0.44	4,00 ± 1,33
	Diet A / day 20	1.30 ± 0.48	1.10 ± 0.32	1.20 ± 0.42	3,60 ± 0,84
	Diet B / day 20	1.22 ± 0.44	1.33 ± 0.50	1.11 ± 0.33	3,73 ± 0,96
	Diet C / day 20	1.10 ± 0.32	1.00 ± 0.00	1.10 ± 0.32	3,20 ± 0,63
	Diet D / day 20	1.30 ± 0.48	1.20 ± 0.42	1.30 ± 0.48	3,80 ± 0,79
	Diet A / day 27	1.00 ± 0.00	1.20 ± 0.42	1.00 ± 0.00	3,20 ± 0,42
	Diet B / day 27	1.00 ± 0.00	1.20 ± 0.42	1.10 ± 0.32	3,30 ± 0,48
	Diet C / day 27	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3,00 ± 0,00
	Diet D / day 27	1.00 ± 0.00	2.00 ± 0.93	1.50 ± 0.53	4,40 ± 0,52
	Diet A / day 40	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3,00 ± 0,00
	Diet B / day 40	1.00 ± 0.00	1.11 ± 0.33	1.11 ± 0.33	3,20 ± 0,42
	Diet C / day 40	1.00 ± 0.00	1.20 ± 0.42	1.00 ± 0.00	3,20 ± 0,42
	Diet D / day 40	1.30 ± 0.48	1.70 ± 0.82	1.50 ± 0.53	4,50 ± 1,43
	Diet A / day 90	1.00 ± 0.00	1.40 ± 0.52	1.10 ± 0.32	3,50 ± 0,53
	Diet B / day 90	1.10 ± 0.32	1.30 ± 0.48	1.20 ± 0.42	3,60 ± 0,97
	Diet C / day 90	1.40 ± 0.70	1.90 ± 0.57	1.30 ± 0.48	3,65 ± 0,48
	Diet D / day 90	1.30 ± 0.48	1.10 ± 0.32	1.10 ± 0.32	3,50 ± 0,85
	Diet A / day 140	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3,00 ± 0,00
	Diet B / day 140	1.11 ± 0.33	1.11 ± 0.33	1.11 ± 0.33	3,33 ± 0,94
	Diet C / day 140	1.00 ± 0.00	1.11 ± 0.33	1.00 ± 0.00	3,11 ± 0,31
	Diet D / day 140	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	3,00 ± 0,00

The scoring for each single parameter is reported in figures 3.14 - 3,19. As we told above the supranuclear vacuoles showed high values (absence or limited presence of vacuoles) in every gut tracts. Vacuoles score don't seem diets related although in distal intestine (hindgut), physiologically designed to absorb nutrients, diet A showed a score slightly lower, until 40 days (Fig. 3.18).

Taking in account only the other three parameters ((Lamina Propria of single folds; Connective Tissue; Mucosal Folds) we recorded following results (better showed in figures 3.16; 3,17 and 3.19):

- In proximal intestine, diets C and D showed higher score respect to the initial time (0 days) and to other diets until 40 day, then values tend to come back to initial score. A and B diets score remain quite steady with a trend to reduce values respect the initial time.
- In medium intestine (Fig.3.17), the scoring didn't show significant difference during the tests and between the diets.
- In distal intestine (Fig.3.19), diet D showed almost ever higher score with a trend to come back to initial value at end of the test (the connective tissue showed higher score variation). For the other diets, the score remained steady until 20 day, then went down until 40 day, to come back close to initial value at end of test.

Except for supranuclear vacuoles where the values were mostly neighbours to maximum value of the arbitrary scale used, each other parameters remained in normal morphology range reported to such scale.

Fig.3.14 Proximal intestine scoring: histogram of single values for each considered parameter (Supranuclear Vacuoles; Lamina Propria of single folds; Connective Tissue; Mucosal Folds)

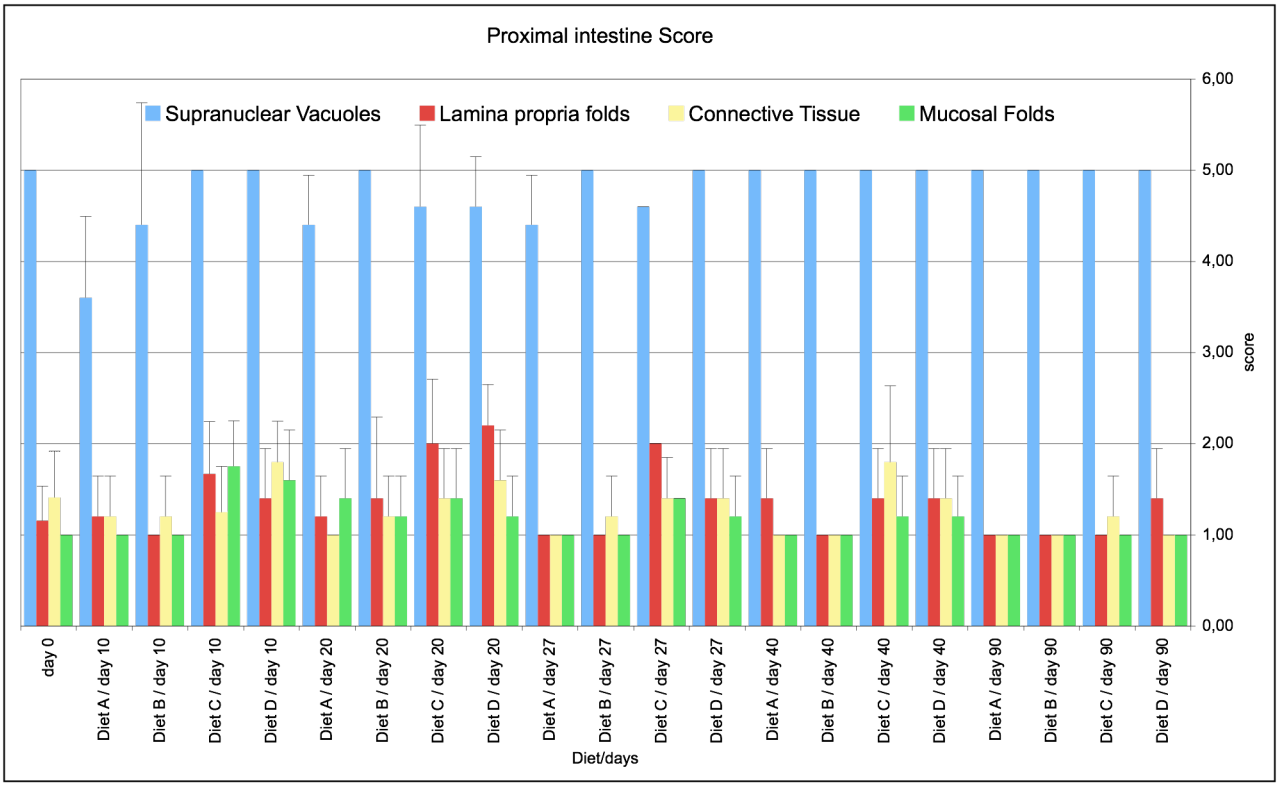


Fig.3.15 Proximal intestine scoring: histogram of single values for each considered parameter (Lamina Propria of single folds; Connective Tissue; Mucosal Folds)

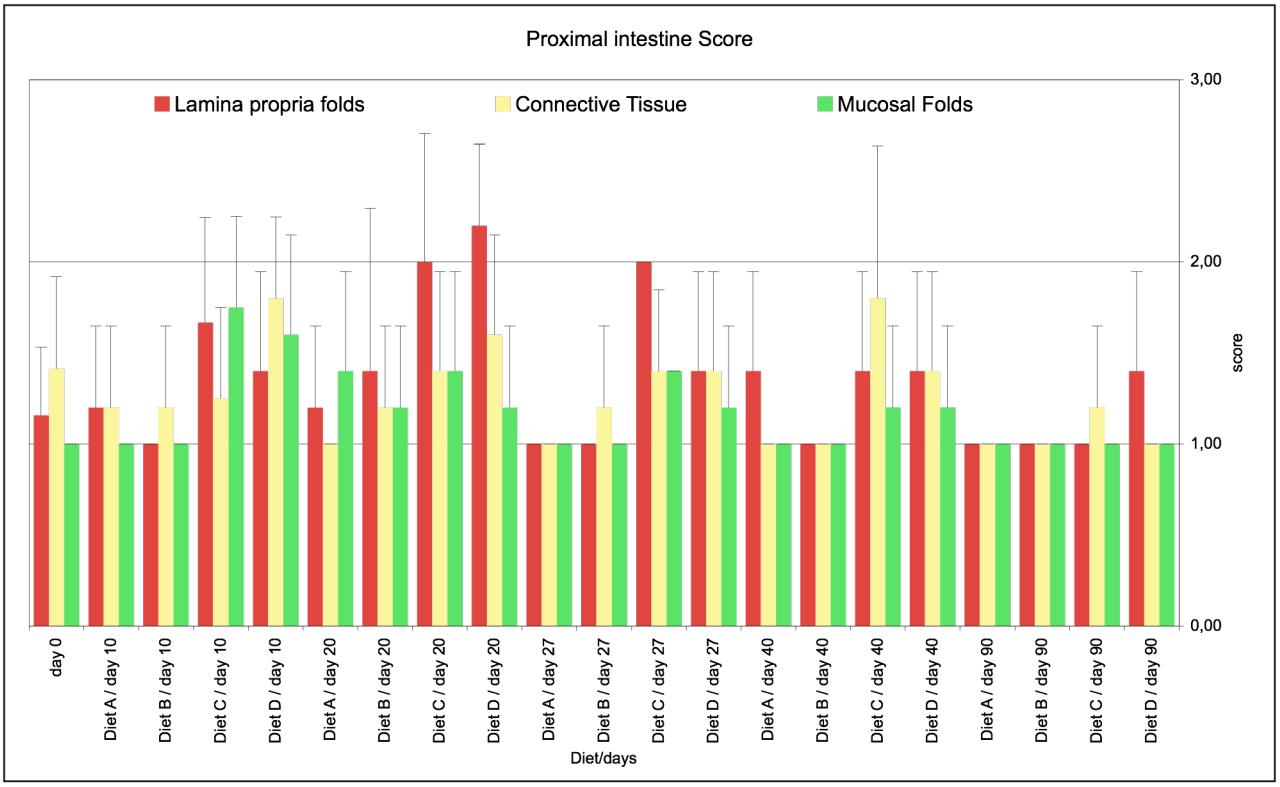


Fig.3.16 Medium intestine scoring: histogram of single values for each considered parameter (Supranuclear Vacuoles; Lamina Propria of single folds; Connective Tissue; Mucosal Folds)

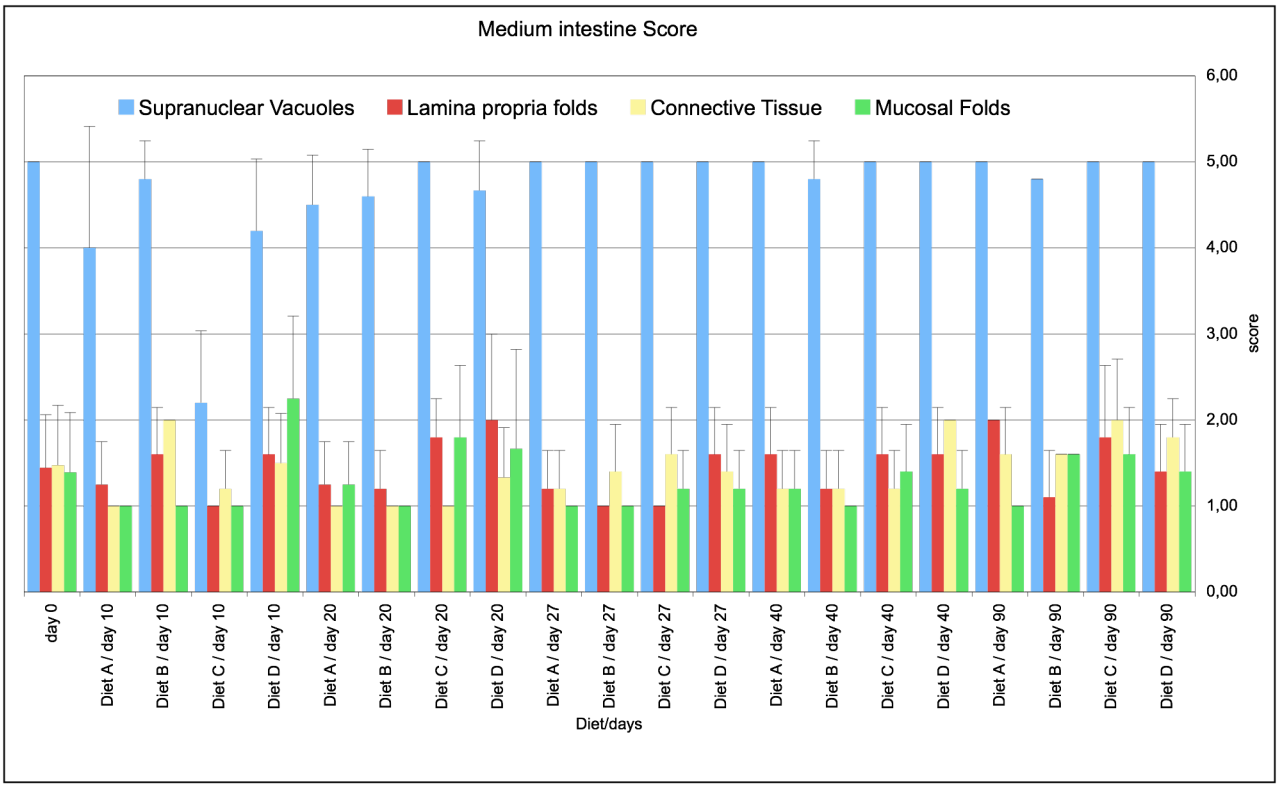


Fig.3.17 Medium intestine scoring: histogram of single values for each considered parameter (Lamina Propria of single folds; Connective Tissue; Mucosal Folds)

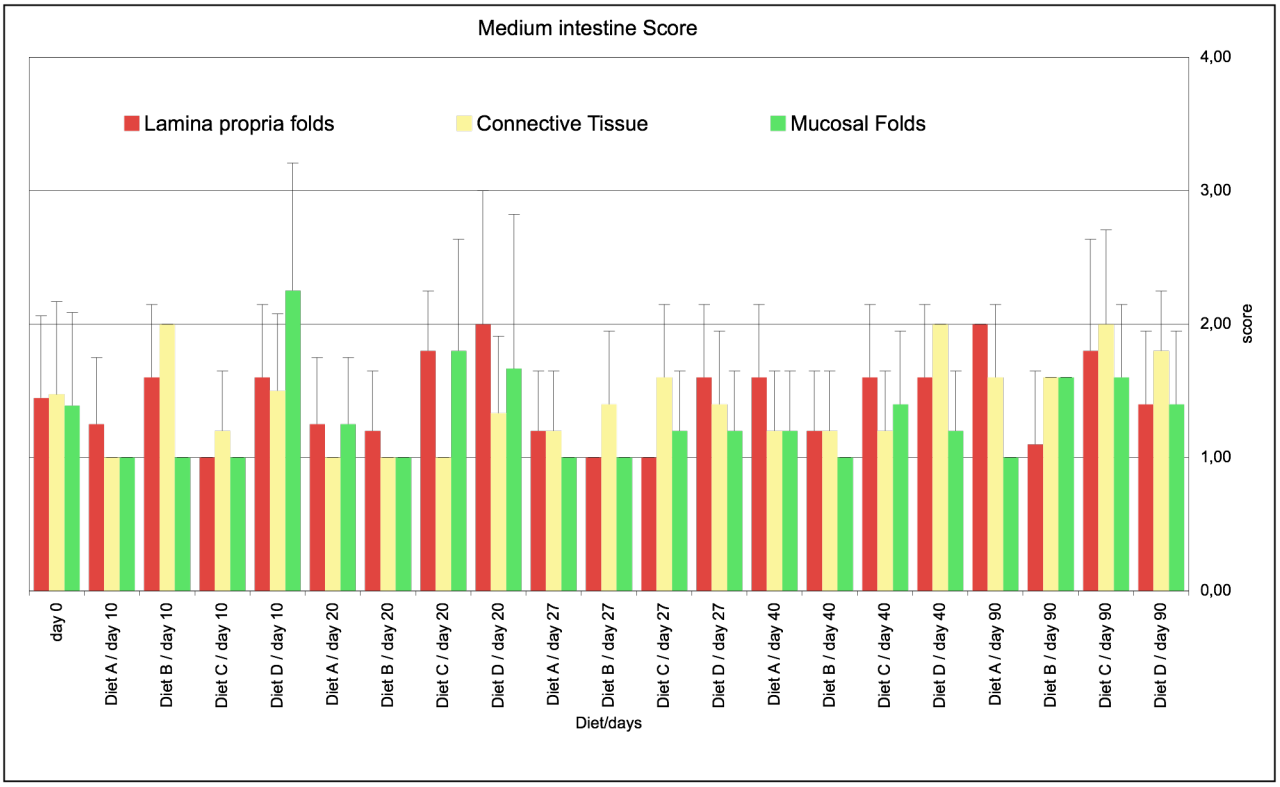


Fig.3.18 Distal intestine scoring: histogram of single values for each considered parameter (Supranuclear Vacuoles; Lamina Propria of single folds; Connective Tissue; Mucosal Folds)

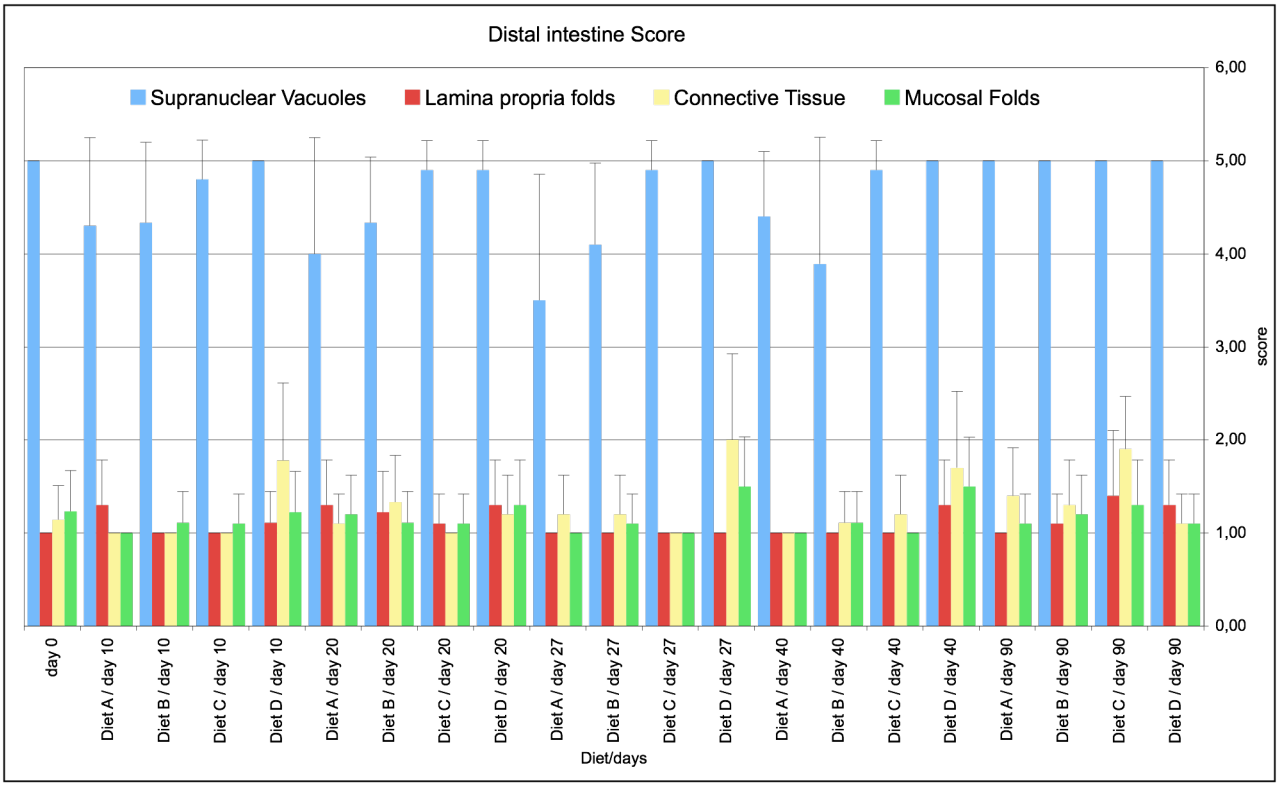
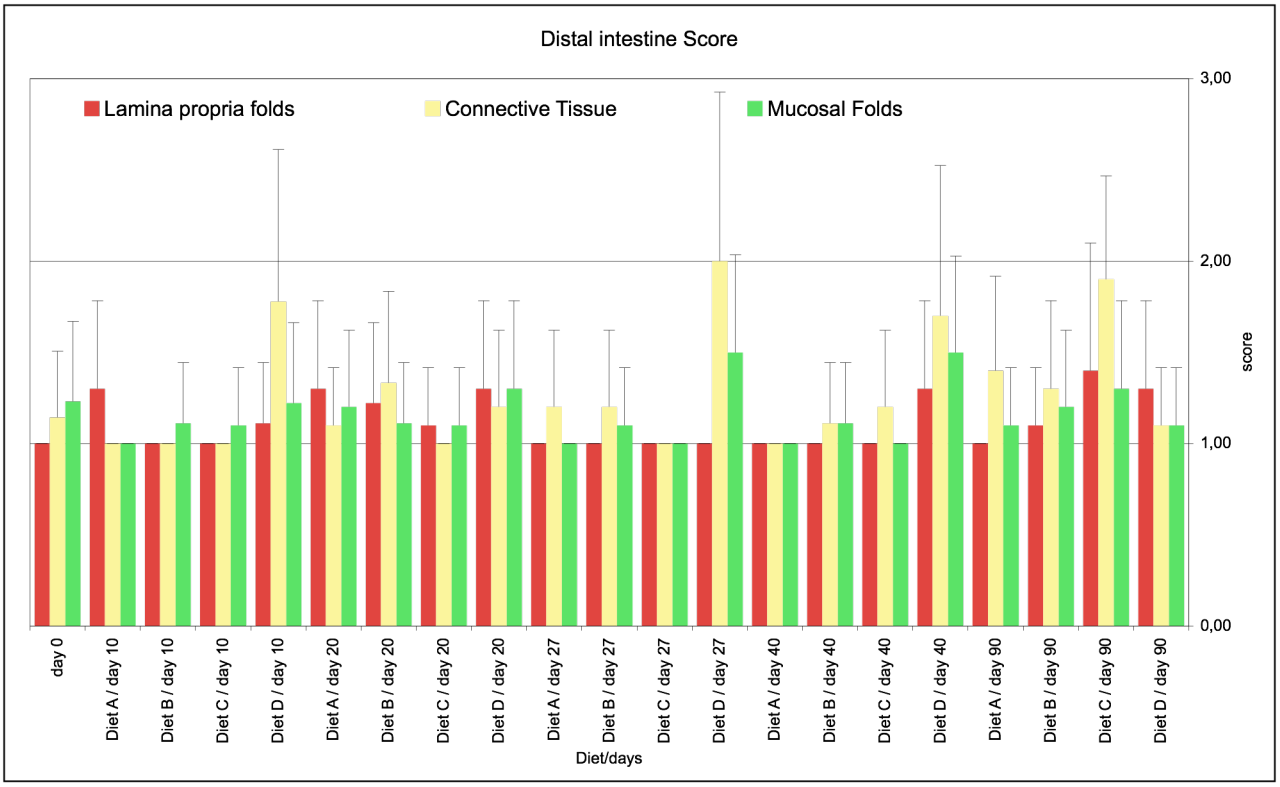


Fig.3.19 Distal intestine scoring: histogram of single values for each considered parameter (Lamina Propria of single folds; Connective Tissue; Mucosal Folds)

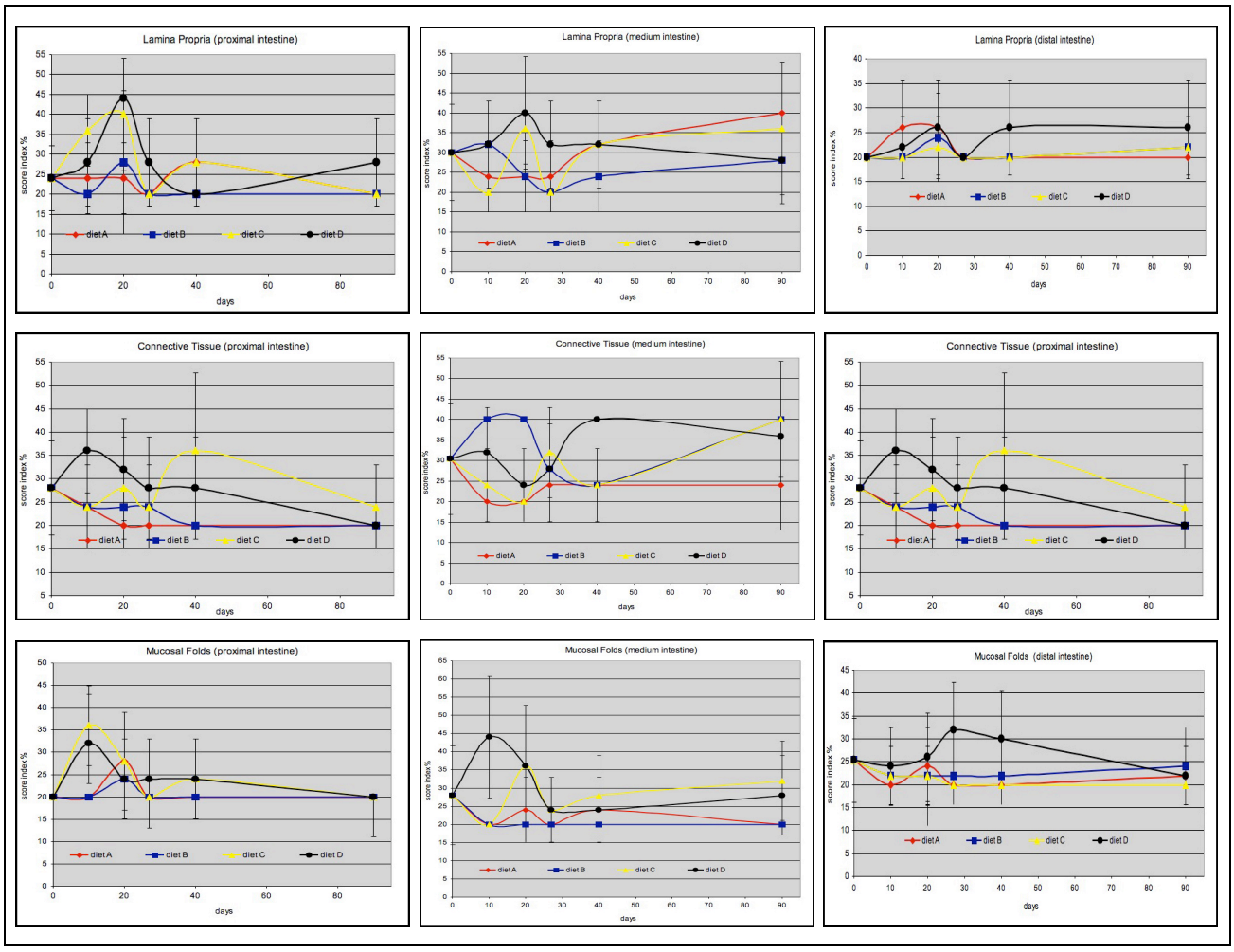


To better evaluate the single parameter weigh and to refine the scoring assessment we created a Score Index expressed in % (SI%),, according with following formula:

SI% = score read value / max possible score value *100

In figure 3.20 and into tables 3.6; 3.7 and 3.8 it was reported the Score Index %, for the three parameters (lamina propria, connective tissue, mucosal folds) and for each diet (A, B, C, D), in proximal, medium and distal intestine tract at time 0, 10, 20, 27, 40 and 90 days.

