UNIVERSITY OF CAMERINO
School of Advanced Studies

Doctorate course in

"LIFE AND HEALTH SCIENCES: ONE HEALTH"

XXXI CYCLE

A scientific contribution towards sustainable aquaculture

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SUMMARY

The present PhD project has been made possible thanks to the support and collaboration of the major rainbow trout (*Oncorhynchus mykiss,* Walb.) farm in Europe. It was initially based on the original research proposal aimed at examining rainbow trout aquaculture in order to find alternative raw materials to be utilized in the formulation of feed for all the stages of breeding of this important salmonid. However, in 2017 the farm which funded the research project began rearing marine fish species in the Adriatic Sea and it was, therefore, decided to widen the scope of the study to include European gilthead sea bream (*Sparus aurata*, L.).

During the course of the PhD triennium various activities relating to the above two species were carried out. With regard to rainbow trout, the first of the tree main studies focused on the effects on broodstocks of a diet which included materials of vegetable origin; the second assessed the supplementation of an innovative emulsifier into the diet of juveniles to improve the assimilation of lipids; the last one examined the qualitative traits of by-products obtained by rainbow trout processing for their possible reutilization.

In particular, a trial was carried out employing 4-year-old broodstocks of rainbow trout – both females and males – fed on a diet which totally substituted fish meal with vegetable meal and partially substituted (50%) fish oil with linseed oil. The effects of this diet, compared to a control diet containing fish meal and fish oil, were evaluated in terms of reproductive performances: quality of eggs, hatching and fingerling survival rate and mean body weight. Based on the results of this trial, it was possible to assume that feedstuffs containing vegetable protein and fat administered to broodstocks, negatively affected the quality of gametes and the progeny of rainbow trout.

The second trial was performed in order to investigate the effects on the growth performance and feed conversion rate of diets supplemented, at two different doses, with

a new emulsifier aimed at increasing lipid assimilation in rainbow trout juveniles. After 90 days of experimental feeding, the overall results of rainbow trout fed on the supplemented diets, can be considered satisfactory and within the normal range for the species size. Concerning the histology of the intestinal tract, a lower degree of irritation was observed in the trout fed on the experimental diets compared to those fed on the diets without the additive. The emulsifier supplemented in the feeds, resulted in beneficial effects together with a better welfare status of the rainbow trout.

The research went on to consider the qualitative traits of by-products obtained by rainbow trout processing, including muscle meat and skin. This issue has become more pressing worldwide due to the ever-increasing amount of waste and wastewater. Recently, attention has been focused on the possibility of extracting precious nutrients from rendered fish proteins. In this study, the proximate composition and the fatty acid profile of rendered rainbow trout were evaluated and compared with the traits of the trimmed fillet. The results showed that rendered fish from rainbow trout processing still contains valuable nutrients, which could be successfully considered as possible feedstuff and also employed in various sectors and in innovative ways as, for example, in the production of finger food and fish burgers.

Concerning sea bream, our research assessed the effects on the growth of juveniles of a partial substitution of fish meal with insect meal in their diet. Furthermore, the possibility of eliminating the use of antibiotics in sea bream rearing was also examined.

In the last few years, insect meal has become one of the most studied sources of protein feedstuffs as an alternative to fish meal. A growing trial was performed in order to evaluate the efficacy of the inclusion of chironomid meal in the feed of gilthead sea bream juveniles. To this aim, samples of chironomid midges at larval stage were collected from aquatic environments, converted into meal and analysed from a qualitative point of view.

Two experimental feeds with a different percentage of replacement were tested and compared to a control diet where the chironomid meal was absent. The experimental diets resulted in excellent palatability and led to satisfactory growth performances.

The research also considered the possibility of rearing gilthead sea bream without the use of antibiotics. In recent years, the issue of antimicrobial resistance has become of prime importance at an international level. The possibility of rearing antibiotic free fish is the challenge we face in the twenty-first century. In this context, the company involved in this Eureka project, has decided to work towards the improvement of rearing techniques, biosafety and the management of environmental parameters, in order to reach high standards of animal welfare, from breeding to transportation and subsequent stages, in the hope that this will result in a successful battle against antibiotic-resistance. To this goal, a trial was performed monitoring the production cycle of gilthead sea bream that were farmed adopting an antibiotic free protocol in an offshore cage plant. Their growth performance, health status and flesh quality were then compared with conspecific wild fish. The antibiotic free sea bream were fed on a diet that contained not only raw materials of aquatic origin – which constituted the main source of essential fatty acids of the omega 3 series – but also those of vegetable origin, in full respect of environmental sustainability. There was a strong similarity between the morphometric parameters and somatic indices of the two fish groups of different origin. Moreover, the antibiotic free sea bream displayed a very low lipid fraction similar to that of the fish captured in the Adriatic Sea, which classified them in the category of lean fish. Data concerning the omega 3 content, demonstrated that the antibiotic free samples could be defined either as “Rich in omega-3”, or as an “Omega-3 source”.

Keywords: rainbow trout, gilthead sea bream, fish farming, fish feeding, sustainability.