Fig. 3.20distal intestine at days 0, 10, 20, 27, 40 and 90, for lamina propria, connective tissue and mucosal fold



In proximal intestine (Tab.3.6):

- for lamina propria the two-way analysis of variance showed a time effect ($F(5,156)=6.19$ $p<0.001$) and a diet effect ($F(3,156)=3.18$ $p=0.02$). An interactive effect exists also between time and diet ($F(15,156)=2.09$ $p=0.012$ n.s.). At day 10, diet C resulted in a significantly higher SC% than diet A. At day 27 and 90 diet D values were higher than in other diets. Diet C showed significant higher SC% at day 10 and 20, compared to values observed at days 0, 27 and 90. The significantly highest SC% for diet D has been recorded at day 20.
- for connective tissue, two-way analysis of variance did not show any significant effects, neither diet nor time related.
- for mucosal folds the two-way analysis of variance showed a time effect $F(5,156)=11.4$ $p<0.001$) as well as a diet effect ($F(3,156)=3.58$ $p=0.015$). An interactive effect between time and diet ($F(15,156)=3.12$ $p<0.001$) has been observed as well. At day 10,

both diets C and D showed higher SI% than the diets A and B that achieved their highest value at day 20 ($p<0.05$). Diet C achieved the highest value at day 10 ($p<0.05$), while the 20 day value was significantly different from those in days 0, 27 and 90.

Tab. 3.6 Score Index % detected in proximal intestine at days 0, 10, 20, 27, 40 and 90, for lamina propria, connective tissue and mucosal fold

			Score Index %					
Gut Trac	time	diet	Lamina Propria		Connective tissue		Mucosal Folds	
			Med	SD	Med	SD	Med	SD
Proximal intestine	0 days	AGROITTICA	24.00	8.21	28.00	10.05	20.00	0.00
	10 days	A	24.00	8.94	24.00	8.94	20.00	0.00
		B	20.00	0.00	24.00	8.94	20.00	0.00
		C	36.00	8.94	24.00	8.94	36.00	8.94
		D	28.00	10.95	36.00	8.94	32.00	10.95
	20 days	A	24.00	8.94	20.00	0.00	28.00	10.95
		B	28.00	17.89	24.00	8.94	24.00	8.94
		C	40.00	14.14	28.00	10.95	28.00	10.95
		D	44.00	8.94	32.00	10.95	24.00	8.94
	27 days	A	20.00	0.00	20.00	0.00	20.00	0.00
		B	20.00	0.00	24.00	8.94	20.00	0.00
		C	20.00	0.00	24.00	8.94	20.00	0.00
		D	28.00	10.95	28.00	10.95	24.00	8.94
	40 days	A	28.00	10.95	20.00	0.00	20.00	0.00
		B	20.00	0.00	20.00	0.00	20.00	0.00
		C	28.00	10.95	36.00	16.73	24.00	8.94
		D	20.00	0.00	28.00	10.95	24.00	8.94
	90 days	A	20.00	0.00	20.00	0.00	20.00	0.00
		B	20.00	0.00	20.00	0.00	20.00	0.00
		C	20.00	0.00	24.00	8.94	20.00	0.00
		D	28.00	10.95	20.00	0.00	20.00	0.00

In medium intestine (Tab.3.7):

- for lamina propria two-way analysis of variance did not show any significant effect, neither diet nor time related. At day 27 diet A and D showed higher SI% than other diets ($p<0.05$).
- for connective tissue two-way analysis of variance showed a significant principal effect time related ($F(5,156)=4.41$ $p<0.001$). At day 10 diet B and D showed higher SI% than

diets C and A ($p<0.05$) while diet D was significantly different from diet A. At day 40 diet D showed SI% achieved values significantly higher than other diets.

- for mucosal folds two-way analysis of variance showed a significant principal effect diet related ($F(3,156)=2.93$ $p=0.03$). At day 10 diet D showed SI% achieved values higher than other diets ($p<0.05$).

Tab. 3.7 Score Index % detected in medium intestine at days 0, 10, 20, 27, 40 and 90, for lamina propria, connective tissue and mucosal fold

			Score Index %					
	time	diet	Lamina Propria		Connective tissue		Mucosal Folds	
			Med	SD	Med	SD	Med	SD
Medium intestine		B	32.00	10.95	40.00	0.00	20.00	0.00
		C	20.00	0.00	24.00	8.94	20.00	0.00
		D	32.00	10.95	32.00	10.95	44.00	16.73
	20 days	A	24.00	8.94	20.00	0.00	24.00	8.94
		B	24.00	8.94	40.00	0.00	20.00	0.00
		C	36.00	8.94	20.00	0.00	36.00	16.73
		D	40.00	14.14	24.00	8.94	36.00	16.73
	27 days	A	24.00	8.94	24.00	8.94	20.00	0.00
		B	20.00	0.00	28.00	10.95	20.00	0.00
		C	20.00	0.00	32.00	10.95	24.00	8.94
		D	32.00	10.95	28.00	10.95	24.00	8.94
	40 days	A	32.00	10.95	24.00	8.94	24.00	8.94
		B	24.00	8.94	24.00	8.94	20.00	0.00
		C	32.00	10.95	24.00	8.94	28.00	10.95
		D	32.00	10.95	40.00	0.00	24.00	8.94
	90 days	A	40.00	0.00	24.00	10.95	20.00	0.00
		B	28.00	10.95	40.00	0.00	20.00	0.00
		C	36.00	16.73	40.00	14.14	32.00	10.95
		D	28.00	10.95	36.00	8.94	28.00	10.95

In distal intestine (Tab.3.8):

- for lamina propria two-way analysis of variance showed there is time effect ($F(5,236)=4.74$ $p<0.001$) and a diet effect ($F(3,236)=2.98$ $p=0.03$). The interactive effect between time and diet was not significant ($F(15,236)=1.43$ n.s.). At day 40 diet D showed SI% significantly higher than other diets ($p<0.05$). Diet A highest SI% was achieved at days 10 and 20. Diet D Si% at days 20, 40 and 90 was higher than those of the day 0 and 27.

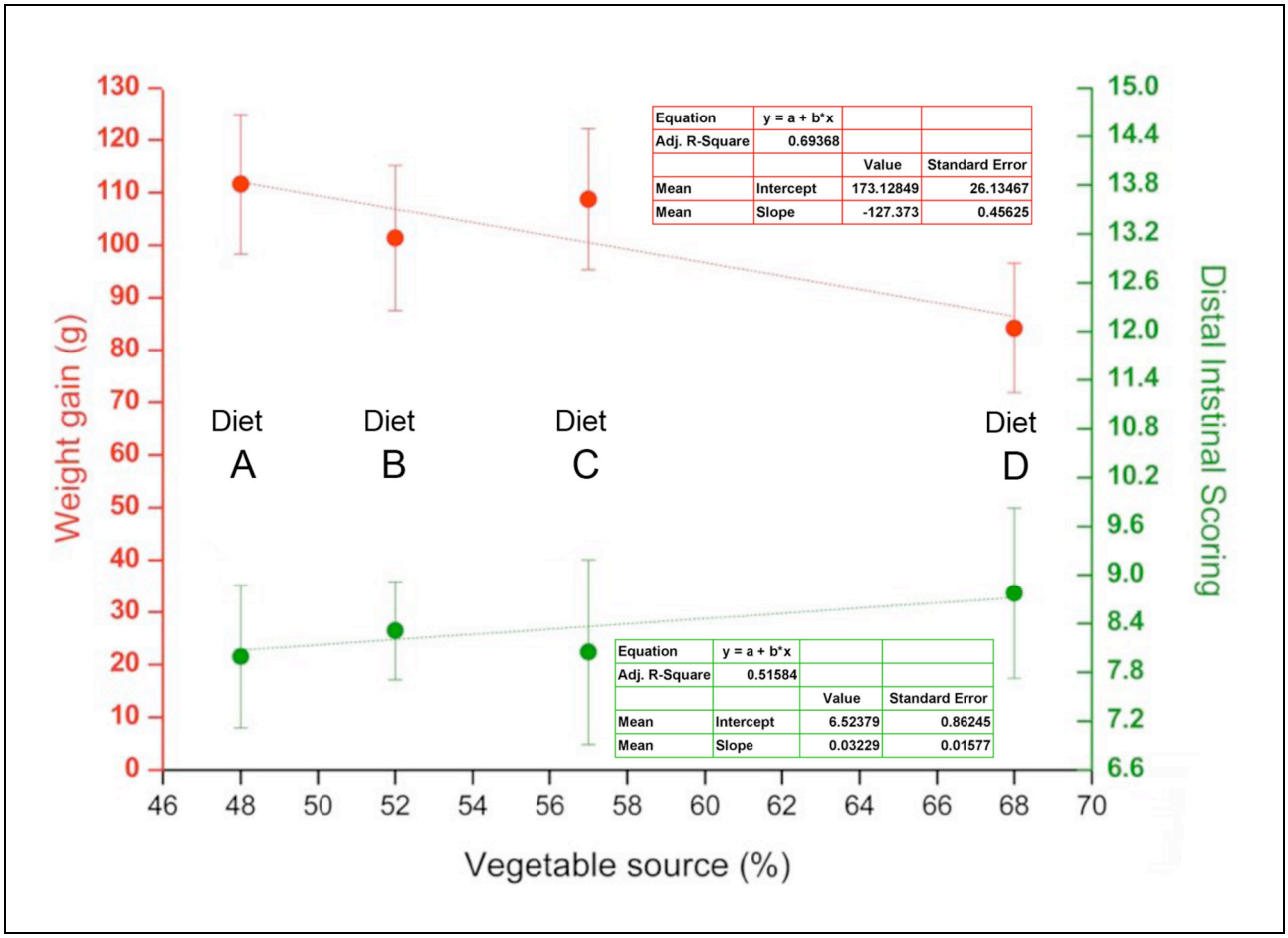
- for connective tissue two-way analysis of variance showed a significant principal effect diet related ($F(3,236)=5.96$ $p<0.001$). An interactive effect between time and diet is also observed ($F(15,236)=2.956$ $p<0.001$). At days 10, 27 and 40 diet D showed SI% significantly higher than other diets ($p<0.05$). Diet D values was higher at day 27 than at days 0 and 90.
- for mucosal folds two-way analysis of variance showed a significant principal effect diet related ($F(3,236)=5.96$ $p<0.001$). At days 27 and 40 diet D recorded SI% significantly higher than other diets.

Tab. 3.8 Score Index % detected in distal intestine at days 0, 10, 20, 27, 40 and 90, for lamina propria, connective tissue and mucosal fold

			Score Index %					
	time	diet	Lamina Propria		Connective tissue		Mucosal Folds	
			Med	SD	Med	SD	Med	SD
Distal intestine		B	20.00	0.00	20.00	0.00	22.00	6.32
		C	20.00	0.00	20.00	0.00	22.00	6.32
		D	22.00	6.32	34.00	16.47	24.00	8.43
	20 days	A	26.00	9.66	22.00	6.32	24.00	8.43
		B	24.00	8.94	28.00	10.95	22.00	10.95
		C	22.00	6.32	20.00	0.00	22.00	6.32
		D	26.00	9.66	24.00	8.43	26.00	9.66
	27 days	A	20.00	0.00	24.00	8.43	20.00	0.00
		B	20.00	0.00	24.00	8.43	22.00	6.32
		C	20.00	0.00	20.00	0.00	20.00	0.00
		D	20.00	0.00	36.00	18.38	32.00	10.33
	40 days	A	20.00	0.00	20.00	0.00	20.00	0.00
		B	20.00	0.00	22.00	6.32	22.00	6.32
		C	20.00	0.00	24.00	8.43	20.00	0.00
		D	26.00	9.66	34.00	16.47	30.00	10.54
	90 days	A	20.00	0.00	28.00	10.33	22.00	6.32
		B	22.00	6.32	26.00	9.66	24.00	8.43
		C	22.00	6.32	30.00	10.54	20.00	0.00
		D	26.00	9.66	22.00	6.32	22.00	6.32

At last, with protein plant source increase in the diet, we recorded a trend to scoring increase (worsening) and, at the same time, a worsening of growth performance; Growth performance and histological scoring in distal intestine were reassumed in figure 3.21.

Fig. 3.21 Weight gain and histological scoring related to increased fish meal substitution with protein plant source.



3.3 Measure of histological changes

In order to confirm the scoring results obtained by arbitrary scale of Tab.2.3, we used "image J" free software to measure same parameters on pictures taken from histological slides. The connective tissue thickness measured at folds base, were reported in table. 3.9 (Proximal intestine) and table 3.10 (Distal intestine) as Lamina Propria (LP) basal thickness.

Tab.3.9 Proximal Intestine measure of connective tissue (basal Lamina Propria) thickness (measured at the folds base)

	Diet A	**Diet B**	**Diet C**	**Diet D**
Time (days)	LP basal (μ)	LP basal (μ)	LP basal (μ)	LP basal (μ)
0	20.5 ± 5.1	20.5 ± 5.1	20.5 ± 5.1	20.5 ± 5.1
40	20.5 ± 5.0	16.7 ± 4.6	30.7 ± 9.2	20.7 ± 5.7
90	37.8 ± 15.9	37.7 ± 18.8	42.9 ± 9.2	43.7 ± 17.3

Tab.3.10 Distal Intestine measure of connective tissue (basal Lamina Propria) thickness (measured at the folds base)

	Diet A	**Diet B**	**Diet C**	**Diet D**
Time (days)	LP basal (μ)	LP basal (μ)	LP basal (μ)	LP basal (μ)
0	23.2 ± 4.6	23.2 ± 4.6	23.2 ± 4.6	23.2 ± 4.6
40	24.9± 5.1	23.7 ± 3.6	30.6 ± 7.3	33.7 ± 10.3

In proximal intestine, connective tissue thickness didn't show any significant difference between the diets during the fist 40 days of test (medium value around 20 μ). Instead the values measured at 90 days were higher for diets C and D (respectively 42,9 ± 9,2 μ and 43,7 ± 17,3 μ) than diets A and B (respectively 37,8 ± 15,9 μ and 37,7 ± 18,8 μ).

On the measures we applied analysis of variance (one way ANOVA) considering, in the model, as main effect that related to the diets. Moreover we applied between diets post-hoc multiple comparisons (Fig.3.21). The one-way ANOVA analysis model was significant ($F(1,443) = 10.64$, $p = 0.0012$) showing diet correlation on LP thickness.

The same measures recorded in table 3.10 for distal intestine showed a similar trend already after 40 days test.

At the beginning, the medium values measured were around 23 μ and after 40 days were achieved 30,6 ± 7,3 μ for diet C and 33,7 ± 10,3 μ for diet D while diets A and B values did not showed significant variations.

ANOVA one-way is significant ($F(1,735) = 240.1$, $p < 0.001$) showing changes due to the diet (Fig. 3.22).

By multiple comparisons we obtained the following results:

The values recorded at day zero (test begining) are significantly lower than those recorded after 40 days for all diets.

At day 40, diets A and B were equivalent and with values significantly lower than of the diets C and D; the latter were significantly different between their.

At end of the test (90 days) in proximal intestine tract, we measured single folds total width of and lamina propia (LP) width, at three different level (Up; Mid; Down) along the folds (Tab.3.11). In the table, epithelium width comes from the subtraction: total fold width - LP width. It is equivalent to width of two epithelium layers.

Tab.3.11 Proximal Intestine at the end of the test (90 days). Single folds total Width of and Lamina Propia (LP) width, measured at three different levels (Up; Mid; Down) along the folds.

Diet	Mesure UP			Mesure MID			Mesure DOWN		
	Width fold Max (μ)	Width LP (μ)	Epithelium (Max-LP=) (μ)	Width fold Max (μ)	Width LP (μ)	Epithelium (Max-LP=) (μ)	Width fold Max (μ)	Width LP (μ)	Epithelium (Max-LP=) (μ)
A	61.56 ±19	25.01±12	36.55±11	68.14±17	28.74±10	39.40±10	76.39±21	34.43±16	41.96±10
B	62.78±20	26.65±13	36.12±10	73.52±19	32.85±13	40.67±11	90.46±33	45.68±28	44.78±10
C	63.17±19	26.26±10	36.90±12	76.72±24	32.81±15	43.92±13	94.49±31	45.68±23	48.81±13
D	64.56±20	27.05±11	37.51±13	75.57±21	33.96±12	41.62±13	94.89±28	48.78±21	46.11±13

On the measures we applied analysis of variance (one way ANOVA) considering, in the model, as main effect that related to the diets. Moreover we applied between diets post-hoc multiple comparisons. The results were summarized in figures 3.23 - 3.31.

Level Up:

1) The width max of fold at this level showed a few variations between the diets (diet A= 61,56 ±19 μ; diet B= 62,78±20 μ; diet C= 63,17±19 μ; diet D= 64,56±20 μ). Analysis of variance was not significant (F (1,222) = 0.739 n.s.). In figure 3.21 were reported the boxplot of the width max of folds at Up level.
2) The width of fold LP at this level showed a few variations between the diets (diet A= 25,01±12 μ; diet B= 26,65±13 μ; diet C= 26,26±10 μ; diet D= 27,05±11 μ). Analysis of variance was not significant (F (1,222) = 0.858 n.s.). In figure 3.22 were reported the boxplot of the width folds LP at Up level.
3) The width of epithelium at this level showed a few variations between the diets (diet A= 36,55±11 μ; diet B= 36,12±10 μ; diet C= 36,90±12 μ; diet D= 37,51±13 μ). Analysis of variance was not significant (F (1,222) = 0.299 n.s.). In figure 3.23 were reported the boxplot of epithelium width at Up level.

Level Mid:

1) The width max of fold at this level showed several values between the diets (diet A= 68,14±17 μ; diet B= 73,52±19 μ; diet C= 76,72±24 μ; diet D= 75,57±21 μ). Analysis of variance was significant to confirm the relationship by diet ((F(1,266)=6.016 p=0.0148). However, any pairwise comparison was not significant. In figure 3.24 were reported the boxplot of the width max of folds at Mid level.
2) The width of fold LP at this level showed several values between the diets (diet A= 28,74±10 μ; diet B= 32,85±13 μ; diet C= 32,81±15 μ; diet D= 33,96±12 μ). Analysis of variance was significant to confirm the relationship by diet (F(1,266)=5.965 p=0.0152). However, any pairwise comparison was not significant. In figure 3.25 were reported the boxplot of the width of fold LP at Mid level.
3) The width of epithelium at this level showed a few variations between the diets (diet A= 39,40±10 μ; diet B= 40,67±11 μ; diet C= 43,92±13 μ; diet D= 41,62±13 μ). Analysis of variance was not significant (F(1,266)=2.467 n.s.). In figure 3.26 were reported the boxplot of epithelium width at Mid level.

Level Down:

1) The width max of fold at this level showed several values between the diets (diet A= 76,39±21 μ; diet B= 90,46±33 μ; diet C= 94,49±31 μ; diet D= 94,89±28 μ). Analysis of variance was significant to confirm the relationship by diet ($F(1,235)=13.76$ $p<0.001$). Noteworthy that diets A, showed values significantly lower than other diets. In figure 3.27 were reported the boxplot of the width max of folds at Down level.
2) The width of fold LP at this level showed several values between the diets (diet A= 34,43±16 μ; diet B= 45,68±28 μ; diet C= 45,68±23 μ; diet D= 48,78±21 μ). Analysis of variance was significant to confirm the relationship by diet ($F(1,235)=12.17$ $p<0.001$). Noteworthy that diet A, showed values significantly lower than other diets. In figure 3.28 were reported the boxplot of the width of folds LP at Down level.
3) The epithelim at this level showed several values between the diets (diet A= 41,96±10 μ; diet B= 44,78±10 μ; diet C= 48,81±13 μ; diet D= 46,11±13 μ). Analysis of variance was significant to confirm the relationship by diet ($F(1,235)=6.276$ $p=0.0129$). Noteworthy that diet A, showed values significantly lower than other diets. In figure 3.29 were reported the boxplot of the epithelium width at Down level.

3.4 Count of cell in gut and liver

At the end of the test (90 days), in addition to the scoring and measures, on histological slide, we counted some cells and calculated their concentration per square millimetre (Tab. 3.12 and 3.13).

Tab.3.12 Proximal Intestine at the end of the test (90 days). Granulocytes count at the folds base (number per square millimetre).

Diet A	Diet B	Diet C	Diet D
Granulocytes (n/mm^2)	Granulocytes (n/mm^2)	Granulocytes (n/mm^2)	Granulocytes (n/mm^2)
301 ± 251	253 ± 157	242 ± 132	314 ± 222

Tab.3.13 Trend of hepatocytes count (number per liver square millimetre).

	Diet A	Diet B	Diet C	Diet D
Time (days)	Hepatocytes (N°/mm^2)	Hepatocytes (N°/mm^2)	Hepatocytes (N°/mm^2)	Hepatocytes (N°/mm^2)
0	4476 ± 342	4476 ± 342	4476 ± 342	4476 ± 342
20	3461 ± 868	3067 ± 519	4144 ± 572	4119 ± 1341
40	3271 ± 625	4542 ± 1497	4970 ± 695	4935 ± 823
90	2738 ± 930	4400 ± 652	5059 ± 868	5850 ± 777

Table 3.12 shows the granulocytes concentration at the base of the folds. There were not significant differences between values recorded (diet A = 301 ± 251 granulocites/mm^2; diet B = 253 ± 157 granulocites/mm^2; diet C = 242 ± 132 granulocites/mm^2; diet D = 314 ± 222 granulocites/mm^2). Analysis of variance was not significant ($F(1,429)=0.055$, n.s.). In figure 3.32 were reported the boxplot of the granulocytes concentration.

Table 3.13 shows the hepatocytes concentration in the liver, at beginning and after 20, 40 and 90 days (end of test). The diet B values remain quite steady during all the test (around 4000 heoatocytec/mm^2); C and D diets showed higher value at 90 day (respectively 5059 ± 868 and 5850 ± 777 heoatocytec/mm^2), instead diet A showed a decrease at the end of test (2738 ± 930 heoatocytec/mm^2).

On the hepatocytes concentration we applied two-way analysis of variance crossing effect due to time trend with effect due to treatments administered (diets A, B, C, and D).

According with following model:

Hepatocytes concentration = Time + Treatment + Time * Treatment + Error

As may be seen in table 3.14 there is no main effect due independently to the time but there is a significant main effect due to treatments plus a significant combined effect due to time and treatment together (interaction).

Tab. 3.14 Results of two-way analysis of variance applied to hepatocytes concentration (significant values highlighted in grey)

Effects	Df	Sum Sq	Mean Sq	F value	p
Time	1	376909	376909	0.632	0.429
Diet	3	17441483	5813828	9.745	1.20e-05
Time*Diet	3	19335512	6445171	10.804	3.79e-06
Residuals	92	54884914	596575		

Therefore we utilized analysis of variance multiple comparisons post-hoc tests: between diets independently from time; inside diets during test time; between diets in each different time.

Results are summarized below (significance level α=0,05):

- Multiple comparisons between diets (time independently). Diet D achieved the higher hepatocytes concentration and showed similar values to diet C like, but hepatocytes concentration was significantly different to the others two diets. Diet A hepatocytes concentration remained lower than those C and B diets, latter values were significantly different.
- Multiple comparisons inter diets (during test time). At beginning (time 0), diet A showed hepatocytes concentration higher than subsequent counts. The decrease recorded at 20 day with diet D was significant. Any comparison between times was no significant for diet C. With diet D, hepatocytes concentration at the end of test was significantly higher than those at the beginning (time 0) and at 20 days.
- Multiple comparisons between diets (in different time). At the beginning and at 20 days there ware no significant differences between diets. At 40 days hepatocytes concentration for diet A was significantly different compared to other diets. At 90 days (end of test) diet A values were significantly different compared to other diets and diet B was significantly different to diet D.

Fig.3.22 Proximal intestine: Boxplot of LP folds thickness measurements at basal level, for each diet (A, B, C, D)

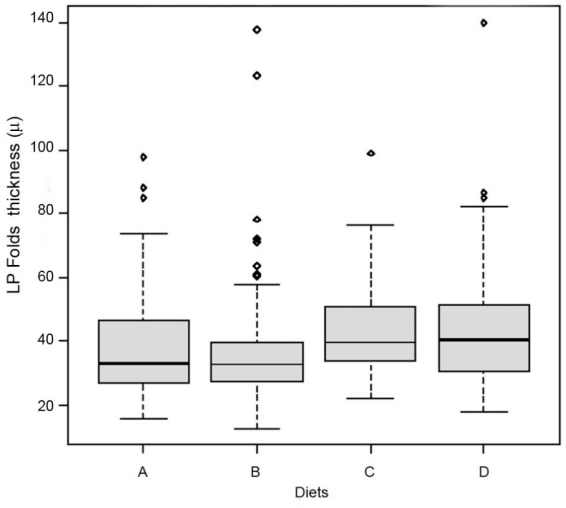


Fig.3.23 Distal intestine: Boxplot of LP folds thickness measurements at basal level, for each diet (A, B, C, D) and initial value (day zero)

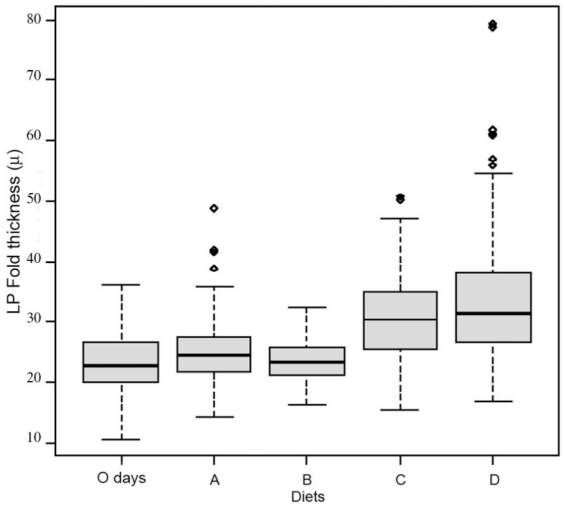


Fig.3.24 Boxplot of folds width max measurements at Up level, for each diet (A, B, C, D)

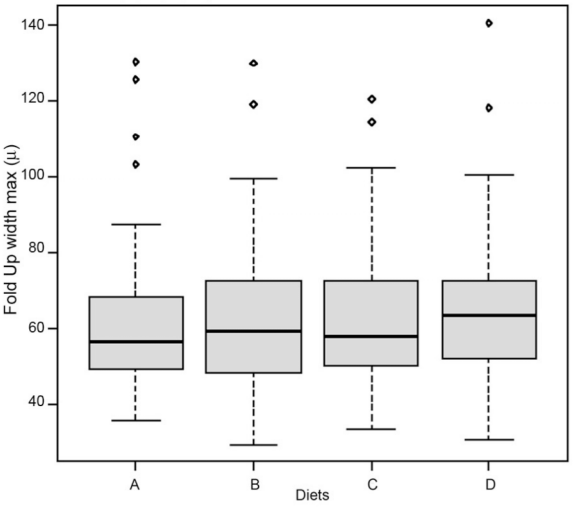


Fig.3.25 Boxplot of folds LP width at Up level, for each diet (A, B, C, D)

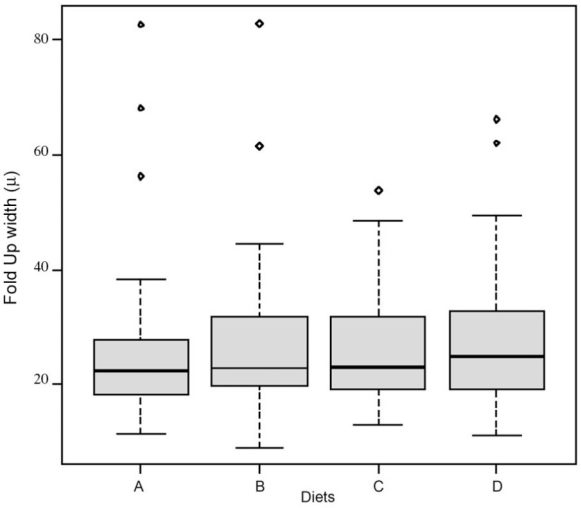


Fig.3.26 Boxplot of epithelium width at Up level, for each diet (A, B, C, D)

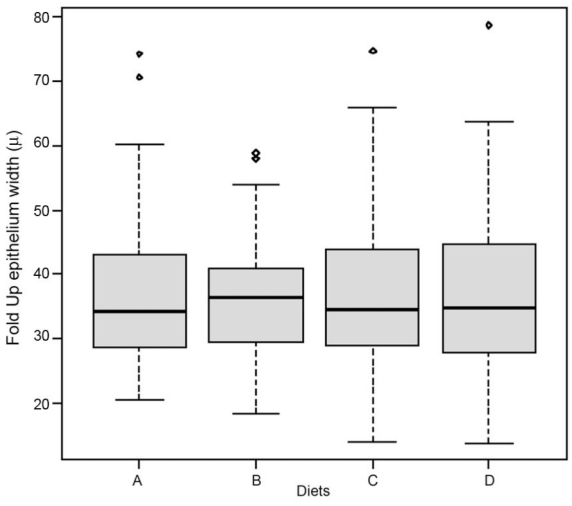


Fig.3.27 Boxplot of folds width max measurements at Mid level, for each diet (A, B, C, D)

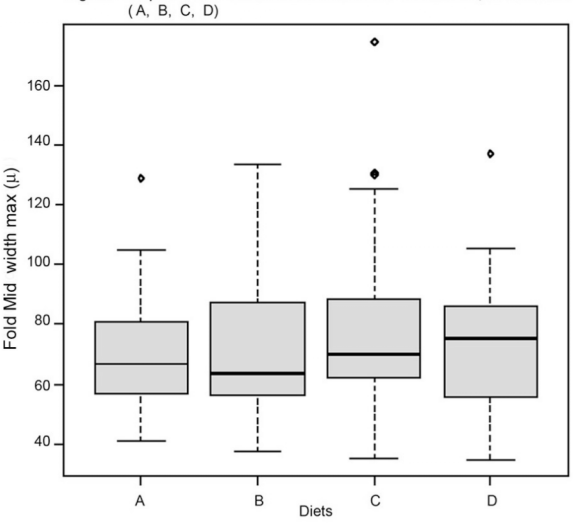


Fig.3.28 Boxplot of folds LP width at Mid level, for each diet (A, B, C, D)

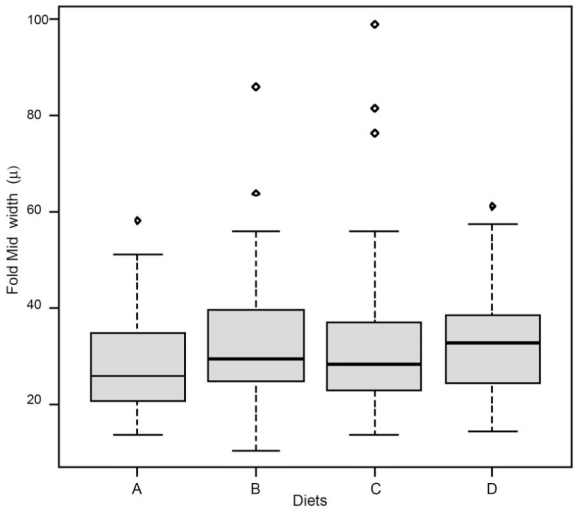


Fig.3.29 Boxplot of epithelium width at Mid level, for each diet (A, B, C, D)

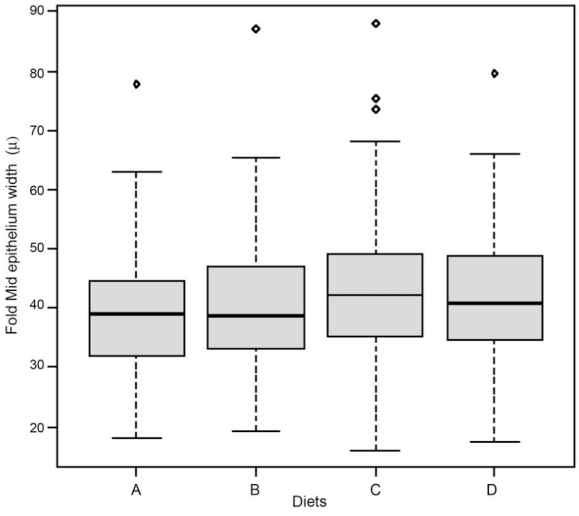


Fig.3.30 Boxplot of folds width max measurements at Mid level, for each diet (A, B, C, D)

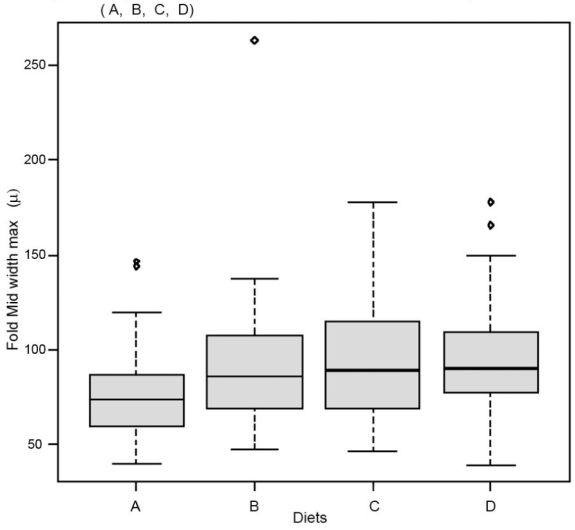


Fig.3.31 Boxplot of epithelium width at Down level, for each diet (A, B, C, D)

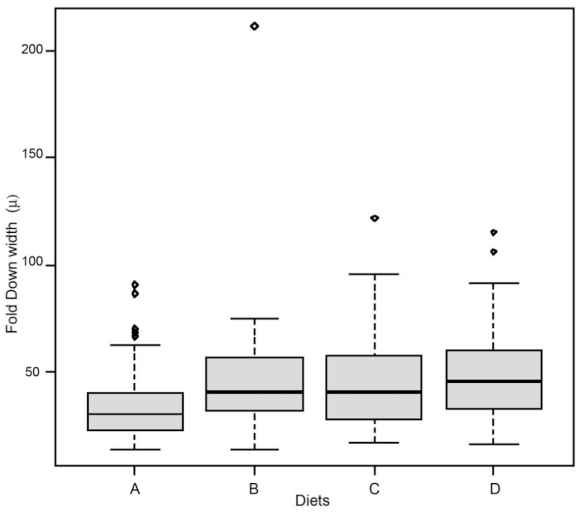


Fig.3.32 Boxplot of epithelium width at Down level, for each diet (A, B, C, D)

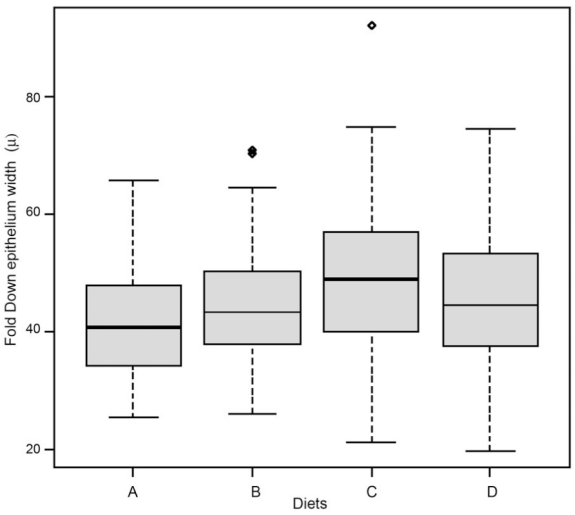
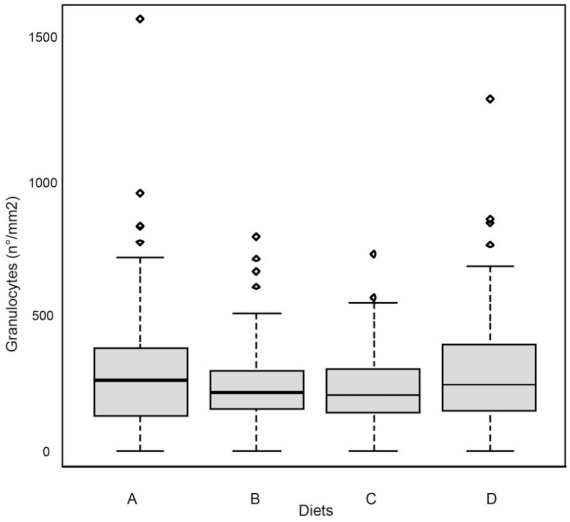


Fig.3.33 Boxplot of granulocytes concentration, for each diet (A, B, C, D)



3.5 Microbial culture from fish gut

Homogenates of distal intestine (hindgut) were diluted (1:9) in sterile BHI (Brain Heart Infusion) broth were spread and then, immediately or after 24 hours incubation in BHI at 20°C onto other different bacterial growth mediums. The microbial culture results (at three different time), were reported below:

Time 0 days (test beginning)

On five pools of fishes (each one 9 fishes), we evaluated the presence or absence of different bacterial strains and when it was possible, we counted the UFC (colony forming unit); such pools were considered representative of population present in each tank at the beginning of the feeding trials:

- *Aeromonas spp* = present in all 5 pools
- Enterobacteriaceae = five UFC count (UFC<10; UFC=1,5x10^3; UFC8,3x10^3; UFC=5x10^3; UFC=3x10^3).
- Faecal streptococci = all counts UFC <10
- Lactobacillaceae = all counts UFC <10
- *Pseudomonas spp* = present in all 5 pools
  Pseudomonas fluorescens = present in 2 of the 5 pools
- Vibrio spp = absent in all 5 pools
  Photobacterium damsela = present in 2 of the 5 pools

Time 30 days

We took a pool of 5 fishes per tank (A0 - A1; B0 - B1; C0 - C1; D0 - D1) for each diet. we evaluated the presence or absence of different bacterial strains and when it was possible, we counted the UFC (colony forming unit) or calculated the MPN/g; the latter is a method to estimate of the "Most Probable Number" of specific bacteria present per gram of gut tract sampled.

Diet A:

- *Aeromonas spp* = present in each tank
  Aeromonas hidrophila = present in each tank
- Enterobacteriaceae = UFC <1 in each tank
- Faecal streptococci = 0,36 MPN/g in tank A0 and 4,3 MPN/g in tank A1
- Lactobacillaceae = UFC <1 in each tank
- *Pseudomonas spp* = UFC <1 in each tank

- *Vibrio spp* = present in each tank
  Vibrio alginolyticus = present in each tank

Diet B:

- *Aeromonas spp* = present in each tank
  Aeromonas hidrophila = present in each tank
- Enterobacteriaceae = UFC <1 in each tank
- Faecal streptococci = 0,92 MPN/g in tank B0 and 2,3 MPN/g in tank B1
- Lactobacillaceae = UFC <1 in each tank
- *Pseudomonas spp* = UFC <1 in each tank
- *Vibrio spp* = present in each tank
  Vibrio alginolyticus = present in each tank
  Vibrio parahaemolyticus = present in tank B1

Diet C:

- *Aeromonas spp* = present in each tank
  Aeromonas hidrophila = present in each tank
- Enterobacteriaceae = UFC <1 in each tank
- Faecal streptococci = 0,92 MPN/g in tank C0 and 0,36 MPN/g in tank C1
- Lactobacillaceae = UFC <1 in each tank
- *Pseudomonas spp* = UFC <1 in each tank
- *Vibrio spp* = present in each tank
  Vibrio alginolyticus = present in each tank
  Vibrio parahaemolyticus = present in tank C0

Diet D:

- *Aeromonas spp* = present in each tank
  Aeromonas hidrophila = present in each tank
- Enterobacteriaceae = UFC <1 in each tank
- Faecal streptococci = 1,5 MPN/g in tank D0 and 0,74 MPN/g in tank D1
- Lactobacillaceae = UFC <1 in each tank
- *Pseudomonas spp* = UFC <1 in each tank
- *Vibio spp* = present in each tank
  Vibrio alginolyticus = present in each tank

Time 90 days (end of test)

We taken a pool of 5 fishes per tank (A0 - A1; B0 - B1; C0 - C1; D0 - D1) for each diet. we evaluated the presence or absence of different bacterial strains and when it was possible, we counted the UFC (colony forming unit) or calculated the MPN/g; the latter is a method to estimate of the "Most Probable Number" of specific bacteria present per gram of gut tract sampled.

Diet A:

- *Aeromonas spp* = absent in each tank
- Enterobacteriaceae = present in each tank
- Faecal streptococci = 0,92 MPN/g in tank A0 and 0,36 MPN/g in tank A1
- Lactobacillaceae = absent in each tank
- *Pseudomonas spp* = *present* in each tank
  Pseudomonas fluorescens = present in tank A0
- *Vibrio spp* = present in each tank

Diet B:

- *Aeromonas spp* = absent in each tank
- Enterobacteriaceae = present in each tank
- Faecal streptococci = 0,92 MPN/g in tank B0 and 0,92 MPN/g in tank B1
- Lactobacillaceae = absent in each tank
- *Pseudomonas spp*
  Pseudomonas fluorescens = present in each tank
- *Vibrio spp* = present in each tank

Diet C:

- *Aeromonas spp* = absent in each tank
- Enterobacteriaceae = present in each tank
- Faecal streptococci = 0,92 MPN/g in tank C0 and 0,92 MPN/g in tank C1
- Lactobacillaceae = absent in each tank
- *Pseudomonas spp*
  Pseudomonas fluorescens = present in each tank
- *Vibrio spp* = present in each tank

Diet D:

- *Aeromonas spp* = absent in each tank
- Enterobacteriaceae = absent in each tank
- Faecal streptococci = 0,36 MPN/g in tank D0 and 0,36 MPN/g in tank D1
- Lactobacillaceae = absent in each tank
- *Pseudomonas spp*

  Pseudomonas fluorescens = present in each tank
- *Vibrio spp* = present in each tank

  Vibrio alginolyticus = present in tank D1

  Vibrio parahaemolyticus = present in tank D1

The result of microbial cultures from distal intestine, were summarized in table 3.15.

Tab.3.15 Distal intestine bacterial cultures. The results were expressed, for single bacterial strain as: presence/absence or Colony Forming Unit (CFU) or Most Probable Number per gram (MPN/g). For each bacteria genus, when isolated, we reported also the specie.

bacteria	time day 0	times day 30				times day 90			
	initial Pool	diets				diets			
		A	B	C	D	A	B	C	D
Aeromonas spp	**present**	**present** *A.hidrophila*	**present** *A.hidrophila*	**present** *A.hidrophila*	**present** *A.hidrophila*	**absent**	**absent**	**absent**	**absent**
Enterobacteriaceae	CFU<9x10^3	CFU <1	CFU <1	CFU <1	CFU <1	**present**	**present**	**present**	**present**
Enterobacteriaceae	CFU<10	0,36 MPN/g 4,3 MPN/g	0,92 MPN/g 2,3 MPN/g	0,92 MPN/g 0,36 MPN/g	1,5 MPN/g 0,74 MPN/g	0,92 MPN/g 0,36 MPN/g	0,92 MPN/g 0,92 MPN/g	0,36 MPN/g 0,36 MPN/g	0,36 MPN/g 0,36 MPN/g
Lactobacillaceae	CFU<10	CFU<10	CFU<10	CFU<10	CFU<10	**absent**	**absent**	**absent**	**absent**
Pseudomonas spp	**Present** *P.fluorescens*	CFU <1	CFU <1	CFU <1	CFU <1	**present**	**present**	**present**	**present**
Vibrio spp	**Absent** *	**Present** *V.Alginolyticus*	**Present** *V.Alginolyticus* *V.parahaemolyticus*	**Present** *V.Alginolyticus* *V.parahaemolyticus*	**Present** *V.Alginolyticus*	**present**	**present**	**present**	**Present** *V.Alginolyticus* *V.parahaemolyticus*
	*Isolated *Photobacterium damsela*								

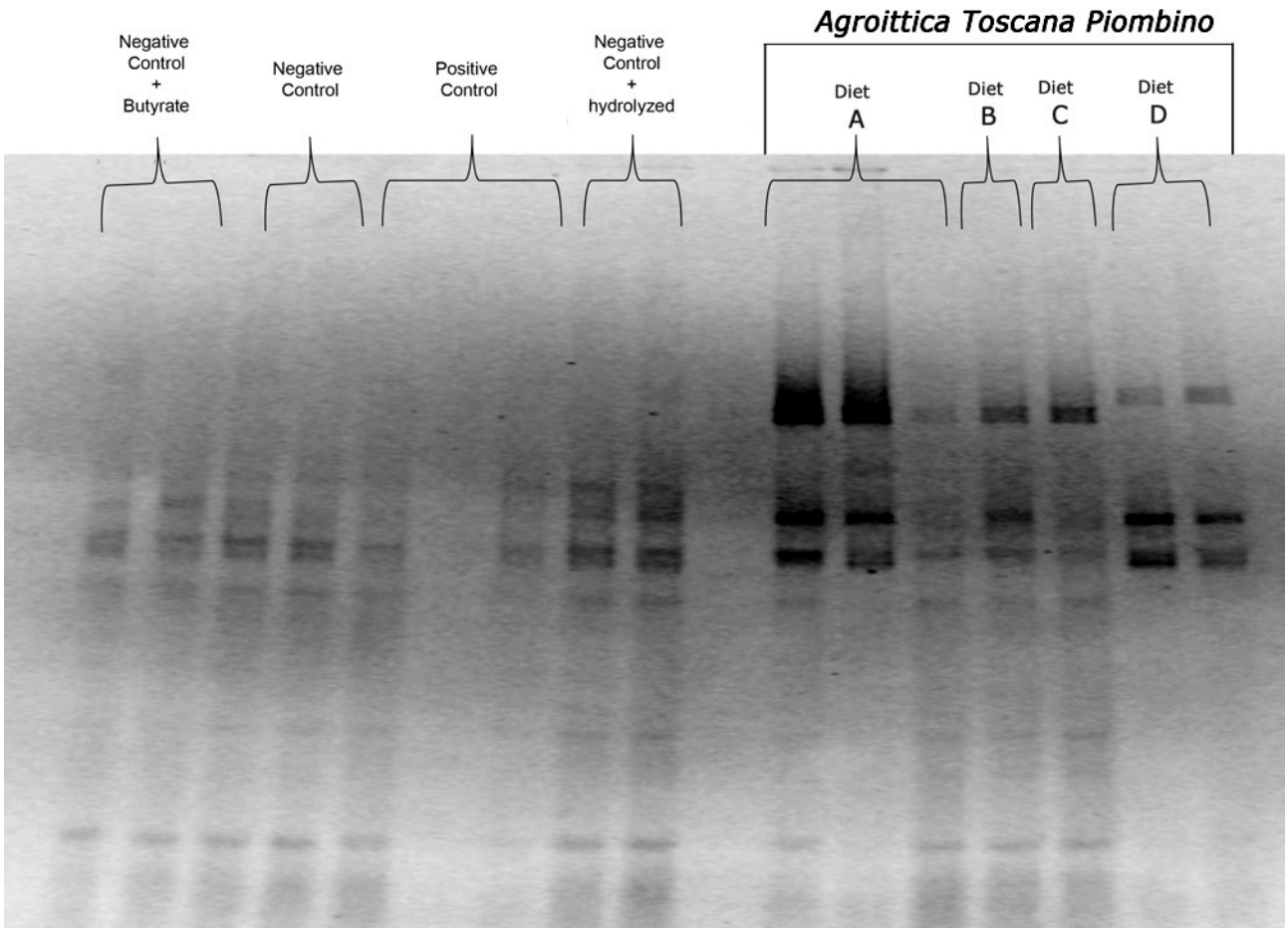
3.6 DNA isolation and PCR-denaturing gradient gel electrophoresis (DGGE)

Considering the fact that a large population of GI bacteria in fish is unculturable the total bacterial load is higher as recorded from the total culturable heterotrophic bacteria. Therefore in figure 3.32 we reported the preliminary results from DNA isolation and PCR-denaturing gradient gel electrophoresis (DGGE analysis) where we can see some difference between the gel bands that could confirm a microbiota biodiversity in fish gut fed by diets at different level of fishmeal replacement with protein plant source.

Beside the 4 feed (A, B, C, and D), samples from a laboratory experiment in which sea bass were fed with experimental feeds that were formulated either as the "D diet" alone or with addiction hydrolyzed shrimp protein or of Na-butyrate.

The figure 3.33 clearly shows differences in the microbiota composition among the different_diets, suggesting the possibility to manipulate gut microbiota biodiversity by tuning the diet composition.

Fig.3.33 DNA isolation and PCR-denaturing gradient gel electrophoresis (DGGE) for gut flora identification.



At the moment only a few bacterial strains were identified (Tab. 3.16) by National Center for Biotechnology Information (NCBI - GenBank). Among sequenced strains, two at least (Aliivibrio sp. and Vibrio sp.) with genes for butyrate synthesis have been found.

Tab.3.16 Bacterial strains isolated by DGGE in seabass gut

Bacteria	NCBI Acc. n°	Butyrate synthesis
Enterobacteriaceae bacterium SR5	JF495478.2	?
Aliivibrio sp.	FR744854.1	YES
Vibrio sp.	JF792689.1	YES
Uncultured spirochete	DQ340184.1	NO
Uncultured spirochete	DQ340184.1	NO
Photobacterium aquimaris	AB428877.1	?
Photobacterium aquimaris	AB428877.1	?
Photobacterium aquimaris	AB428877.1	?

3.7 Molecular Biomarkers (mRNA levels with Real-Time PCR method)

With regard the molecular biomarkers, we report, for each diet, time 0 and day 40 values (Tab. 3.17). Moreover table 3.18 shows the "Condition factor" (K=body weight x 100/standard body length3) recorded also at days 90 and 140 (after 50 days of recovering period) but biomarkers are still under analysis. "Condiction factor" showed a significant difference in fish diet D fed at day 40 with lower value than fish fed other diets; at day 90 (end of test) there wasn't significant difference between the four diets, whereas at the end of recovering period fish from diet A and B showed "condition factor" values significantly higher than fish from C and D diets. There wasn't significant difference between fish fed diets A and B as well as fed diets C and D.

Tab. 3.17 Biomarkers value (mRNA copy n°/100 ng RNA) recorded at the beginning of test (T0) and day 40 for each diet (A, B, C, D) in several body districts: PepT1 (oligopeptide transporter) in proximal and distal intestine, SLC6A19 (intestinal neutral amino acids transporter) in proximal and distal intestine; Myosin (muscular protein) as growth indicator in muscle; IGF1 (Insulin-like Growth Factor 1) in liver; Dicentracine (antimicrobial peptide) as Innate Immunity indicator in kidney.

	Beginning	Time: 40 days fed diets			
BIOMARKERS	T0	A	B	C	D
	(mRNA copy n°/100g RNA)				
PepT1 (Prox Int.)	$1.27x10^8$ ± $2.84x10^7$	$8.45x10^7$ ± $5.85x10^7$	$1.80x10^8$ ± $3.63x10^7$	$1.38x10^8$ ± $5.81x10^7$	$1.38x10^8$ ± $7.82x10^7$
PepT1 (Distal Int.)	$6.37x10^5$ ± $1.93x10^5$	$3.44x10^5$ ± $1.40x10^5$	$3.90x10^5$ ± $1.44x10^5$	$4.75x10^5$ ± $1.38x10^5$	$5.68x10^5$ ± $2.16x10^5$
SLC6A-19 (Prox Int.)	$5.57x10^6$ ± $1.42x10^6$	$2.44x10^6$ ± $1.89x10^6$	$4.84x10^6$ ± $7.40x10^6$	$3.40x10^6$ ± $1.71x10^6$	$3.31x10^6$ ± $1.76x10^6$
SLC6A-19 (Distal Int.)	$1.54x10^7$ ± $8.78x10^6$	$6.91x10^6$ ± $4.27x10^6$	$6.76x10^6$ ± $5.27x10^6$	$5.69x10^6$ ± $3.42\ x10^6$	$4.18x10^6$ ± $4.42\ x10^6$
Myosin (Muscle)	n.r.	$7.05\ x10^9$ ± $3.72x10^9$	$1.23\ x10^{10}$ ± $4.62x10^9$	$1.25\ x10^{10}$ ± $3.32x10^9$	$9.27\ x10^9$ ± $3.99x10^9$
IGF1 (Liver)	$5.60x10^7$ ± $2.16x10^7$	$4.53x10^7$ ± $1.90x10^7$	$6.26x10^7$ ± $8.54x10^6$	$3.98x10^7$ ± $1.49x10^7$	$5.47\ x10^7$ ± $2.16x10^7$
Dicentracine (Kidney)	n.r.	$4.91x10^5$ ± $1.01x10^5$	$2.89x10^5$ ± $1.06x10^5$	$2.48x10^5$ ± $4{,}33x10^4$	$3.19\ x10^5$ ± $1.09x10^5$

Tab.3.18 Condition factor (K=body weight x 100/standard body length3) recorded for fish fed diet A. B. C and D at days 0. 40. 90 and 140 (after 50 days of recovering period).

	TEST			**RECOVERING**
Diet	**Day 0**	**Day 40**	**Day 90**	**Day 140**
Diet A	1.94 ± 0.25	1.86 ± 0.12	2.27 ± 0.27	2.31 ± 0.46*
Diet B	1.94 ± 0.25	1.85 ± 0.14	2.23 ± 0.29	2.26 ± 0.16*
Diet C	1.94 ± 0.25	1.87 ± 0.14	2.23 ± 0.44	2.53 ± 0.20
Diet D	1.94 ± 0.25	1.63 ± 0.28*	2.19 ± 0.38	2.43 ± 0.33
	P<0.05	* significant difference	No significant difference	* significant difference

PepT1 showed significant difference comparing proximal and distal intestine: at time 0 (diet supplied from Agroittica Toscana) pepT1 level (expressed in mRNA copy n°/100g RNA) was clearly higher ($1.27x10^8 \pm 2.84x10^7$) in proximal tract than distal one ($6.37x10^5 \pm 1.93x10^5$).

Although there is no significant difference between diets A, B, C and D, inside of the same gut tract, PepT1, for such diets, always showed values lower than T0 ones (Fig. 3.34), in proximal intestine (dietA=$8.45x10^7 \pm 5.85x10^7$, dietB=$1.80x10^8 \pm 3.63x10^7$, dietC=$1.38x10^8 \pm 5.81x10^7$, dietD=$1.38x10^8 \pm 7.82x10^7$) as well as in distal intestine (dietA=$6.37x10^5 \pm 1.93x10^5$, diet B=$3.90x10^5 \pm 1.44x10^5$, dietC=$4.75x10^5 \pm 1.38x10^5$, dietD=$5.68x10^5 \pm 2.16x10^5$).

SLC6A-19 values, in proximal intestine (Fig. 3.35), for diets A, C and D (respectively: $2.44x10^6 \pm 1.89x10^6$, $3.40x10^6 \pm 1.71x10^6$, $3.31x10^6 \pm 1.76x10^6$) were significantly lower than T0 level ($5.57x10^6 \pm 1.42x10^6$); there are no significant differences between the three diets. Diet B ($4.84x10^6 \pm 7.40x10^6$) showed the highest value between each diet although there is no significantly difference with T0 as well as diets C and D. In distal intestine, SLC6A-19 showed high variability and no significant differences between each measurement (T0=$1.54x10^7 \pm 8.78x10^6$, diet A=$6.91x10^6 \pm 4.27x10^6$, diet B=$6.76x10^6 \pm 5.27x10^6$, diet C=$5.69x10^6 \pm 3.42\ x10^6$, dietD=$4.18x10^6 \pm 4.42\ x10^6$).

Fig. 3.34 Intestinal oligopeptide transporter (PepT1) in proximal and distal intestine at day 0 and day 40 for each diet (A. B. C. D)

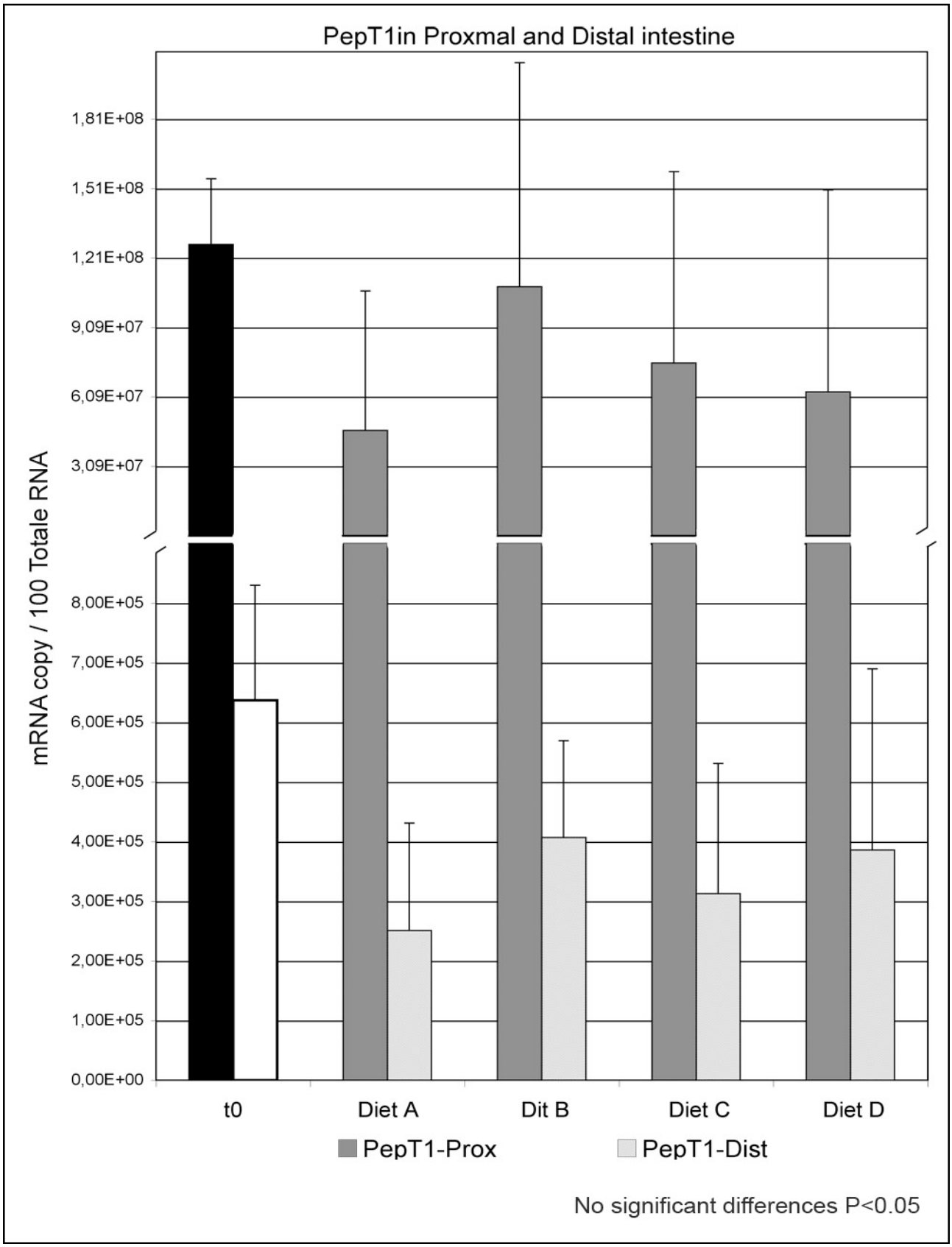
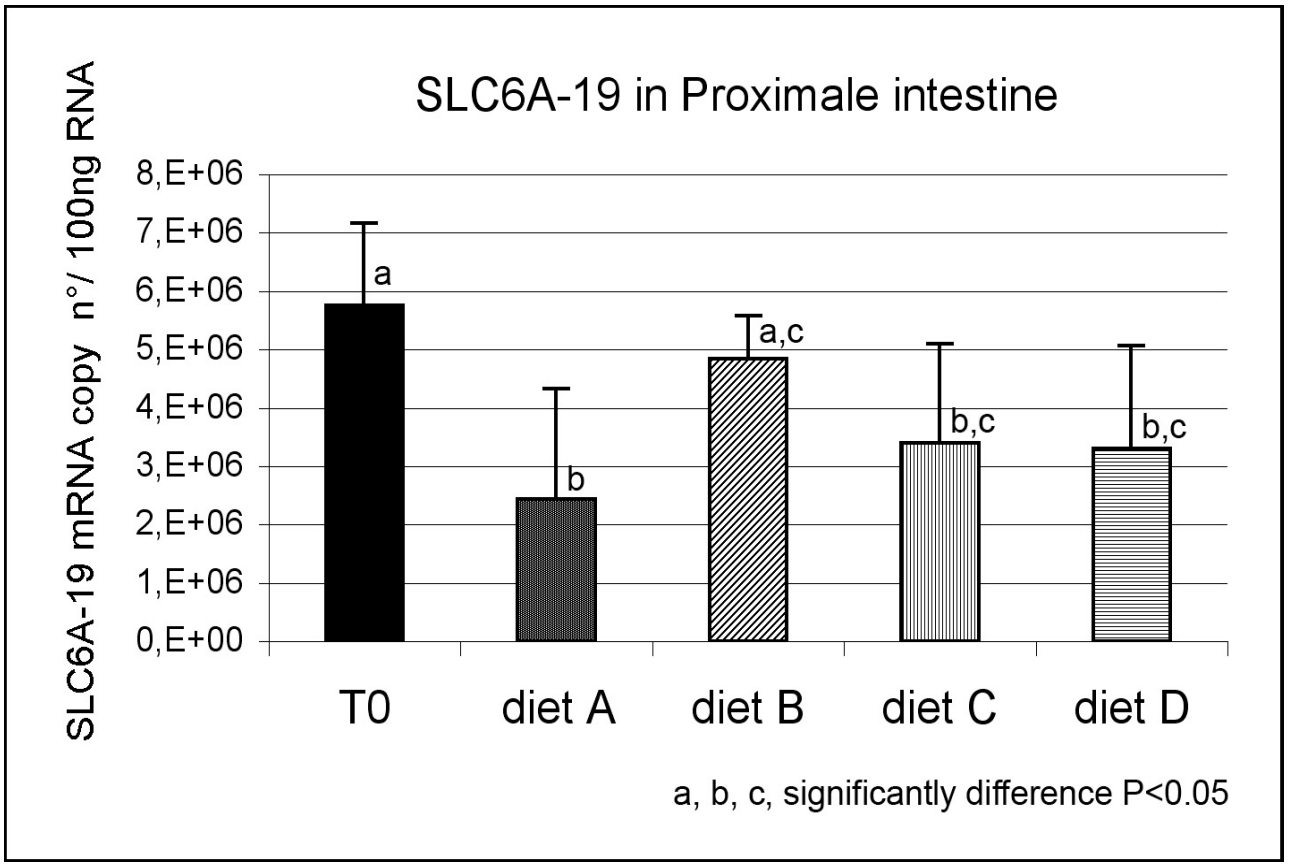


Fig. 3.35 Neutral amino acids transporter (SLC6A-19) in proximal intestine at day 0 and day 40. for each diet (A. B. C. D)



We compared also intestinal oligopeptide transporter and neutral aminoacids transporter, in proximal intestine with protein concentrate (PC) in the feed.

PepT1 (Fig. 3.36) appeared higher at T0 than by every diet at day 40, although there were not significant differences between all measurements; fish fed diet A (high source of oligopeptide) showed highest value. SLC6A-19 (Fig. 3.37) value was significantly higher at the beginning (TO) than in fish fed by other diets; diet A showed lowest value although there were not significant difference with other tested diets.

Myosin (muscular protein) at day 40 into muscle, as growth indicator, showed a value range among 7.05 x109±3.72x109 and 1.25 x1010±3.32x109 without significant differences between the diets (Fig. 3.38).

Also the Insulin-like Growth Factor 1(IGF1) did not show any significant difference between the diets at T0 and after day 40 (Fig. 3.39); recorded values were: T0=$5.60x10^7$±$2.16x10^7$, dietA=$4.53x10^7$±$1.90x10^7$, dietB=$6.26x10^7$±$8.54x10^6$, dietC=$3.98x10^7$±$1.49x10^7$ and dietD=$5.47\ x10^7$±$2.16x10^7$.

Fig. 3.36 Intestinal oligopeptide transporter (PepT1) in proximal intestine vs Protein Concentrate (PC) at day 0 and day 40. for each diet (A. B. C. D)

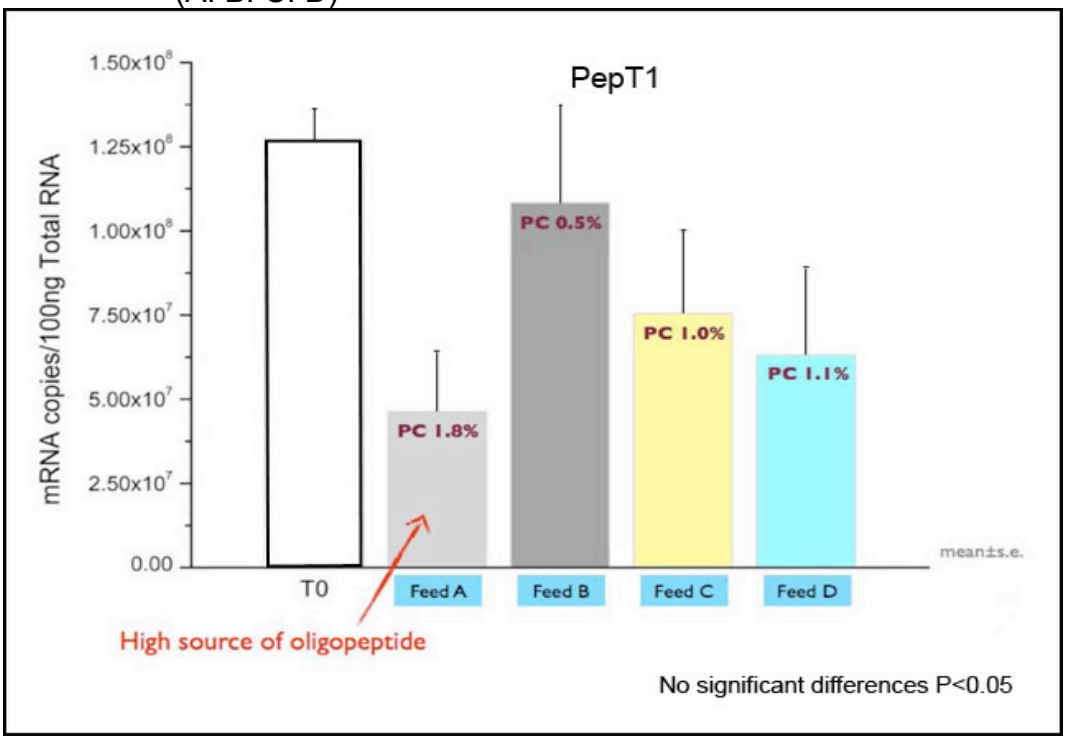


Fig. 3.37 Neutral aminoacids transporter (SLC6A-19) in proximal intestine vs Protein Concentrate (PC) at day 0 and day 40. for each diet (A. B. C. D)

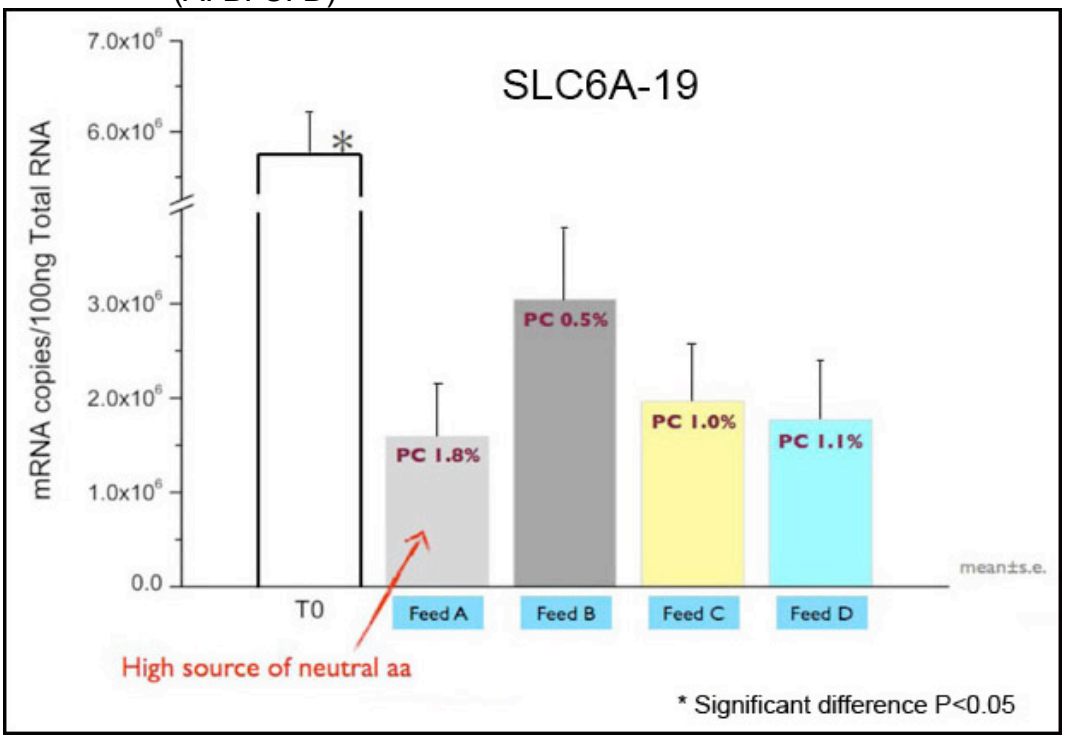


Fig. 3.38 Myosin (muscular protein) in the muscle for each diet (A. B. C. D) at day 40.

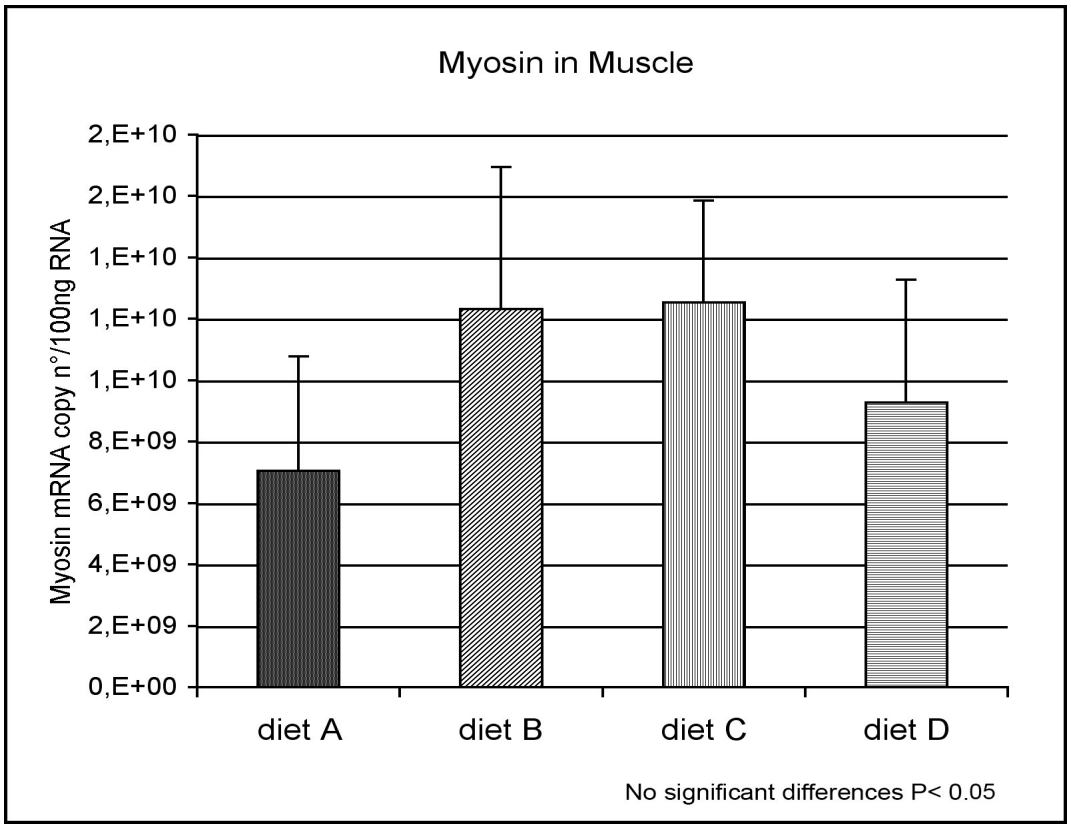
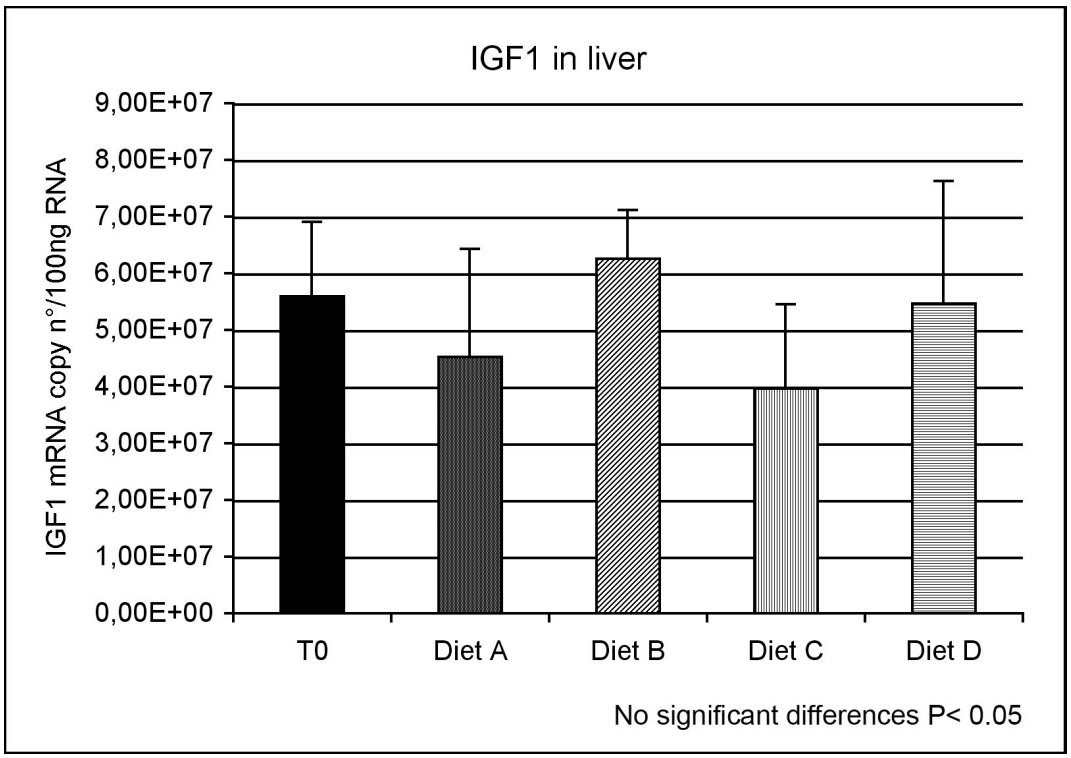
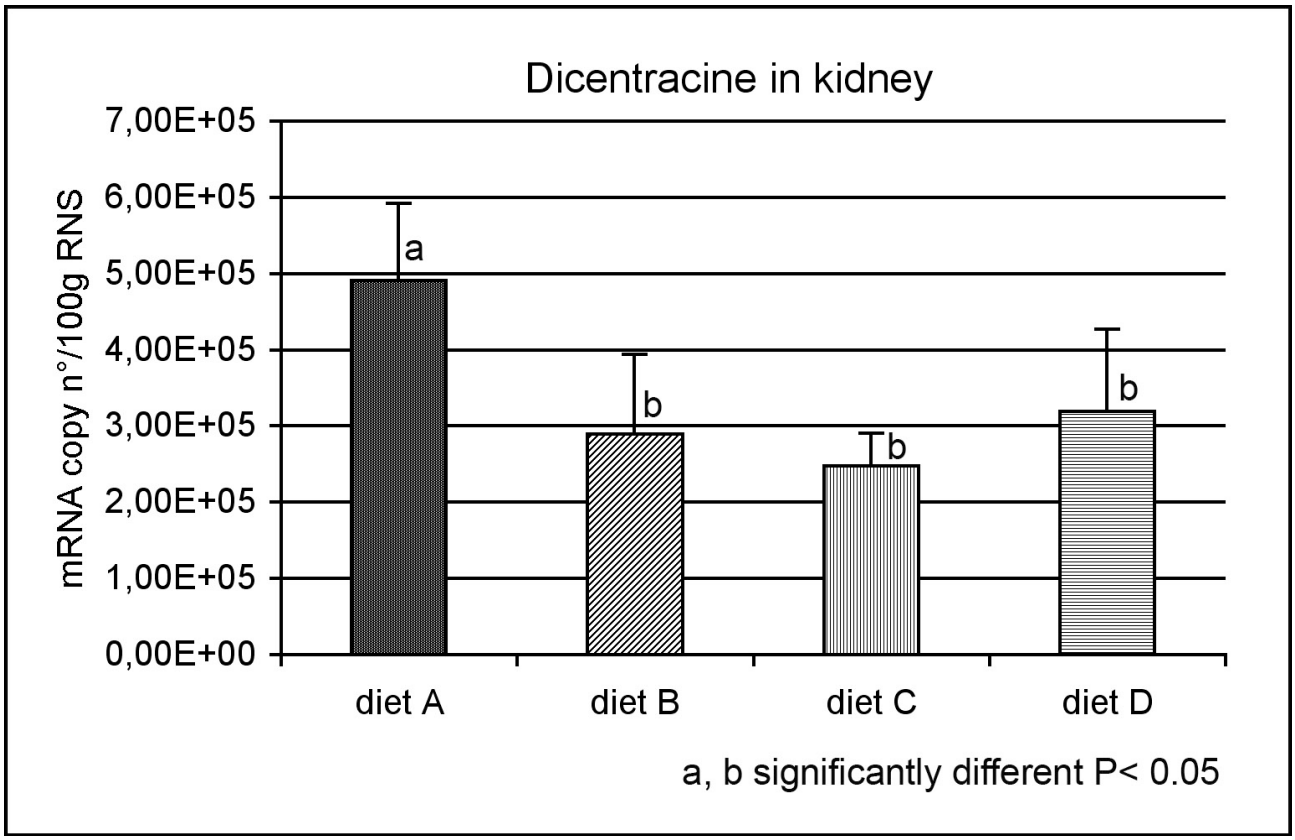


Fig. 3.39 Insulin-like Growth Factor 1(IGF1) into the liver. for each diet (A. B. C. D) at day 40.



Fish fed diet A (Fig. 3.40), at day 40, showed significantly higher level ($4.91x10^5 \pm 1.01x10^5$) of dicentracina (antimicrobial peptide) into kidney whereas there were not significant difference between fish fed diets B ($2.89x10^5 \pm 1.06x10^5$), C ($2.48x10^5 \pm 4{,}33x10^4$) and D ($3.19\ x10^5 \pm 1.09x10^5$).

Fig. 3.40 Dicentracine (antimicrobial peptide) in the kidney. for each diet (A. B. C. D) at day 40.



4.0 Discussion

Fish fed for 90 days with diets A, B, C and D increased their weight significantly with respect to the beginning of the trial. However diet D performed worst than the other three ones.

Some studies with rainbow trout, as well as European seabass, have suggested that the major problem connected with poor growth of fish fed fish meal-free, plant protein- based diets is caused by poor feed intake (Gomes et al., 1995; Dias, 1999). In European seabass fed diets containing very high levels of single protein sources such as soy protein concentrate or corn gluten meal, there was a decrease in voluntary feed intake (VFI), which was improved by supplementation with an attractant mix (Dias et al., 1997).

In our trial, operators in charge of daily ratio administration noted that fishes fed diet D (the poorest ones) showed a lazy predatory behaviour throughout the test, while other diets palatability, especially in the early stages of administration, increased with increasing fishmeal content.

Other data indicate that when the same protein sources replaced about 60% of fish meal, adequately supplemented with limiting amino acids such as lysine or methionine, there was no need for an attractant mix such as squid extract (Tibaldi et al., 1999).

The diet D lack in growing was more clear during the first 40 days. We could relate the growth gap with the feeding procedure (twice a day until apparent satiety): it is reasonable to assume that a high level of protein from plant source reduced the feed palatability, therefore the feed intake will be lower than administered, this resulting in feed waste. To support this hypothesis, we can see the FCR value, which, for diet D, was very higher (more than 35%) in respect to the other diets.

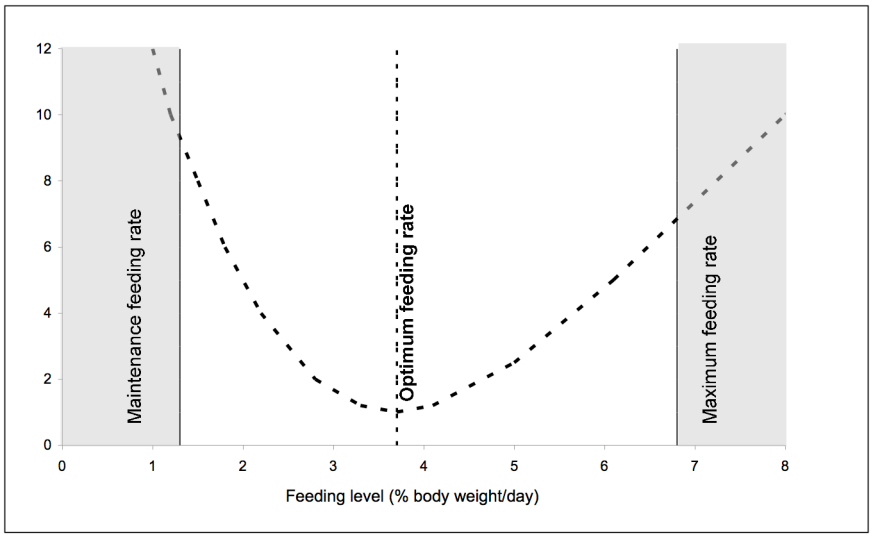
It is possible to hypothesize an adaptation of the animals to new food also due to hyperphagia. A sort of adaptation was confirmed by FCR, which, during the period comprised between day 40 and 90, showed similar values for each diet without significant differences. Consequently, at day 90, diet D global gap was reduced to 20%. However the better FCR values at the end of trial was shown from diets B and C (1.07 both), whereas diets A and D showed values of about 10% higher.

During 50 days of recovering, when all fish received diet A, the weight of fish previously fed diet D, remained the lowest, but the gap still decreased.

By assuming that the FRC increase and growth lack showed by fish fed diet D could be due to reduced palatability with consequent undernourishment, we can also assume that the higher diet A palatability (the highest fishmeal levels) might lead to overfeeding and

worsening conversion rate (Fig.4.1)

Fig.4.1 Theoretical relationship between Food Conversion Rate (FCR) and feeding rate in fish (from de Silva and Anderson, 1995, modified)



Optimal dietary protein concentrations for fish are requested by a delicate balance between the dietary protein-to-energy ratio, plus protein quality (amino acid balance), and non-protein energy sources (i.e., amount of fat in relation to carbohydrates) (Millikin, 1982). Fish consume protein for growth, reproduction, and other normal physiological function, they obtain, by protein digestion, amino acids, which are absorbed from the intestine tract and used as building blocks for protein synthesis. In addition, amino acids serve as precursors of non-protein nitrogenous high molecular weight compounds (such as nucleic acid, porphyrine and creatine), and substrates for energy. Fish require a well-balanced mixture of nonessential and essential amino acids though they do not have a true protein requirement: fish need the same 10 essential amino acids as warm-blooded animals (Wilson and Halver, 1986). Inadequate protein content in the diet results in a reduction in growth and loss of weight. When excess protein is supplied in the diet, only a fraction is used for protein synthesis, and the remainder is converted into energy.

The digestion is described as the mechanical and chemical breakdown of food into metabolisable parts, which can be used by the organism and in such mechanism, pepsin plays a pre-eminent role in the proteins digestion. Pepsin is secreted as an inactive zymogen, called pepsinogen, and its conversion into the active form is catalyzed by the action of the acid. The “stomach acid” working in all monogastric animals is hydrochloric

acid, which is able to lower the pH in the stomach to levels between pH 1-3. Like every enzyme, pepsin has certain optimal conditions in which it is working best. The optimal pH for pepsin activity is 2.0. At higher pH-levels the activity is severely reduced (Lückstädt, 2012). The reduction of pH in the feed and stomach largely depends on the buffering capacity of feed ingredients. Animal protein (e.g. fishmeal), extensively used in aquaculture diets, has a 15 fold higher buffering capacity compared to cereals (Bucking and Wood, 2009). Therefore high levels of protein from plant source should contribute to maintain low pH increasing proteins digestion (Marquez et al., 2013). Obviously we have to ensure to match diet composition (essential amino acids and other specific elements) with fish needs. With regard to protein requirements, significant interspecies differences appear to exist in fish. These variations can be attributed to a number of factors such as differences in basal diet composition, size and age of fish, genetic difference, and feeding rate (Fournier et al., 2002).

Also the use of organic acids (Short Chain Fatty Acids, SCFA) or acid salts as feed additives, studied in numerous publications over the past half-century in animal nutrition (Cole et al., 1968; Suryanarayana, 2012), can reduces the pH in the stomach, improving protein digestion by increasing the rate of proteolysis of large protein molecules.

Given the increase in aquaculture production and the implications of poor protein utilization on nitrogenous losses in effluents, there is an increasing need to optimize the supply of protein and nonessential amino acids. Thus, studies have focused on understanding the mechanism underlying protein digestion and absorption in fish. Cellular transport of amino acids and small peptides is a crucial in assimilation by the intestine. Furthermore, intestinal peptide transport is of major nutritional significance because many amino acids are more rapidly and efficiently absorbed in peptide form (Gilbert et al., 2008).

Our anatomical investigation showed some increase of perivisceral fat (possible overfeeding indicator) in fishes fed diet A, although viscerosomatic index did not showed significant differences between all diets and during the test time.

The highest fat level in the liver of fishes fed diets A and C could be confirmed by hepatosomatic index value, which is higher than in fish fed diets B and D. With diet A (highest palatability), the fishes can increase their metabolism with consequent liver enlargement (hypertrophy); Although it did not find reflected in literature, we could hypothesize a relationship between hepatocytes hypertrophy and haemoglobin presence in diet C.

The hepatocytes hypertrophy in fishes fed diet A, could be confirmed by hepatocytes

concentration per square millimeter: the number of hepatocytes per square millimeter lower than other diets could be due to an increased volume of cells.

During the recovering period (all fish fed diet A), animals previously fed diets A and B increased their weight with respect to those, which were previously fed diets C and D. We have to emphasize the recover of fish previously fed diet D; indeed, such fishes did not show significant difference in mean weight with respect to those previously fed diet C.

Observing the growth performances, we could say that diets A - B and C are more suitable for intensive aquaculture than diet D, which lower growth performances produced.

We never detected specific, diets related diseases or noteworthy pathological lesions, therefore we confirm the viability for commercial aquaculture of all tested aquafeeds; the farmer will choose according to his productive program, also considering the aquafeed costs which will drop as protein plant source increases. In every case we would like to underscore that it is possible to reduce the fishmeal quantity at values near to 20% without significant reduction of growth performances. Moreover increasing fishmeal substitution by vegetable protein could reduce aquafeed buffering capacity, consequently we might expect an improving protein digestion with FCR reduction. We will need further studies to asses the undigested large protein molecules, that we might observe at the gut end in relationship to diet composition

The scoring assessment of histological changes showed a quick response to the diets administration (10 - 20 days after the beginning). The score increased or decreased with to respect the beginning (day 0), until day 40, thereafter for every diet the scoring tended to initial values.

Considering the fishmeal percentage in aquafeed as a quality index, the results of this study showed that fish fed diets with high level of fishmeal had a better score than those fed aquafeed with low level of fishmeal. We have to take in consideration that the score at day 0 was related to those standard aquafeed utilized in "Agroittica Toscana" farm. Therefore, as into above mentioned, diet A quality in our study is better than the other diets; diet D would be the worst one while diets B and C showed a quality similar to the standard diet used in the farm.

As previously highlighted, the supranuclear vacuoles presence or absence inside enterocytes is conditioned by many factors, such as extended fasting, pathologic syndromes or specific diseases that could give enteritis.

Our results showed that in sea bass the vacuoles were not in close relationship with diets: therefore, in scoring, we took into account only the other three parameters (Lamina

Propria of single folds; Connective Tissue; Mucosal Folds).

It is possible that the seabass rearing temperature which is higher than that of the salmonids one, can influence the increasing of metabolism therefore vacuoles are quickly absorbed. Actually, in the majority of cases, the supranuclear vacuoles were absent (score value =5), with an inverse trend respect to the other parameters, which almost always showed values close to the normality range. However all recorded histological changes were reversible, this supporting the viability of each tested diet in commercial aquaculture.

We saw a rapid scoring change, started about 10 days after a new diet administration and lasted more than a month; the same thing was detected even with diet A during recovering period.

The graphs in figure 3.20 shows a fluctuation in the score index (IS%) during the first month, that could be interpreted as a sort of adaptive response to the new feed, followed by a steady state. Also to diet D, which had the highest level of fishmeal substitution with plant sources, shows such a fluctuation followed by a trend of recovering. One may wander how such histological dynamics follows the intestinal microbiota evolution as an "holistic adaptation" of fish to the diets, as a secondary effect resulting to faster dynamics of bacterial communities modifications, into the different gut tracts.

In any case it would possible to use the scoring as gut function index or, in other words, as "welfare index" where low score means better diet, and high score means worst diet. Then fish showed an adaptation to diet change.

Such adaptation capacity could be another animal welfare index that may be interpreted as a good allostatic resource (Korte et al., 2007 review). For example, a healthy population will give a quick scoring feedback to diet changes and, during about three months, it will reveal its adaptation capacity with a trend to return to the initial score. Diet changes could cause stress, and healthy animals will react with their physiological compensation capacity.

Although we did not saw high scoring level during all tests, except for supranuclear vacuoles as explained above, if we will use a diet not properly balanced or containing pro-inflammatory compounds we should find a high score and see real histological lesions (not simple changes), up to acclaimed pathology (i.e. severe enteritis). For instance IL-16 is a chemoattractant cytokine with various effects on cellular activities and diseases (Wang et al., 2013). The authors administered dextran sodium sulphate to induce colitis in *Tetraodon nigroviridis*, used as fish model, to study inflammatory bowel disease (IBD). In this species revealed that IL-16 levels significantly increase accompanied by elevations in

PepT1 in the colon. They saw remarkable intestinal inflammation with typical ulcerative colitis–like features, including histological damage, inflammatory cell infiltration. Also in that case the severity of the intestinal inflammation was determined based on histopathological scoring applied in a blinded manner.

Genetic, environmental factors, dysregulation of immune system, intestinal microbes and oxidative stress in the human specie are the most important factors that play the role in the pathogenesis of inflammatory bowel disease (IBD) (Moeinian et al., 2013).

As previous studies showed (Nikfar et al., 2008; Segain el al., 2000), butyrate, L-Carnitine, and probiotics have the potential to control inflammation by reduction of main inflammatory cytokines, the nuclear factor-kappa B (NF-kB) and tumor necrosis factor-a (TNF-a).

Butyric acid is a main end-product of anaerobic bacterial fermentation of carbohydrates in the rumen or fore stomach animal species and in the colon of omnivorous species, including humans. Among the so-called Short Chain Fatty Acids (SCFA), butyrate has received particular attention for its multiple beneficial effects from the intestinal tract to the peripheral tissues. A great number of studies have clarified the effects of butyrate in the intestine of animals and humans. It plays a regulatory role in the transepithelial fluid transport, promoting sodium and water absorption (Bond and Levitt, 1976), ameliorates mucosal inflammation and oxidative status, reinforces the epithelial defense barrier, modulates visceral sensitivity and intestinal motility (Berni Canani et al., 2011; Scheppach 1994) and also provides energy to colonocytes (Bergman, 1990). Butyrate is part of a well-known class of epigenetic substances known as histone deacetylase inhibitors (Berni Canani et al., 2012). When used as a feed additive, it also shows positive influence on body weight gain, feed utilization and composition of intestinal microflora, as well as trophic effects on the intestinal epithelium, through the increase in the villi length and crypt depth in growing pigs (Galfi and Bokori, 1990; Kotunia et al., 2004). Although such effects have been demonstrated in several species, very few studies have assessed them in fish.

Butyrate has been shown also to exert anti-inflammatory effects in several cell types as well as intestinal biopsy specimens (Tedelind et al., 2007).

In contrast with so far reported, Dalmasso et al. (2008) found that butyrate, both in vivo and in vitro, induced human PepT1 (hPepT1) expression/activity consequently there should be inflammatory effect. We can speculate that inflammatory action is due to proinflammatory bacterial peptides, which are transported by PepT1. Usually in mammal there is not PepT1 into colon where instead many strains of bacteria proinflammatory bacterial peptides producers are present. Also in fish we saw (Scollo et al., 2012) gut

inflammation when there was to high PepT1 level into of distal intestine tissue but the true anomaly was the PepT1 presence in that intestine tract, consequently the inflammation arise from bacterial tripeptides transport. It has been reported that PepT1 transports pro-inflammatory bacterial peptides, such as muramyl dipeptide (MDP) (Vavricka et al., 2004) or N-formyl-methionyl-leucylphenylalanine (fMLP) (Buyse et al., 2002), which participate in intestinal inflammation.

Within the small intestine, PepT1 has a differential pattern of expression. Along the vertical axis, PepT1 is most abundant at the villous tip and expression decreases towards the crypt (Ogihara et al., 1999). Along the longitudinal axis, the density of PepT1 increases from duodenum to ileum (Tanaka et al., 1998). PepT1 is generally not expressed in the esophagus, stomach or normal colon; however, hPepT1 expression has been observed in inflamed colon from patients with inflammatory bowel disease (IBD) (Merlin et al., 2001).

Taking in account that fish physiology, at regard PepT1, is not so far to that human, it should be normally expected PepT1, only in proximal and medial tract of fish gut (Terova et al., 2009), however it was recorded significant PepT1 level in distal intestine and recto concurrently to inflammation of that gut tract.

Therefore we could speculate that in physiological conditions, PepT1 should be present only into the first tract of intestine where normally the bacteria are few. Instead PepT1 abundance into distal intestine, where bacterial level is high, may represent an anomaly of bowel function. In this case PepT1 will transport also pro-inflammatory bacterial peptides, which participate in intestinal inflammation.

Moreover we should suppose that, if we add butyrate into the diet, this should give PepT1 up regulation (consequently increase peptides adsorption), but we should haven't PepT1 in distal intestine and recto.

In our results, oligopeptides transporters (PepT1) and neutral amino acids transporter (SLC6A19) seem to respond both a diet with high fishmeal inclusion and a different source of oligopeptides (i.e. soybean protein concentrate); they showed inverse relationship with increasing of protein concentrate into diets. It is reasonable to suppose a low need of transporters when the supply of digestible protein concentrate is high.

We did not find great differences, diet related, between the other molecular biomarkers: we did not observe any significant change concerning growth indicators (Myosin in muscle and IGF1 in liver), and this could further support the thesis that each diet was viable for commercial use; the innate immunity indicator (Dicentracine in kidney) would seem to show that high levels of fishmeal can increase the innate immunity, but diet A showed a

significantly greater value compared to each other diet only at day 40. However, the molecular biomarkers should be considered useful tools to support the assessment of animal welfare.

As expected, decreasing fishmeal inclusion and substituting it with vegetable protein source, fish growth decreased. This aspect was more evident with 68% of vegetable inclusion.

Although histological changes were reversible and all diets showed viability for commercial use, the scoring values showed a direct relationship with increase of protein plant source in the diet. This was mostly evident in the first period (10 - 40 days) after the feeding change where score worsening corresponds to high level of vegetable proteins.

It is noteworthy that histological changes, always, were more evident in fish fed lowest and highest vegetable inclusion (respectively 48% = high quality diet and 68% = low quality diet). Moreover histological changes began quickly after diet change with scoring increase or decrease according to higher or lower diet quality, compared to that previously administered. The scoring increased or decreased until days 40 then tended to come back to initial values (days 90). We can suppose that fish healthy are be able to adapt to diet sudden changes in about two months.

Scoring system set up it be itself a noteworthy result, in figure 4.2 we report the form to fill out for an histological assessment by our scoring system.

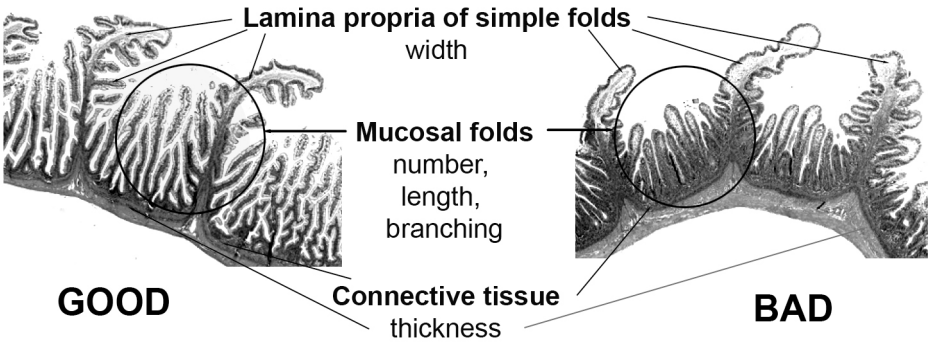
We tried also to take in account the relationship between diet and intestinal mibrobiota (Ringo and Olsen, 1999; Ringo et al., 2006; Silvi et al., 2008). Although our results are altogether preliminary and there is still no complete identification of the bacterial strains, the DGGE bands showed some clear difference between the diets to mean a relationship between diet composition and microbic biodiversity into gut.

It is crucial emphasize that the gut microbiota of fish constitutes a great number of cells and these cells and their metabolites play important roles in host digestive function, gastric development, mucosal tolerance, immunity and disease resistance. In fact, in the absence of gut microbiota the digestive tract fails to differentiate fully, lacks brush border intestinal alkaline phosphatase activity, presents immature patterns of glycan expression, displays reduced epithelial proliferation and a reduction of goblet and enteroendocrine cells (Rawls et al., 2004; Bates et al., 2006).

It is of utmost importance for the health of the animal that microbes that improve gut function and the immune apparatus dominate these microbial communities. Therefore, great efforts have been made to find dietary supplements that ensure that benign or

beneficial microbes dominate the gut microbiota of fish (Merrifield et al., 2010; Ringo et al., 2010; Dimitroglou et al., 2011).

Fig.4.2 Form for scoring system evaluations



ID sampling:	Date:		
Score Appearance (*)	Gut tract (**)	Score for single reading	
Lamina propria of simple folds 1 There is a very thin and delicate core of connective 2 The lamina propria appears slightly more distinct and robust in some of the folds. 3 There is a clear increase of lamina propria in most of the simple folds. 4 There is a thick lamina propria in many folds. 5 There is a very thick lamina propria in many folds.			
Connective tissue (between base of folds and stratum compactum) 1 There is a very thin layer of connective tissue between the base of folds and the stratum compactum. 2 There is a lightly increased amount of connective tissue beneath some of the mucusal folds. 3 There is a clear increase of connective tissue beneath most of the mucosal folds. 4 A thick layer of connective tissue is beneath many folds. 5 An extremely thick layer of connective tissue is beneath some folds.			
Mucosal folds 1 Simple and complex folds (CFs) appear long and thin. Thin side branches on the CF. 2 Simple mucosal folds have medium length. CFs are still long but appear thicker. 3 Simple folds have short to medium length. Side branches on CF are stubby. 4 Thick CFs are prevalent. Simple folds are short. Almost no side branches are on the CF. 5 Both complex and simple folds appear very stubby.			

(*) description corresponding to score. For each parameter a score of "1 - 2" represented normal morphology, while a score of "5" was given to morphological symptoms of severe enteritis.
(**) ID = Distal Intestine ; IM = Mid Intestine IP = Proximal Intestine

Our finds, within the few bacteria strains identified, showed that two (*Aliivibrio sp.* and *Vibrio sp.*) are able to synthesize butyrate which, as previously reported, plays a key role on body weight gain, feed utilization and on trophism of intestinal epithelium.

The gastrointestinal tract contributes to body health in many ways; of particular interest in this context is the emerging concept of "gut health" (Berni Canani et al., 2012) and the mechanisms that can explain it. “Gut health” is a term increasingly used in human medicine literature. It covers multiple positive aspects of the gastrointestinal tract, such as the absence of gastrointestinal illness, normal and stable intestinal microbiota, and effective immune status, that are also able to determine a state of well-being (Bischoff, 2011). There is now ample evidence that two functional entities are crucial to achieve and maintain "gut health" (Meddings, 2008; Groschwitz and Hogan, 2009). These entities are the intestinal microbiota and the intestinal barrier. Any impairment of the intestinal microbiota biodiversity, for example, deriving from an unbalanced diet such as a carbohydrate-rich diet, could affect gut functionality and consequently “gut health” (Sonnenburg, 2010).

Thus, "gut health" could offer a new approach for preventive medicine if we learn more about how to achieve and maintain it, for instance prebiotics and probiotics may support "gut health". In this context intestinal microflora biodiversity plays an important role in SCFAs production (Berni Canani et al., 2011).

In our case we focused attention on four "markers" to have a global picture of nutritional welfare and consequently of physiological efficiency, fish diet related: i) zoothechnical markers show a general picture on growth performance like FCR and SGR; ii) the scoring extent histological changes occurring with feed changing and their reversibility to initial score value gives an idea of adaptive capacity to new diet; iii) molecular biomarkers give information about cellular functioning until nuclear level, in particular we saw that PepT1 is produced according absorption needs then high levels of PepT1 could mean diets compound are not easily absorbable; moreover high presence of PepT1 in distal intestine could promote the transport of pro-inflammatory bacterial peptides produced by gut microbiota; iiii) microbiological markers allow to evaluate microbiota biodiversity in which in which may be to isolate probiotic bacteria able to produce some SCFAs, especially butyrate.

More recently, Licciardi et al. (2010) hypothesized that the epigenetic mechanisms elicited by probiotics through the production of SCFAs, especially butyrate, are the key to understand how they mediate their numerous health promoting effects from the gut to the

peripheral tissues. Butyrate could have a pivotal role in the context of "gut/body health". Its production is dependent on diet and intestinal microflora composition, but it should be also able, if administered, to modulate intestinal microflora through regulation of lumen pH and to exert many beneficial extraintestinal effects through epigenetic mechanisms.

Published data on the effect of butyrate on growth performance and intestinal metabolism in fish are limited. Owen et al. (2006) evaluated the addition of 0.2 % sodium butyrate to diets differing in their major protein source (fishmeal or defatted soya) provided to the tropical catfish (*Clarias gariepinus*). Both specific growth rates and food conversion rates measured in fish fed the fishmeal-based diet containing sodium butyrate were improved (by 4.7 and 4.1 %, respectively) when compared to those in fish fed a non supplemented diet or a defatted soya-supplemented diet.

In a recent study (Zheng, 2009), tilapia (*Oreochromis niloticus*) fingerlings showed significant increases in weight and feed efficiency when fed on a diet containing 0.05 % butyrate. Positive results were also obtained in this study with carp (Cyprinus carpio), being growth rate and protein efficiency increased in fish receiving a feed containing 0.1% sodium butyrate by 6.1 and 4.54%, respectively, when compared to the control group. De Wet and Viljoen (2012) also reported an enhancement in growth and feed efficiency resulting from the combination of butyrate and nucleotides in feeds for Oreochromis mossambicus. A positive effect of butyrate on transcriptomic activity of some pivotal genes at intestinal level has been suggested in a study carried out on sea bass by Scollo et al. (2012).

More study are needed to explain in detail the action mechanism on gut cells of SCFA and butyrate in particular as anti-inflammatory and promoter of peptides transport as well as his effect on fish growth. Moreover we will need further investigations to know the better way to utilize butyrate, if by direct administration or by promotion of his production via gut anaerobic bacteria.

Finally, we would try to do a global assessment of animal welfare. Taking in account someone of the considered parameters, we can build an assessment schedule assigning to each parameter a value positive (+1) or negative (-1) according on own specific evaluation; the sum of any single score will give a quite objective indication, in terms of well-being. The values close zero will be of little significance, instead high values towards positive or negative could give clearer idea on animal welfare.

In Tab.4.1 we report an example of a "welfare assessment schedule", taking in account: global scoring system (normal= +1 / altered = -1); time of response to diets change by

histological changes (quick = +1 / slow = -1); adaptation to new diet by return to initial values of score (yes = +1 / no = -1); presence or absence of PepT1 high values into distal intestine (yes = +1 / no = -1). Clearly the "welfare assessment schedule" may be further developed adding more parameters to refine the assessment; for instance other molecular biomarker.

Tab.4.1 Hypothesis of an "welfare assessment schedule" The values close zero will be of little significance, instead high values towards positive or negative could give clearer idea on animal welfare

welfare assessment schedule			
Unit	Parameter description	Parameters evaluation	score (+1/-1)
Fish fed diet XXXX	**Global score summation** (∑ medium value of single score: Lamina Propria;Connective Tissue; Mucosal Folds)	Value 1-6 = score +1 Values >6 = score -1	-1
	Responce to diets change by histological changes (time)	Quick (10-20 days) score +1 Slow (> 20 days) score -1	+1
	Adaptation to new diet: return to initial values of score (return to initial values of score within three months from diet change)	Yes = score +1 No = score -1	+1
	PepT1 (high values into distal intestine)	Absence = score +1 Presence = score -1	+1
		Global well-being	+2

5.0 Conclusion

The great importance of biodiversity concept, in fishing resources for feed as well as in maintaining the fish gut microbiota in good condition is evident for this study. The present work has enabled to evaluate the zootechnical performances of four diets at different level fishmeal substitution with protein vegetable source. The result showed that seabass may be fed with aquafeed containing around 20% of fishmeal without relevant difference in growth performance from feed richer in FM. Although fish fed diet D showed the worst growing values, all tested diets were available to meet aquaculture commercial farm needs.

It was set up a scoring system to assess histological changes diet related in different gut tracts, such system showed that histological changes occurred early (10 days) after new diet administration. The score increase or decrease until 40- 50 day then the values tend to come back at initial level. We speculate that healthy fish, when such changes are reversible, is able to respond quickly at diet variation to return at initial condition in one or two month.

This could mean that other factors acts on the histological aspect of fish gut: excluding specific disease or pathologic syndromes, we have to take in account the rearing condition as biomass density, oxygen saturation, temperature, water flow and so on. In certain rearing condition, healthy fish should reach a balance corresponding to an own histological shape into the gut. Quick changes of diet could cause stress to which fish respond with compensatory response due to adaptation mechanism.

Further studies should consider the microbiota dynamics first and how it responds to changing diet, then the comunication among bacteria and intestine tracts should be analyzed, focusing the attention on the products of bacterial fermentation, among which the SCFA.

We can confirm also the influence of the diet on microbiota composition into the gut and diet interaction with molecular biomarkers, as intestinal peptide (PepT1) and neutral aminoacid (SLC6A19) transporters into proximal intestine.

In detail it seems existing an inverse relationship between protein concentrate in diets and PepT1 into proximal intestine.

The histological scoring method, if shared, could help the comparison of histological changes recorded by different authors.

With regard to "welfare assessment schedule" further investigations are needed to select appropriate parameters and their evaluation.

Further investigation are also needed to better explain the butyrate and other SCFA action mechanism and/or his administration route or how to manipulate its production by gut microbiota.. Taking in account that butyrate usually is product by so called "probiotic" bacteria, we should detect the compounds ("prebiotic"), to add to the diet, which promote probiotic bacteria.

However the attention should be focused on the assessment of nutritional welfare and consequent physiological efficiency.

6.0 Acknowledgement

This work has been partially funded under the EU ARRAINA project N. 288925: Advanced Research Initiatives for Nutrition and Aquaculture. The views expressed in this work are the sole responsibility of the authors and do not necessary reflect the views of the European Commission.

It would not have been possible to complete this study without the collaboration of several people. At every time, during these last three years, I had the fortune to meet excellent collaborators, qualified technicians in fish farm and feed industry as well as scientists, that allowed me to conclude this study.

All the experimental tests would not have been possible without provisions from "Agroittica Toscana" facilities in Piombino (Italy), for which I send a particular thank to Dr. Antonio Tonelli and Dr. Sabrina Clementi, and their staff.

The diets were supplied by "Naturalleva" that gave also an important technical support thanks to Dr. Fabio Brambilla, nutritionist and Dr. Ernesto Franzolini, pathologist and zootechnic.

The histological microbiological analysis has been carried on at the labs of Istituto Zooprofilattico Sperimentale (IZS) delle Regioni Lazio e Toscana, in Rome (Italy). I would like to thank the colleague and friend Dr. Teresa Bossu, fish pathologist, and her staff with particular regard to Sonia Amiti. A special thank to Dr. Cristiano Cocumelli and his staff, because without his histopathological experience it would not have been possible to do a correct assessment of histological changes and set the "scoring system".

The molecular analyses were performed in the University of Insubria labs (Department of Biotechnology and Molecular Sciences) under the supervision of Professor Genciana Terova: my gratitude goes to her and her staff, especially to Dr. Simona Rimoldi, Dr. Giuseppe Scollo and Micaela Antonini.

Data processing would not have been possible without the support of Dr. Mariagrazia Finoia, Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA), who contributed to statistical elaboration.

Last, but by no means least, I say "thank you" to my tutor and friend Professor Marco Saroglia, who for the second time supported me in a thesis work: first time 27 years ago for my degree in veterinary medicine, and now for the pHD. I could not wish for a better or friendlier supervisor.

7.0 References

Allan G.L. (2004). Review of fish nutrition and feeding. In: Edwards P. and Allan G.L., eds., Feeds and feeding for inland aquaculture in Mekong region countries. Canberra, ACIAR Technical Reports No. 56, 37 – 48.

Adermann K., Raida M., Paul Y., Abu-Raya S., Bloch-Shilderman E., Lazarovici P., Hochman J., Wellhoner H. (1998). Isolation, characterization and synthesis of a novel paradaxin isoform. FEBS Lett 1998, 435:173–177

Asfie M., Yoshijima T., Sugita H. (2003). Characterization of the goldfish fecal microflora by the fluorescent in situ hybridization method. Fish. Sci. 69, 21–26.

Bals R., Weiner D.J., Moscioni A.D., Meegalla R.L., Wilson J.M. (1999). Augmentation of innate host defense by expression of a cathelicidin antimicrobial peptide. *Infect Immun* 1999, 67:6084–6089

Barrows F.T., Bellis Krogdahl. A., Silverstain J.F., Herman E., Sealey W.M., Rust M.B., Gatlin III D.M. (2008) Report of the plant products in aquafeed strategic planning workshop1: An integrated, interdisciplinary research roadmap for increasing utilization of plant feedstuffs in diets for carnivorous fish. *Reviews in Fishery Science* 16, 449-455.

Basurco B, and Abellán E (1999). Finfish species diversification in the context of Mediterranean marine fish farming development. In: Marine Finfish Diversification: Current Situation and Prospects in Mediterranean Aquaculture. Options Mediterranéennes (eds. E. Abellan and B. Basurco), FAO, serie B: Etudes et Recherches, no. 24, pp. 9–25.

Bates J.M., Mittge E., Kuhlman J., Baden K.N., Cheesman S.E., Guillemin K. (2006). Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. Developmental Biology 297, 374–386.

Bauer E., Williams B.A., Smidt H., Mosenthin R., Verstegen M.W.A. (2006). Influence of dietary components on development of the microbiota in single-stomached species. Nutr. Res. Rev. 19, 63-78.

Bergman E.N. (1990). Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 70(2):567–590

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

Berni Canani R., Di Costanzo M., Leone L. (2012). The epigenetic effects of butyrate: potential herapeutic implications for clinical practice. Clin Epigenetics 4(1):4. http://www.clinicalepigeneticsjournal.com/content/4/1/4

Birkemo G.A., Lüders T., Andersen O., Nes I.F., Nissen-Meyer J. (2003). Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Biochim Biophys Acta* 2003, 1646:207–215

Bischoff S.C. (2011). 'Gut health': a new objective in medicine? *BMC Med* 2011, 9:24.
Bond J.H., Levitt M.D. (1976). Fate of soluble carbohydrate in the colon of rats and man. J Clin Invest 57(5):1158–1164.

Bone Q: Locomotor muscle (1978). In *Fish Physiology, vol.7*. Edited by Hoar WS, Randall DJ. New York, London: Academic Press;1978:361-424.

Brechtel P.J. (1986). Muscle development and contractile proteins. In *Muscle as Food.* Edited by Brechtel PJ. New York: Academic Press; 1986:1-35.

Bucking C. and Wood C.M. (2009). The effect of postprandial changes in pH along the gastrointestinal tract on the distribution of ions between the solid and fluid phases of chyme in rainbow trout. Aquacult. Nutr. 15, 282–296.

Buyse M., Tsocas A., Walker F., Merlin D., Bado A. (2002). PepT1-mediated fMLP transport induces intestinal inflammation in vivo. Am J Physiol Cell Physiol 283: C1795–1800.

Cahu C.L., Zambonino Infante J.L., Takeuchi T. (2001). Nutrients affecting quality in marine larval fish development. In: Hendry CI, van Stappen G, Willie W, Sorgeloos, P (Eds.), Larvi 2001. European Aquaculture Society, Special Publication no. 30. Oostende, Belgium, pp. 94– 95.

Carter C., Owen S., He Z., Watt P., Scrimgeour C., Houlihan D., Rennie M. (1994). Determination of protein synthesis in rainbow trout, *Onchorhynchus* m*ykiss*, using a stable isotope. *J Exp Biol* 1994, 189:279-284.

Chehade M., Mayer L. (2005). Oral tolerance and its relation to food hypersensitivities. J Allergy Clin. Immunol. 115, 3-12.

Clements K.D. (1997). Fermentation and the gastrointestinal microorganisms in fishes. In: Mackie, R.I., White, B.A. (Eds.), Gastrointestinal Microbiology. Gastrointestinal Ecosystems and Fermentation, vol. 1. Chapman and Hall, New York, pp. 156–198

Clements K.D. and Raubenheimer D. (2006). Feeding and Nutrition. In D.H. Evans and J.B. Claiborne (Eds.). *The Physiology of Fishes.* 3rd ed. Boca Raton, FL, USA, Taylor and Francis Group.

Cole D.J.A.R., Beal M. and Luscombe J.R. (1968). The effect on performance and bacetrial flora of lactic acid, propionic acid, calcium propionate and calcium acrylate in the drinking water of the weaned pigs. Veterinary. Record 459-464.

Cole A.M., Weis P., Diamond G. (1997). Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. *J Biol Chem* 1997, 272:12008–12013

Corrales J., Gordon W.L., Noga E.J. (2009). Development of an ELISA quantification of the antimicrobial peptide piscidin 4 and its application to assess stress in fish. *Fish Sellfish Immunol* 2009, 27:154–163

Corrales J., Mulero I., Mulero V., Noga E.J. (2010). Detection of antimicrobial peptides related to piscidin 4 in important aquacultured fish. *Dev Comp Immunol* 2010, 34:331–343

Cowey C.B., Walton M.J. (1988). Studies on the uptake of (^{14}C) aminoacids derived from both dietary (^{14}C) protein and dietary (^{14}C) aminoacids by rainbow trout *Salmo gairdneri* Richardson. *J Fish Biol* 1988, 33:293-305.

Crissman J.W., Goodman D.G., Hildebrandt P.K., et al. (2004). Best practices guideline: toxicologic histopathology. Toxicol Pathol.;32: 126–131.

Criteria for safe use of plant ingredients in diets for aquacultured fish. (2009) Opinion of the Panel on Animal Feed of the Norwegian Scientific Committee for Food Safety *05.02.2009.* http://www.vkm.no/dav/1232b28f3b.pdf

Dalmasso G., Nguyen H.T.T., Yan Y., Charrier-Hisamuddin L., Sitaraman S.V., Merlin D. (2008). Butyrate Transcriptionally Enhances Peptide Transporter PepT1 Expression and Activity. PLoS ONE www.plosone.org June 2008 Volume 3 Issue 6 e2476

D'Anatro N., Vidal I., González-Bergonzoni F., Teixeira de Mello J., Tana D., Naya E. (2013). Geographic and seasonal variation analysis of digestive morphology in the catfish *Iheringichthys labrosus* along lower Río Uruguay. Dove Press journal: Open Access Animal Physiology 13 August 2013

Dawson K.A., Pirvulescu M. (1999). Alltech's Asia Pacific Lecture Tour. Alltech, Sidney, pp 75–83; 1999.

De Paula Silva F.C., Nicoli J.R., Zambonino-Infante J.L., Kaushik S. and Gatesoupe F.J. (2009). Influence of the dieton the microbial diversityof faecal and gastrointestinal contents in gilthead sea bream(Sparus aurata) and intestinal contents in gold¢sh(Carassius auratus). FEMS Microbiol Ecol 78 (2011) 285–296

De Paula Silva F.C., Nicoli J.R., Zambonino-Infante J.L., Kaushik S. and Gatesoupe F.J. (2011). Infuence of the dieton the microbial diversityof faecal and gastrointestinal contents in gilthead sea bream(Sparus aurata) and intestinal contents in goldfish(Carassius auratus). FEMS Microbiol Ecol. 78 (2011) 285–296

De Schryver P., Kumar Sinha A., Kunwar P.S., Baruah K., Verstraete W., Boon N., De Boeck G., Bossier P. (2010). Poly-*β*-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, Dicentrarchus labrax. Appl Microbiol Biotechnol 86:1535–1541.

De Schryver P., Dierckens K., Thi Q.Q. B., Amalia R., Marzorati M., Bossier P., Boon N. and Verstraete W. (2011). Convergent dynamics of the juvenile European sea bass gut microbiota induced by poly-b-ydroxybutyrate. Environmental Microbiology (2011) **13**(4), 1042–1051

De Silva S.S. and Anderson T.A. (1995). Fish Nutrition in Aquaculture. Chapman & Hall aquaculture series 1

Desmet V.J. (2003). Milestones in liver disease: scoring chronic hepatitis. J. Hepatol 38:382–386

De Wet F., Viljoen S. (2012). Evaluating calcium butyrate and nucleotides for use in diets of mozambique tilapia Oreochromis mossambicus. Aquaculture Europe 2012. Book of abstracts. Prague

Dias J., Gomes E.F., Kaushik S.J. (1997). Improvement offeed intake trough supplementation with an attractant mix in European seabass fed plant-protein rich diets. Aquat. Living Resour. 10, 385–389.

Dias J. (1999). Lipid deposition in rainbow trout (Oncorhynchus mykiss) and European seabass (Dicentrarchus labrax L.): nutritional regulation of hepatic lipogenesis. Dr thesis, Univ. Porto (Portugal) and Univ. Bordeaux I (France). 190 pp.

Dimitroglou A., Merrifield D. L., Carnevali O., Picchietti S., Avella M., Daniels C. L., Güroy D., Davies S. J. (2011). Microbial manipulations to improve fish health and production - a Mediterranean perspective. *Fish* & *Shellfish Immunology,* 30, 1-16.

Dobly A., Martin S.A.M., Blaney S.C., Houlihan D.F. (2004). Protein growth rate in rainbow trout (*Oncorhyncus mykiss*) is negatively correlated to liver 20S proteasome activity. *Comp Biochem Physiol A* 2004, 137:75-85.

Douglas SE, Gallant JW, Liebscher RS, Dacanay A, Tsoi SCM: Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. *Dev Comp Immunol* 2003, 27:589–601

Duan C. (1998). Nutritional and developmental regulation of insulin-like growth factors in fish. Journal of Nutrition 128, 306S-314S.

Dunham R.A., Warr G.W., Nichols A., Duncan P.L., Argue B., Middleton D., Kucuktas H. (2002). Enhanced bacterial disease resistance of transgenic channel catfish *Ictalurus punctatus* possessing cecropin genes. *Mar Biotechnol (NY)* 2002, 4:338–344

Elworthy S., Hargrave M., Knight R., Mebus K., Ingham P.W. (2008). Expression of multiple slow myosin heavy chain genes reveals a diversity of zebrafish slow twitch muscle fibers with differing requirements for Hedgehog and Prdm1 activity. Development 2008, 135:2115-2126.

Fernandes J.M., Molle G., Kemp G.D., Smith V.J. (2004). Isolation and characterization of oncorhyncin II, a histone H1-derived antimicrobial peptide from skin secretions of rainbow trout, *Oncorhynchus mykiss*. *Dev Comp Immunol* 2004, 28:127–138

Fernandes J.M., Ruangsri J., Kiron V. (2010). Atlantic cod piscidin and its diversification through positive selection. *PLoS One* 2010, 5:e9501

Florini J.R., Ewton D.Z., Coolican S.A. (1996). Growth hormone and the insulin-like growth factor system in myogenesis. Endocrine Reviews 17, 481-517.

Forman B.M., Chen J., Evans R.M. (1997). Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proceeding of National Academy of Sciences of the United States of America, 94: 4312–4317.

Fournier V., Gouillou-Coustans M.F., Metailler R., Vachot C., Guedes M.J., Tulli F., Oliva-Teles A, Tibaldi E., and Kaushik, S.J. (2002). Protein and arginine requirements for maintenance and nitrogen gain in four teleosts. British Journal of Nutrition 87, 459-469.

Galfi P., Bokori J. (1990). Feeding trial in pigs with a diet containing sodium n-butyrate. Acta Vet Hung 38(1–2):3–17

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

Gaucher L., Vidal N., D'Anatro A., Naya D.E. (2012). Digestive flexibility during fasting in the characid fish Hyphessobrycon luetkenii. *J Morphol.* 2012;273(1):49–56.

Gauvry L., Fauconneau B. (1996). Cloning of a trout fast skeletal myosin heavy chain expressed both in embryo and adult muscles and in myotubes neoformed in vitro. *Comp Biochem Physiol B* 1996, 115:183-190.

Gawlicka A.K. and Horn M.H. (2005). Storage and absorption in the digestive system of carnivorous and herbivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects. *Physiol Biochem Zool.* 2005;78(6):879–892.

German D.P., Neuberger D.T., Callahan M.N., Lizardo N.R., Evans D.H. (2010). Feast to famine: the effects of food quality and quantity on the gut structure and function of a detritivorous catfish (Teleostei: Loricariidae). *Comp Biochem Physiol A Mol Integr Physiol.* 2010;155(3):281–293.

Gibson-Corley K.N., Olivier A. K., and Meyerholz D. K.(2013). Principles for Valid Histopathologic Scoring in Research. Veterinary Pathology Online First, published on April 4, 2013 as doi:10.1177/0300985813485099-

Gilbert E.R., Li H., Emmerson D.A., Webb Jr. K.E., and Wong E.A. (2008). Dietary protein quality and feed restriction influence abundance of nutrient transporter mRNA in the small intestine of broiler chicks. The Journal of Nutrition 138, 1-10.

Giuliani A., Pirri G., Nicoletto S.F. (2007). Antimicrobial peptides: an overview of a promising class of therapeutics. *Cent Eur J Biol* 2007, 2:1–33

Godon J.J., Zumstein E., Dabert P., Habouzit F. and Moletta R. (1997). Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. Appl. Environ Microb 63: 2802–2813.

Gomes E.F., Rema P., Kaushik S.J. (1995). Replacement of fish meal by plant proteins in the diet of rainbow trout (Oncorhynchus mykiss): digestibility and growth performance. Aquaculture 130, 177–186.

Groschwitz K.R., Hogan S.P. (2009). Intestinal barrier function: molecular regulation and disease pathogenesis. *J Allergy Clin Immunol* 2009, 124:3-20.

Hancock R.E.W., Chapple D.S.(1999). Peptides antibiotics. *Antimicrob Agents Chemother* 1999, 43:1317–1323

Hashim R. (2006). Alternative Ingredients for Aquafeeds: The Feasibility and Economic Equation. Paper presenteded as Guest Speaker at the National Fisheries Symposium (NaFIS) 26 – 28 june 2006, Kuching, Sarawak, Malaysia

Hevroy E.M., Jorda A.E.O., Hordvik I., Espe M., Hemre G.I., Olsvik P.A. (2006). Myosin heavy chain mRNA expression correlates higher with muscle protein accretion than growth in Atlantic salmon, *Salmo salar*. *Aquaculture* 2006, 252:453-461.

Houlihan D.F., Hall S.J., Gray C. (1989). Effects of ration on protein turnover in cod. Aquaculture 1989, 79:103-110.

Houlihan D.F., Carter C.G., McCarthy I.D. (1995). Protein synthesis in fish. In *Biochemistry and Molecular Biology of Fishes, vol. 4. Metabolic Biochemistry.* Edited by Hochachka PW, Mommsen TP. Amsterdam: Elsevier Biomedical; 1995:191-200.

Hu F.B., Willet W.C. (2002). Optimal diets for prevention of coronary heart disease. Journal of the American Medical Association, 288: 2569–2578.

Kim K., Kayes T.B., Amundson C.H. (1987). Effects of dietary tryptophan levels on growth, feed/gain, carcass composition and liver glutamate dehydrogenase activity in rainbow trout (*Salmo gairdneri*). *Comp Biochem Physiol B* 1987, 88:737-741.

Knudsen D., Urán P., Arnous A. Koppe. W. Frokiaer H. (2007). Saponin containing subfractions of soybean molasses induce enteritis in the distal intestine of Atlantic salmon. J. Agric Food Chem 55: 2261-2267.

Knudsen D., Jutfelt F., Sundh H., Sundell K, Koppe W., Frokiaer, H. (2008). Dietary soya saponins increase gut permeability and play a key role in the onset of soyabean-induced enteritis in Atlantic salmon (Salmo salar L.). Br J Nutr 100: 120-129.

Korte S.M., Berend B., Koolhaas J.M. (2007). A new animal welfare concept based on allostasis. Physiology & Behavior 92 (2007) 422–428

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

IFFO (International Fishmeal Fish Oil Organization). Position Statement Version1: (2012) October.http://www.iffo.net/downloads/Position%20Papers/Demonstrating%20responsible%20marine%20feed%20IFFO.pdf

Jackson A. Fish In- Fish Out, Ratios Explained (2009). Aquaculture Europe 2009, Volume 34 (3)

Johansen K.A., Overturf K. (2006). Alterations in expression of genes associated with muscle metabolism and growth during nutritional restriction and refeeding in rainbow trout. *Comp Biochem Physiol B* 2006, 144:119-127.

Johnston I. A. (1999). Muscle development and growth: Potential implications for flesh quality in fish. Aquaculture 177, 99-115.

Johnston I.A. (2001). Genetic and environmental determinants. In *Muscle Development and Growth*. Edited by Johnston, L.A. Fish Physiology series Vol 18. San Diego: Academic Press; 2001:141-186.

Lauth X., Shike H., Burns J.C., Westerman M.E., Ostland V.E., Carlberg J.M., Van Olst J.C., Nized V, Taylor S.W., Shimizu C., Bulet P. (2002). Discovery and characterization of two isoforms of moronecidin, a novel antimicrobial peptide from hybrid striped bass. *J Biol Chem* 2002, 277:5030–5039

Licciardi P.V., Wong S.S., Tang M.L., Karagiannis T.C. (2010). Epigenome targeting by probiotic metabolites. *Gut Pathog* 2010, 2:24.

Liepke C., Baxmann S., Heine C., Breithaupt N., Ständker L., Forssmann W.G. (2003). Human hemoglobin-derived peptides exhibit antimicrobial activity: a class of host defense peptides. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003, 5:345–356

Leroith D., Bondy C., Yakar S., Liu J.L., Butler A. (2001). The somatomedin hypothesis:2001. Endocrine Reviews **22**, 53-74.

Lückstädt C. (2012). Acidification in monogastric fish: limits and potentials. AQUA Culture Asia Pacific Magazine November/December 2012, 38-39

Márquez L., Overland M., Martínez-Llorens S., Morken T., Moyano F.J. (2013). Use of a gastrointestinal model to assess potential amino acid bioavailability in diets for rainbow trout (Oncorrhynchus mykiss), Aquaculture 384–387 (2013) 46–55

Martins D.A., Estévez A., Stickland N.C., Simbi B.H., Yùfera M. (2010). Dietary Lecithin Source Affects Growth Potential and Gene Expression in *Sparus aurata* Larvae. *Lipids* 2010, 45:1011-1023.

McNamara P.T., Caldarone E.M., Buckeley L.J. (1999). RNA/DNA ratio and expression of 18S ribosomal RNA, actin and myosin heavy chain messenger RNAs in starved and fed larval Atlantic cod (*Gadus morhua*). *Marine Biology* 1999, 135:123-132.

Méndez E., Smith A., Figueiredo-Garutti M.L., Plana J.V., Navarro I. (2001). Receptors for insulin-like growth factor-I (IGF-I) predominate over insulin receptors in skeletal muscle throughout the life cycle of brown trout, Salmo trutta. General and Comparative Endoccrinology 122, 148-157.

Merlin D., Si-Tahar M., Sitaraman S.V., Eastburn K., Williams I. et al. (2001). Colonic epithelial hPepT1 expression occurs in inflammatory bowel disease: transport of bacterial peptides influences expression of MHC class 1 molecules. Gastroenterology 120: 1666–1679.

Merrifield D.L., Dimitroglou A., Foey A., Davies S.J., Baker R.R., Bogwald J., Castex M., Ringo E. (2010). The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* 302, 1-18.

Meddings J.(2008). The significance of the gut barrier in disease. *Gut* 2008, 57:438-440.

Merrifield L.D.L., Olsen R.E., Myklebust R. and Ringo E. (2011). Dietary Effect of Soybean (Glycine max) Products on Gut Histology and Microbiota of Fish, Soybean and Nutrition,

Park I.Y., Park C.B., Kim M.S. (1998). Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. *FEBS Lett* 1998, 437:258–262

Parish C.A., Jiang H., Tokiwa Y., Berova N., Nakanishi K., McCabe D., Zuckerman W., Xia M.M., Gabay J.E. (2001). Broad-spectrum antimicrobial activity of hemoglobin. *Bioorg Med Chem* 2001, 9:377–382

Prof. Hany El-Shemy (Ed.), ISBN: 978-953-307-536-5, In Tech, Available from: http://www.intechopen.com/books/soybean-andnutrition/dietary-effect-of-soybean-glycine-max-products-on-gut-histology-and-microbiota-of-fish

Millikin M.R. (1982). Qualitative and quantitative nutrient requirements of fishes: a review.Fishery Bulletin 80, 655-686.

Mitchell S.O., Baxter E.J., Holland C., Rodger H.D. (2012). Development of a novel histopathological gill scoring protocol for assessment of gill health during a longitudinal study in marine-farmed Atlantic salmon (Salmo salar). Aquacult Int 20:813–825

Mock A., Petrs G. (1990). Lysozyme activity in rainbow trout *Oncorhynchus mykiss* (Walbaum), stressed by handling, transport and water pollution. *J Fish Biol* 1990, 37:873–885

Moeinian M., Ghasemi-Niri S.F., Mozaffari S., Abdollahi M. (2013). Synergistic effect of probiotics, butyrate and L-Carnitine in treatment of IBD. Journal of Medical Hypotheses and Ideas (2013) 7, 50–53

Moriyama S., Ayson F.G., Kawauchi H. (2000). Growth regulation by insulin-like growth factor-I in fish. Bioscience Biotechnology and Biochemistry 64, 1553-1562.

Musaro A., McCullagh K.J., Naya F.J., Olson E.N., Rosenthal N. (1999). IGF-I induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATcl. Nature 400, 581-585.

Navarrete P., Espejo R., Romero J. (2009). Molecular analysis of microbiota along the digestive tract of juvenile Atlantic salmon (*Salmo salar* L.). *FEMS Microbial Ecology* 57, 550-561.

Navarrete P., Magne F., Mardones P., Riveros M., Opazo R., Suau A., Pochart P. and Romero J. (2010). Molecular analysis of intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*). *FEMS Microbial Ecology* 71, 148-156.

Nayak S.K. (2010). REVIEW ARTICLE Role of gastrointestinal microbiota in fish. Aquaculture Research, 2010, 41, 1553^1573

Nikfar S., Rahimi R., Rahimi F., Derakhshani S., Abdollahi M. (2008). Efficacy of probiotics in irritable bowel syndrome: a metaanalysis of randomized, controlled trials. Dis Colon Rectum 2008;51(12):1775–80.

Noga E.J., Fan Z., Silphaduang. U. (2001). Histone-like proteins from fish are lethal to the parasitic dinoflagellate *Amyloodinium ocellatum*. *Parasitology* 2001, 123:57–65

Noga E.J., Fan Z., Silphaduang U. (2002). Host site of activity and cytological effects of histone-like proteins on the parasitic dinoflagellate *Amyloodinium ocellatum*. *Dis Aquat Organ* 2002, 52:207–215

Noga E.J., Silphaduang U. /2003). Piscidins: a novel family of peptide antibiotics from fish. *Drugs News Perspect* 2003, 16:87–92

Noga E.J., Ullal A.J., Corrales J., Fernandes J.M. (2011). Application of antimicrobial polypeptide host defenses to aquaculture: exploitation of downregulation and upregulation responses. *Comp Biochem Physiol Part D Genomics Proteomics* 2011, 6:44–54

Nubel U., Engelen B., Felske A., Snaidr J., Wieshuber A., Amann RI, Ludwig W and Backhaus H (1996) Sequence heterogeneities of genes encoding 16S rRNAs in Paenibacillus polymyxa detected by temperature gradient gel electrophoresis. J Bacteriol 178: 5636–5643.

Ogihara H., Saito H., Shin B.C., Terado T., Takenoshita S., et al. (1996). Immunolocalization of H+/peptide cotransporter in rat digestive tract. Biochem Biophys Res Commun 220: 848– 852.

Olsson J., Quevedo M., Colson C., Svanbäck R. (2007). Gut length plasticity in Eurasian perch: into the bowels of resource polymorphisms. *Biol J Linnean Soc.* 2007;90(3):517–523.

Overturf K., Hardy R.W. (2001). Myosin expression levels in trout muscle: a new method for monitoring specific growth rates for rainbow trout *Oncorhynchus mykiss* (Walbaum) on varied planes of nutrition. *Aquaculture Res* 2001, 32:315-322.

Owen M.A.G., Waines P., Bradley G., Davies S. (2006). The effect of dietary supplementation of sodium butyrate on the growth and microflora of Clarias gariepinus (Burchell 1822). XII international symposium fish nutrition and feeding, May 28–June 1, Book of abstracts: 149

Peragòn J., Barroso J.B., Garcia-Salguero L., dela Higuera M., Lupianex J.A. (2000). Dietary alterations in protein, carbohydrates and fat increase liver protein-turnover rate and decrease overall growth rate in the rainbow trout. *Mol Cell Biochem* 2000, 209:97-104.

Pond D., Stone M., Alderman D.J. (2006). Comparison of conventional and molecular techniques to investigate the intestinal microflora of rainbow trout (Oncorhynchus mykiss). Aquaculture 261 -2006- 194–203

Rana K.J., Siriwardena S. and Hasan M.R. (2009). Impact of rising feed ingredient prices on aquafeeds and aquaculture production. FAO Fisheriess and Aquaculture Techmical Paper 541

Randle P.J. (1954). Plasma-insulin activity in hypopituitarism. Lancet 1, 809-810.

Rawls J.F., Samuel B.S., Gordon J.I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Acadamey of Science* USA 101, 4596–4601.

Ringo E. (1993). Does chromic oxide (Cr2O3) affect faecal lipid and intestinal bacterial flora in Arctic charr, Salvelinus alpinus (L.)? Aquac. Fish. Manage. 24, 767– 776.

Ringo E. and Olsen R.E. (1999). The effect of diet on aerobic bacterial flora associated with intestine of Arctic charr (*Salvelinus alpinus* L.). Journal of Applied Microbiology 1999, 86, 22–28

Ringo E., Olsen R.E., Mayhewc T.M., Myklebustd R. (2003) Electron microscopy of the intestinal microflora of fish. Aquaculture 227 (2003) 395–415

Ringo E., Sperstad S., Myklebust R., Mayhew T.M. and Olsen R.E. (2006). The effect of dietary inulin on aerobic bacteria associated with hindgut of Arctic charr (Salvelinus alpinus L.) Aquaculture Research, 2006, 37, 891^897

Ringo E., Sperstad S., Myklebust R., Refstie S., Krogdahl A. (2006). Characterisation of the microbiota associated with intestine of Atlantic cod (Gadus morhua L.) The effect of fish meal, standard soybean meal and a bioprocessed soybean meal. Aquaculture 261 (2006) 829–841

Ringo E., Lovmo L., Kristiansen M., Bakken M., Salinas I., Myklebust R., Olsen R.E., Mayhew T.M. (2010). Lactic acid bacteria *vs*. pathogens in the gastrointestinal tract of fish: a review. *Aquaculture Research* 41, 451-467.

Romero J. and Navarrete P. (2006). 16S rDNA-based analysis of dominant bacterial populations associated with early life stages of coho salmon (*Oncorhynchus kisutch*). *FEMS Microbial Ecology* 51, 422-430.

Robinette D., Wada S., Arroll T., Levy M.G., Miller W.L., Noga E.J. (1998).Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. *Cell Mol Life Sci* 1998, 54:467–475

Robinette D.W., Noga E.J. (2001). Histone-like protein: a novel method for measuring stress in fish. *Dis Aquat Organ* 2001, 44:97–107

Ronnestad I., Tonheim S.K., Fyhn H.J., Rojas-Garcia C.R., Kamisaka Y., Koven W., et al., (2003). The supply of amino acids during early feeding stages of marine fish larvae: a review of recent findings. Aquaculture; 227 (1-4): 147-164.

Rossi F., Chini V., Cattaneo A.G., Bernardini G., Terova G., Saroglia M., Gornati R. (2007). EST-based identification of gene expressed in perch (*Perca fluviatilis*, L.). *Gene Expr* 2007, 14:117–127

Salerno G., Parriello N., Roch P., Cammarata M. (2007). cDNA sequence and tissue expression of an antimicrobial peptide, *dicentracin*; a new component of the moronecidin family isolated from head kidney leukocytes of sea bass, *Dicentrarchus labrax*. *Comp Biochem Physiol Part B* 2007, 146:521–529

Salzman N.H., Ghosh D., Huttner K.M., Paterson Y., Bevins C.L. (2003). Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* 2003, 422:522–526

Sangaletti R., Terova G., Peres A., Bossi E., Corà S. and Saroglia M. (2009). Functional expression of the oligopeptide transporter PepT1 from the sea bass (Dicentrarchus labrax). Pflugers Arch - Eur J Physiol 2009 - 459:47–54.

Sansonetti P.J. (2004). War and peace at mucosal surfaces. Nat. Rev. Immunol. 4, 953-964.

Sargent J.R., Bell J.G., Bell M.V., Henderson R.J. and Tocher D.R., (1995). Requirement criteria for essential fatty acids. Journal of Applied Ichthyology 11, 183-198.

Sargent J.R., Tocher D.R. and Bell J.G. (2002). The lipids. In: Halver J., Hardy E. (Eds.), Fish Nutrition, (Third edition) Academic, San Diego, pp. 181-257.

Sargent J.R., Tocher D.R., and Bell J.G. (2002). The lipids. In: Halver, J., Hardy, E. (Eds.),Fish Nutrition, (Third edition) Academic, San Diego, pp. 181-257.

Sarmasik A., Warr G., Chen T.T. (2002). Production of transgenic medaka with increased resistance to bacterial pathogens. *Mar Biotechnol (NY)* 2002, 4:310–322

Sarmento A., Marques F., Ellis A.E., Afonso A. (2004). Modulation of the activity of sea bass (*Dicentrarchus labrax*) head-kidney macrophages by macrophage activating factor(s) and lipopolysaccharide. *Fish Shellfish Immunol* 2004, 16:79–92

Sawamura D., Goto M., Shibaki A., Akiyama M., McMillan J.R., Abiko Y., Shimizu H. (2005). Beta defensin-3 engineered skin shows highly protective effect for bacterial infection. *Gene Ther* 2005, 2:857–861

Scheppach W. (1994). Effects of short chain fatty acids on gut morphology and function. Gut 35(1):S35–S38.

Segain J.P., Raingeard de la Ble´ tie`re D., Bourreille A., Leray V., Gervois N., Rosales C. et al. (2000). Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut 2000;47(3):397–403.

Scollo G., Terova G., Rimoldi S., Bernardini G., Antonini M., Saroglia M. (2012). Does butyrate have a role in the protection of fish intestine? Results from a preliminary study on European sea bass (D. labrax). Aquaculture Europe. Book of abstracts. Prague

Shamblott M.J., Chen T.T. (1993). Age related and tissue-specific levels of five forms of insulin-like growth factor mRNA in a teleost. Molecular Marine Biology and Biotechnology 2, 351-361.

Shepherd C.J. and Jackson A.J. (2012). Global fishmeal and fish oil supply - inputs, outputs, and markets 6th World Fisheries Congress, Edinburgh 2012.

Silphaduang U., Colorni A., Noga E.J. (2006). Evidence for widespread distribution of piscidin antimicrobial peptidesin teleost fish. *Dis Aquat Organ* 2006, 72:423–432

Silvi S., Nardi M., Sulpizio R., Orpianesi C., Caggiano M., Carnevali O. and Cresci A. (2008). Microbial Effect of the addition of Lactobacillus delbrueckii subsp. Delbrueckii on the gut microbiota composition and contribution to the well-being of European sea bass (Dicentrarchus labrax, L.). Ecology in Health and Disease. 2008; 20: 53 59

Smith V.J., Fernandes J.M., Jones S.J., Kemp G.D., Tatner M.F. (2000). Antibacterial proteins in raimbow trout, *Oncorhynchus mykiss. Fish Shellfish Immunol* 2000, 10:243–260

Sonnenburg E.D., Zheng H., Joglekar P., Higginbottom S.K., Firbank S.J., Bolam D.N., Sonnenburg J.L. (2010). Specificity of polysaccharide use in intestinal Bacteroides species determines diet-induced microbiota alterations. *Cell* 2010, 141:1241-1252.

Suryanarayana M.V.A.N., Suresh J. and Rajasekhar M.V. (2012). Organic acids in swine feeding - A Review

Synthesis Of Mediterranean Marine Finfish Aquaculture – A Marketing And Promotion Strategy (2010). by Lara Barazi-Yeroulanos. Study and reviews n°88. FAO General Fisheries Commission for the Mediterranean.
http://www.fao.org/docrep/013/i1696e/i1696e00.htm

Tacon A.G.J., Hasan M.R., Subasinghe R.P. (2006). Use of fishery resources as feed inputs to aquaculture evelopment: trends and policy implications. FAO Fisheries Circular No. 1018. FAO, 2006 Rome. 99 pp.

Tacon G.J. and Matian M. (2008). Global overview on the use of fishmeal and fish oil in industrially compounded aquafeeds: Trends and future prospects, Aquaculture 285 (2008) 146–158

Tacon G.J. (2013). Prospects for the use of rendered products in compound aquafeeds**.** 4th Global feed and food congress 2013 – Sun City, South Africa

Tanaka M., Kawai S., Seikai T., Burke J.S. (1996). Development of the digestive organ system in Japanese flounder in relation to metamorphosis and settlement. Mar. Freshw. Behav. Physiol. 28, 19-31.

Tanaka H, Miyamoto K.I., Morita K., Haga H., Segawa H., et al. (1998). Regulation of the PepT1 peptide transporter in the rat small intestine in response to 5-fluorouracil-induced injury. Gastroenterology 114: 714–723.

Tedelind S., Westberg F., Kjerrulf M., Vidal A. (2007). Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. World J. Gastroenterol 13: 2826–2832.

Terova G., Corà S., Verri T., Rimoldi S., Bernardini G., Saroglia M. (2009). Impact of feed availability on PepT1 mRNA expression levels in sea bass (Dicentrarchus labrax). Aquaculture 294 -2009- 288–299

Terova G., Forchino A., Rimoldi S., Brambilla F., Antonini M., Saroglia M. (2009). Bio-Mos®: an effective inducer of *dicentracin* gene expression in European sea bass (*Dicentrarchus labrax*). *Comp Biochem Physiol Part B* 2009, 153:372–377

The State of World Fisheries and Aquaculture 2012. FAO Fisheries and Aquaculture Department Rome, 2012. http://www.fao.org/docrep/016/i2727e/i2727e.pdf

Tibaldi E., Tulli F., Amerio M. (1999). Feed intake and growth responses of sea bass (D. labrax) fed different plant-protein sources are not affected by supplementation with a feeding stimulant. In: Piva G, Bertoni G, Satoh S, Bani P, Calamari L (Eds.), Recent Progress in Animal Production Science: I. Proc. A.S.P.A. XIII Congress, Piacenza, Italy, 21–24 June 1999. Assn. Sci. Anim. Production, Italy, pp.752–754.

Ullal A.J., Litaker R.W., Noga E.J. (2008). Antimicrobial peptides derived from hemoglobin are expressed in epithelium of channel catfish (*Ictalurus punctatus*, Rafinesque). *Dev Comp Immunol* 2008, 32:1301–1312

Urán P., Rombout J.H.W.H., Koppe W., Obach A., Jensen L., Schrama J.W., Verreth J.A.J. (2004). Effects of soybean meal on intestinal morphology of Atlantic salmon (*Salmo salar* L.). In Abstracts Aquaculture Europe, Barcelona; Special Publication No. 34; European Aquaculture Society: Belgium, 2004; pp 803-804.

Uràn P.A., Goncalves A.A., Taverne-Thiele J.J., Schrama J.W., Verreth J.A.J., Rombout J.H.W.M. (2008). Soybean meal induces intestinal inflammation in common carp (Cyprinus carpio L.). Fish & Shellfish Immunology 25, 751e760

Vavricka S.R., Musch M.W., Chang J.E., Nakagawa Y., Phanvijhitsiri K. et al. (2004). hPepT1 transports muramyl dipeptide, activating NF-kappaB and stimulating IL-8 secretion in human colonic Caco2/bbe cells. Gastroenterology 127: 1401–1409.

Verri T., Terova G., Dabrowski K., Saroglia M. (2011). Peptide transport and animal rowth: the fish paradigm. *BiologyLetters* **7**, 597-600.

Verri T., Terova G., Romano A., Barca A., Pisani P., Storelli C., Saroglia M. (2012). The Solute Carrier (SLC) Family Series in Teleost Fish. In: Functional Genomics in Aquaculture. (Marco Saroglia and Zhanjiang (John) Liu, Eds), pp. 219-320. John Wiley & Sons, Ltd Publication, New Delhi, India.

Yazawa R., Hirono I., Aoki T. (2006). Transgenic zebrafish expressing chicken lysozyme show resistance against bacterial diseases. *Transgenic Res* 2006, 15:385–391

Yin Z.X., He W., Chen W.J., Yan J.H., Yang J.N., Chan S.M., He J.G. (2005). Cloning, expression and antimicrobial activity of antimicrobial peptide, epinecidin-1, from the orange-spotted grouper, *Epinephelus coioides*. *Aquaculture* 2005, 253:204–211

Wang C, Harris W.S., Chung M., Lichtenstein A.H., Balk E.M., Kupelnick B., Jordan H.S., Lau J. (2006). Omega-3 fatty acids from fish oil supplements, but not alpha-linolenic acid benefit cardiovascular disease outcomes in primary&secondary prevention. studies: a systematic review. American Journal of Clinical Nutrition, 84: 5–17.

Wang P., Lu Y., Wen Y., Yu D., Ge L., Dong W., Xiang L., and Shao J. (2013). IL-16 Induces Intestinal Inflammation via PepT1 Upregulation in a Pufferfish Model: New Insights into the Molecular Mechanism of Inflammatory Bowel Disease. *J Immunol* 2013; 191:1413-1427; Prepublished online 1 July 2013
http://www.jimmunol.org/content/191/3/1413

Weatherley A.H., Gills H.S. (1987). The biology of fish growth London: Academic Press; 1987

Weber T.E., Bosworth B.G. (2005). Effect of 28 day exposure to cold temperature or feed restriction on growth, body composition and metabolism an channel catfish. *Aquaculture* 2005, 246:483-492.

Weifeng M., Yapin W., Wenbo W., Bo W., Jianxin F., Zuoyan Z. (2004). Enhanced resistance to Aeromonas hydrophila infection and enhanced phagocytic activities in human lactoferrin-transgenic grass carp (*Ctenopharyngodon idellus*). *Aquaculture* 2004, 242:93–103

Wilson P.R., and Halver J.E. (1986). Protein and amino acid requirements of fishes. Annual Review of Nutrition 6, 225-244.

Zheng (2009). The effect of sodium butyrate on the growth performance and intestinal mucous structure of fresh water fish. PhD thesis
http://www.globethesis.com/?t=214336 0272979206

8.0 Appendix

Tab.1.A Contribution of aquaculture to the world total fish production** (excluding aquatic plant)
(source FAO 2011)

	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Aquaculture (Million tonnes)	34.6	36.8	38.9	41.9	44.3	47.3	49.9	52.9	55.7	59.0	62.7
Contribution to total (%)	27.6%	28.8%	30.6%	31.1%	32.4%	34.4%	35.5%	37.0%	38.2%	39.9%	40.1%
Capture (Million tonnes)	90.7	91.0	88.3	92.7	92.5	90.2	90.7	90.1	90.0	89.0	93.5
Contribution to total (%)	72.4%	71.2%	69.4%	68.9%	67.6%	65.6%	64.5%	63.0%	61.8%	60.1%	59.9%
Total fish production	**125.4**	**127.8**	**127.2**	**134.6**	**136.8**	**137.5**	**140.7**	**143.0**	**145.7**	**148.0**	**156.2**

** Total fish production includes production destined for human consumption and for non-food uses (such as reduction for fish meal and oil, etc.).

Fig.1.A Growth real and pojected, in world capture fisheries production, aquaculture and global population (data from various source)

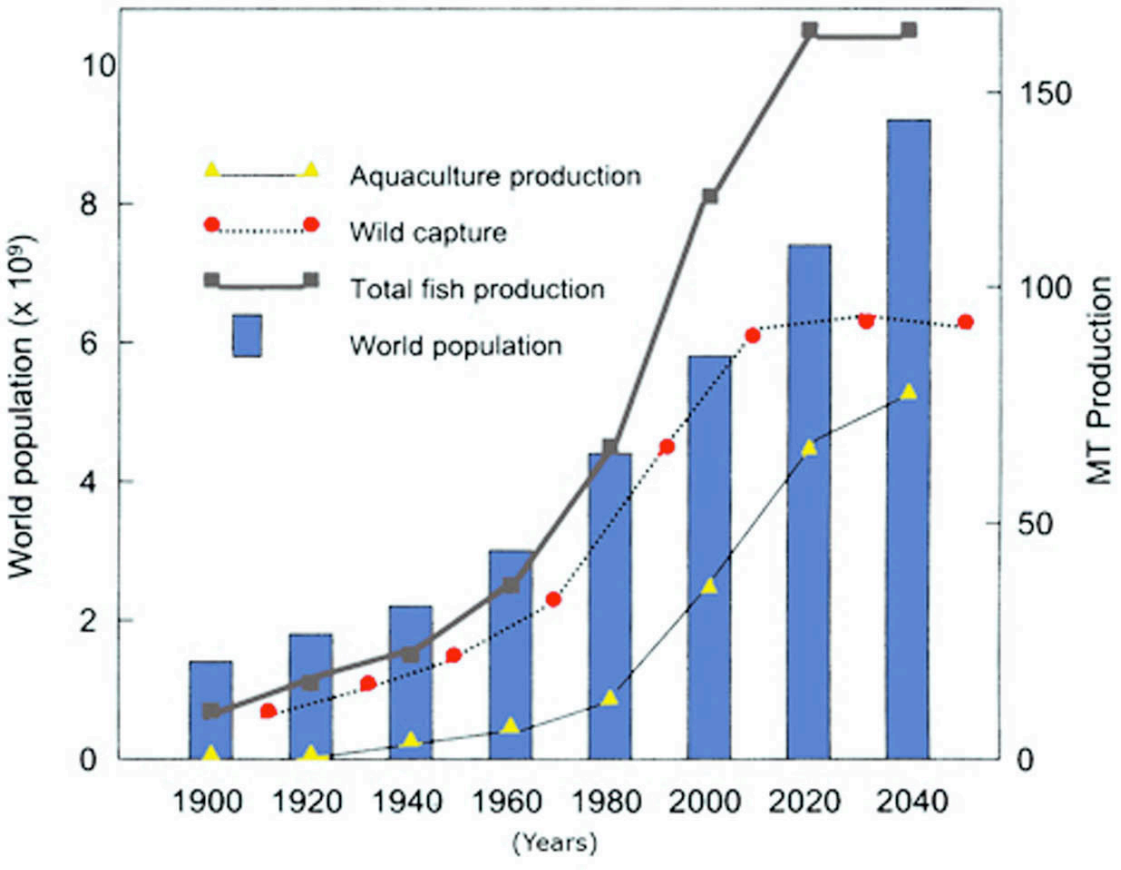


Fig.2.A Fishmeal (FM) anf Fish Oil (FO) production (source IFFO 2011)

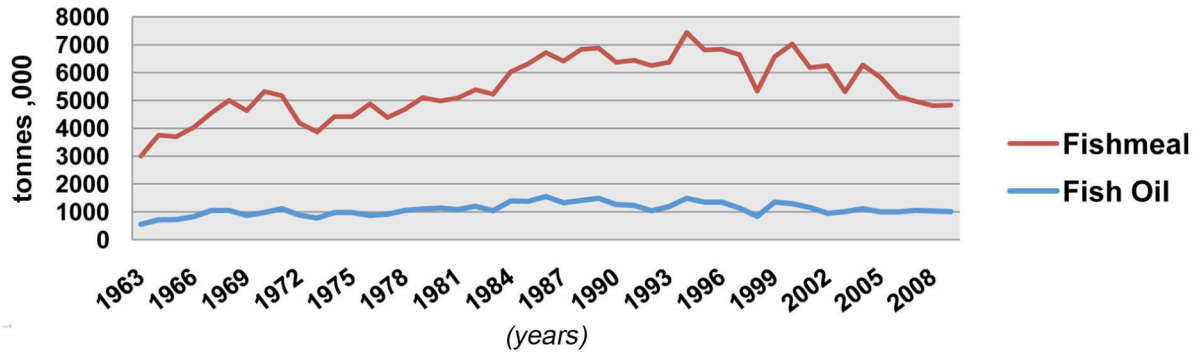


Fig.3.A Fishmeal usage (source IFFO 2011)

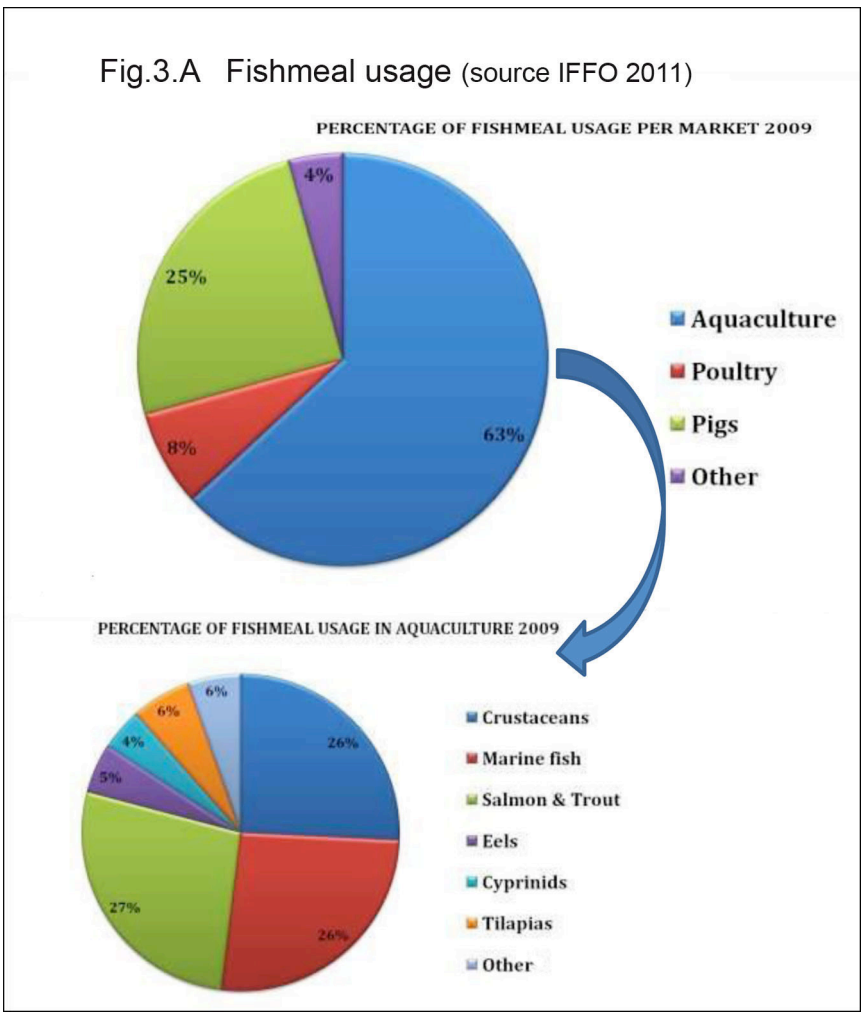
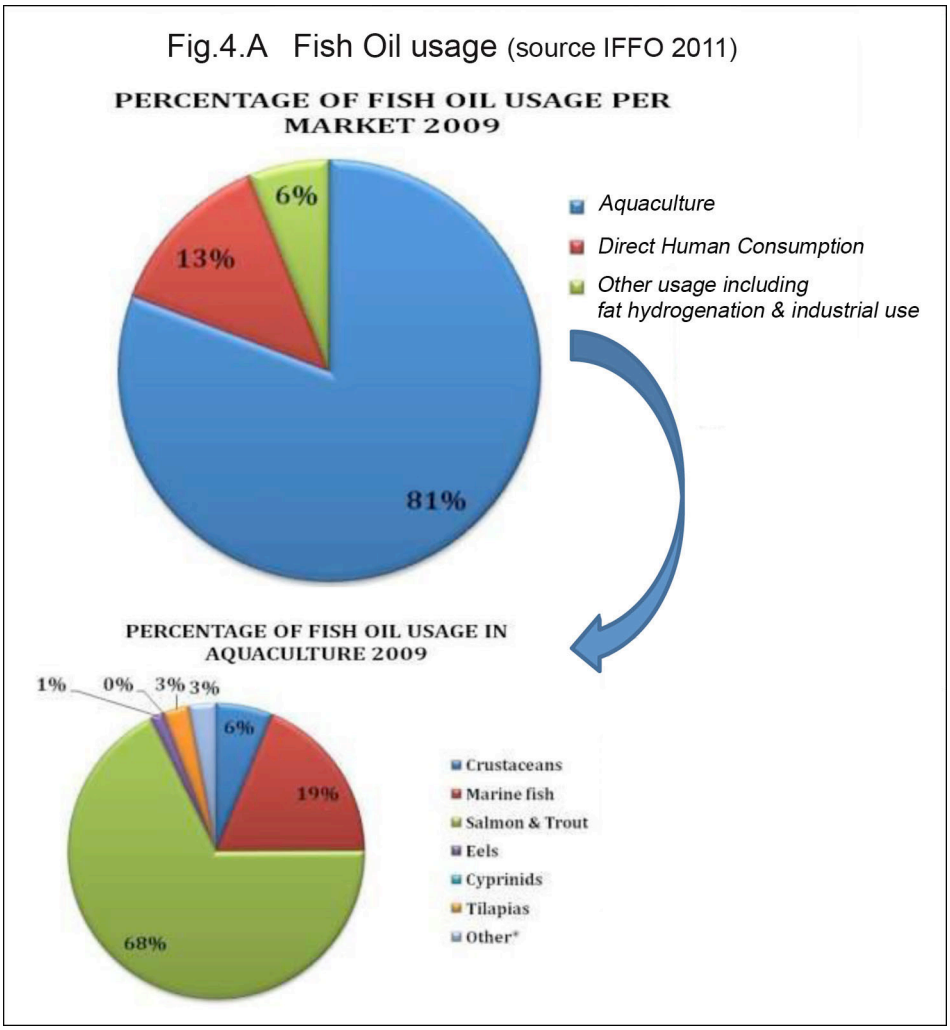


Fig.4.A Fish Oil usage (source IFFO 2011)



Tab.2.A Aquaculture production of European seabass, 2003 - 2007 (source FAO)

	2003			2004			2005			2006			2007		
	Volume (tonnes)	Value (€ '000)	Value (€/kg)	Volume (tonnes)	Value (€ '000)	Value (€/kg)	Volume (tonnes)	Value (€ '000)	Value (€/kg)	Volume (tonnes)	Value (€ '000)	Value (€/kg)	Volume (tonnes)	Value (€ '000)	Value (€/kg)
Greece	42 000	218 820	5.21	34 000	170 000	5.00	35 000	168 000	4.80	45 000	202 500	4.50	48 000	245 760	5.12
Turkey	20 982	89 391	4.26	26 297	103 999	3.95	37 290	165 164	4.43	38 408	160 033	4.17	41 900	172 808	4.12
Spain	4 529	25 589	5.65	4 700	26 085	5.55	5 492	25 263	4.60	8 930	40 185	4.50	10 480	52 190	4.98
Italy	9 600	62 400	6.50	9 700	65 520	6.75	9 100	61 200	6.73	9 300	66 000	7.10	9 900	70 300	7.10
France	3 500	21 280	6.08	4 000	29 680	7.42	4 300	29 498	6.86	5 600	41 104	7.34	4 800	32 832	6.84
Croatia	1 500	7 380	4.92	1 500	7 472	4.98	1 800	9 725	5.40	2 000	10 382	5.19	2 500	13 213	5.29
Cyprus	447	2 578	5.77	698	4 223	6.05	583	3 673	6.30	589	3 758	6.38	740	4 579	6.19
Egypt	1 789	6 816	3.81	1 812	6 541	3.61	4 192	13 372	3.19	4 372	15 564	3.56	4 598	11 817	2.57
Tunisia	458	1 823	3.98	466	1 901	4.08	525	2 447	4.66	493	2 278	4.62	683	2 998	4.39
Morocco	389	1 631	4.19	370	1 576	4.26	845	1 898	2.25	36	136	3.77	79	357	4.52
Malta	101	1 052	10.42	129	1 215	9.42	205	1 504	7.34	153	1 224	8.00	75	1 103	14.71
Albania	48	190	4.00	113	473	4.20	137	603	4.40	185	851	4.60	209	1 001	4.80
Montenegro	4	36 550	8.50	6	49	8.50	8	64	8.50	9	72	8.50	39	279	7.25
Algeria	3	9 840	3.28	3	9 840	3.28	3	9 840	3.28	0	423	3.00	1	3 308	4.00
Israel	251	1 365	5.44	169	933	5.52	6	33	5.52	36	182	5.05	26	157	6.04
Lebanon															
Libyan Arab Jamahiriya															
Slovenia															
Syrian Arab Republic															
Total	**85 601**	**450 192**	**5.26**	**83 962**	**429 507**	**5.12**	**99 486**	**492 283**	**4.95**	**115 111**	**544 692**	**4.73**	**124 029**	**612 702**	**4.94**

Notes:

Albania production is reported for both species. It is assumed here that it is divided equally between both species.
Egypt: Official GAFRD numbers are used. The author of the Country report estimates that production is overstated by about 7000 tonnes.

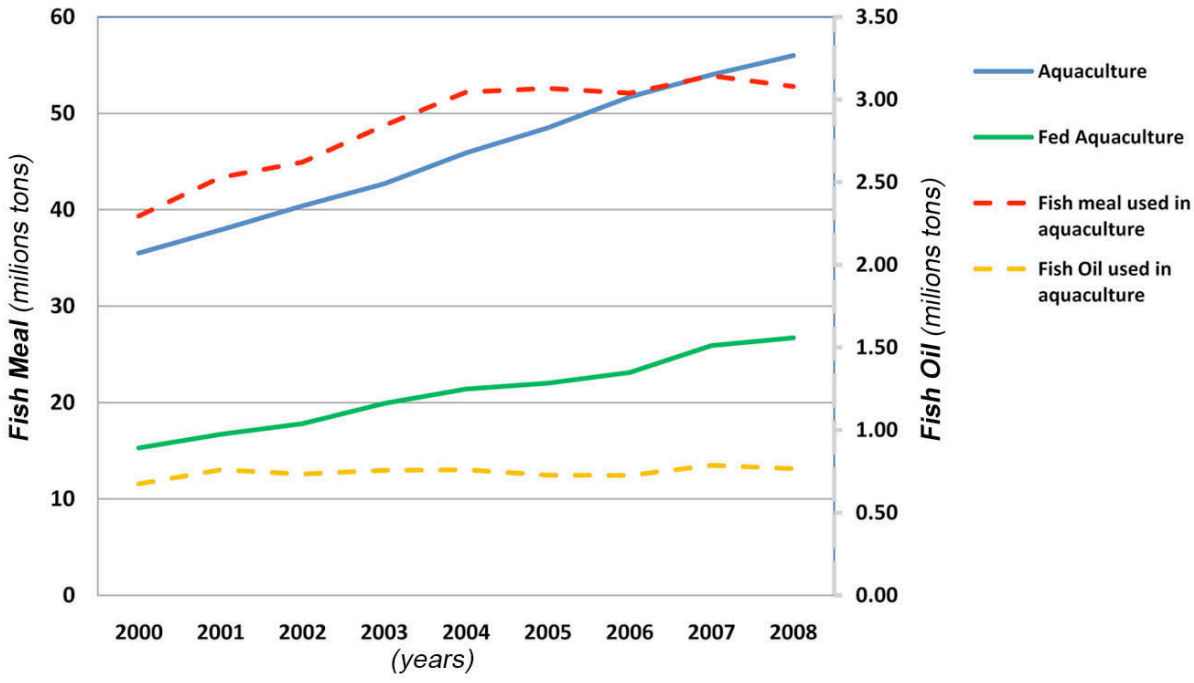
Tab.3.A Aquaculture production of gilthead seabream 2003 - 2007 (Suorce FAO)

	2003			2004			2005			2006			2007		
	Volume (tonnes)	Value (€ '000)	Value (€/kg)	Volume (tonnes)	Value (€ '000)	Value (€/kg)	Volume (tonnes)	Value (€ '000)	Value (€/kg)	Volume (tonnes)	Value (€ '000)	Value (€/kg)	Volume (tonnes)	Value (€ '000)	Value (€/kg)
Greece	55 000	268 400	4.88	48 000	223 680	4.66	50 000	226 000	4.52	66 000	284 460	4.31	79 000	282 030	3.57
Turkey	16 735	66 346	3.96	20 435	86.589	4.24	27 634	129 104	4.67	28 463	122 549	4.31	33 500	134 379	4.01
Spain	12 442	54 745	4.40	13 034	63 215	4.85	15 577	67 916	4.36	20 220	89 170	4.41	22 320	95 976	4.30
Italy	9 000	52 200	5.80	9 050	54 740	6.05	9 500	59 500	6.26	9 500	64 000	6.74	9 800	63 700	6.50
France	1 100	5 390	4.90	1 600	10 544	6.59	1 900	10 640	5.60	2 200	8 932	4.06	1 400	9 226	6.59
Croatia	1 000	5 611	5.61	1 000	5 541	5.54	1 200	6 859	5.72	1 500	8 582	5.72	1 500	8 894	5.93
Cyprus	1 181	5 852	4.96	1 356	7 288	5.37	1 465	7 859	5.36	1 879	10 251	5.46	1 404	8 081	5.76
Egypt	2 424	8 605	3.55	2 465	7 370	2.99	4 398	14 557	3.31	4 433	17 643	3.98	4 205	19 595	4.66
Tunisia	528	2 101	3.98	679	2 770	4.08	705	3 285	4.66	650	3 003	4.62	900	3 951	4.39
Morocco	378	1 152	3.05	350	1 081	3.09	332	2 842	8.56						
Malta	827	3 141	3.80	784	2 985	3.81	645	2 896	4.49	894	4 228	4.73	1 097	4 750	4.33
Albania	48	190	4.00	113	473	4.20	137	603	4.40	185	851	4.60	209	1 001	4.80
Montenegro	4	37 400	8.50	6	49	8.50	8	64	8.50	9	72	8.50	38	276	7.25
Algeria	1	3 280	3.28	1	3 280	3.28	1	3 280	3.28	1	3 100	5.00			
Israel	2 546	11 890	4.67	2 860	13 556	4.74	3 185	15 065	4.73	2 641	12 281	4.65	2 204	11 527	5.23
Lebanon															
Libyan Arab Jamahiriya															
Slovenia															
Syrian Arab Republic															
Total	**103 214**	**488 940**	**4.74**	**101 732**	**483 161**	**4.75**	**116 687**	**550 470**	**4.72**	**138 574**	**629 122**	**4.54**	**157 577**	**643 386**	**4.08**

Tab.4.A FIFOs for farmed species 2000 and 2010 (source IFFO 2012)

Farmed Fed Category	2000	2010
Eels	3.0	1.8
Salmonids (including trout)	2.6	1.4
Marine fish	1.5	0.9
Crustacea including shrimps & crabs	0.9	0.4
Tilapia	0.3	0.2
Other fed freshwater fish (e.g. catfish & pangasius)	0.6	0.2
Fed Cyprinids	0.1	0.1
Total for fed Aquaculture	**0.6**	**0.3**

Fig.5.A Developement of aquaculture production and usage of feedstuff, fishmeal and fish oil (source FAO & IFFO 2011)



9.0 Publications

Ingle E. and Paoloni C. (2011). L'allevamento del riccio di mare (Paracentrotus lividus): dalla riproduzione controllata alla produzione commerciale III Incontro tecnico-scientifico S.I.P.I. "Nuove frontiere in maricoltura: la diversificazione produttiva" On XVII Convegno nazionale S.I.P.I. (Società Italiana Patologia Ittica), book of abstracts, Ostuni (BR) Italy.

Bossù T., Amiti S., Di Giamberardino F., Cardeti G., Dante G., **Ingle E.** (2011). Prove di patogenicità in vitro su ceppi di *Vibrio alginolyticus*. XVII Convegno nazionale S.I.P.I. (Società Italiana Patologia Ittica), book of abstracts, Ostuni (BR) Italy.

Bossù T., and **Ingle E.** (2012). Acquacoltura in acqua dolce: principali specie di interesse per il mercato. AQUAMED 2012, Milano.

Saroglia M. and **Ingle E.** (2012). Sostenibilità della troticoltura in Umbria. API informa anno XXVII – N° 6 – 2012, pp 3 - 4

Ingle E. and Scollo G. (2012). La piscicoltura: una concreta risorsa per l'Umbria. IL PESCE N° 4 – 2012

Brambilla F., **Ingle E.**, Franzolini E., Rimoldi S., Scollo G., Terova G.,Saroglia M. (2012). Effects of different levels of vegetable meal inclusion in replacement for fish meal in practical diets for sea bass (*D. labrax*) on gut histology and nutrigenomic activity. On Aquaculture Europe, book of abstracts. Prague 2012

Ingle E., Terova G., Cocumelli C., Brambilla F., Antonini M., Rimoldi S., Ceccotti C., Saroglia M. (2013). Evaluation of histological changes, diet related in gastrointestinal (GI) tract of sea bass (*Dicentrarchs labrax*). XX ASPA 2013 book of abstract, Bologna, Italy

Ingle E., Bossù T., Cocumelli C., Brambilla F., Amiti S., Boria P., Ascione F., Saroglia M. (2013). "Gut health": analisi di alcuni indicatori del benessere nutrizionale e della conseguente risposta fisiologica in spigole (*D. labrax*) alimentate con diverse diete. XIX Convegno nazionale S.I.P.I. (Società Italiana Patologia Ittica), book of abstracts, Siracusa Italy.

Di Sirio A., **Ingle E.**, Cocumelli C., Marozzi S., Lanni L., Amiti S., De Santis L., Bossù T. (2013). Isolamento ed identificazioni di Micobatteri in telostei di acqua dolce e salmastra, Convegno nazionale S.I.P.I. (Società Italiana Patologia Ittica), book of abstracts, Siracusa Italy.

Ingle E., Terova G., Cocumelli C., Brambilla F., Antonini M., Rimoldi S., Ceccotti C., Saroglia M. (In Prep). Dietary related histological and molecular changes in the gastrointestinal tract of European seabass, fed different levels of substituition of fish meal with plant proteins."