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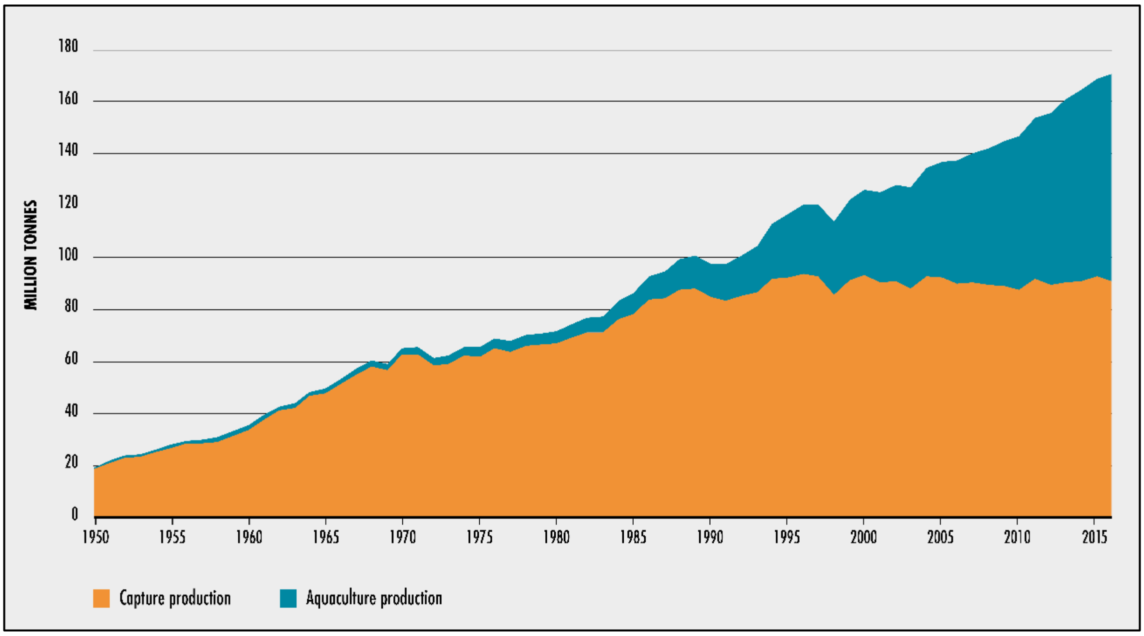
1. INTRODUCTION - ITALY IN THE INTERNATIONAL SCENARIO OF AQUACULTURE

Total fish production peaked at about 171 million tons in 2016 of which 88 percent (over 151 million t) was utilized for direct human consumption thanks to relatively stable capture fisheries production, reduced wastage and aquaculture growth. In 2016, the greatest part of the 12 percent used for non-food purposes (about 20 million t), was reduced to fishmeal and fish oil (74 percent or 15 million t), while the rest was mainly utilized for direct feeding in aquaculture and for livestock and fur animals.

Global aquaculture production in 2016 was 110.2 million tons. With capture fishery production relatively static since the late 1980s, aquaculture has been responsible for the continuing impressive growth in the supply of fish for human consumption (Figure 1.1). In per capita terms, fish consumption has grown from 9.0 kg in 1961 to 20.2 kg in 2015, at an average rate of about 1.5 percent per year. Preliminary estimates for 2016 and 2017 point to further growth to about 20.3 and 20.5 kg, respectively (FAO, 2018).

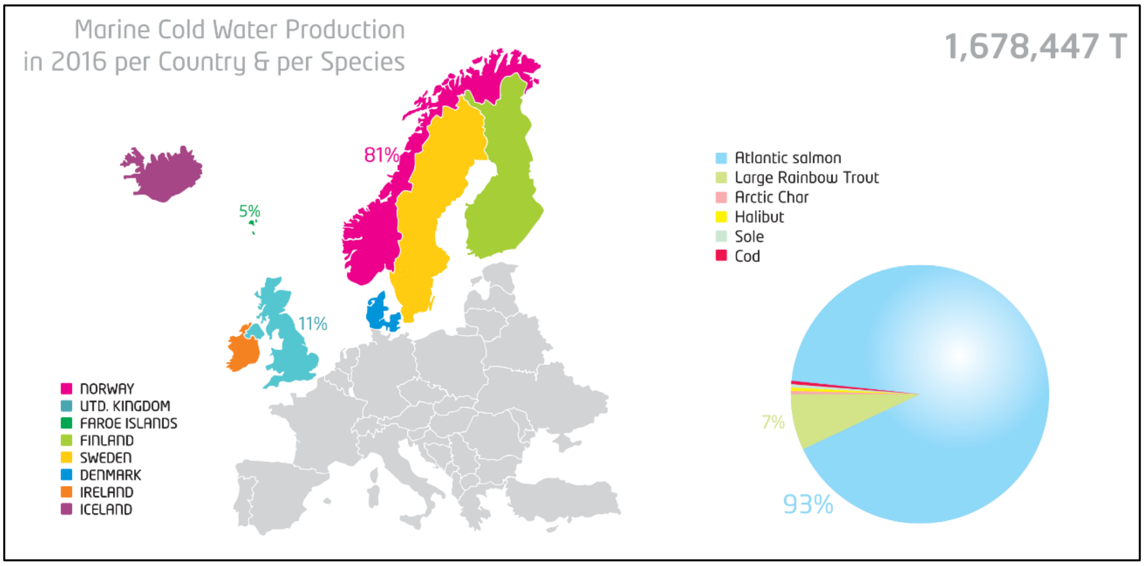
Figure 1.1: World capture fisheries and aquaculture production (Source: FAO, 2018).

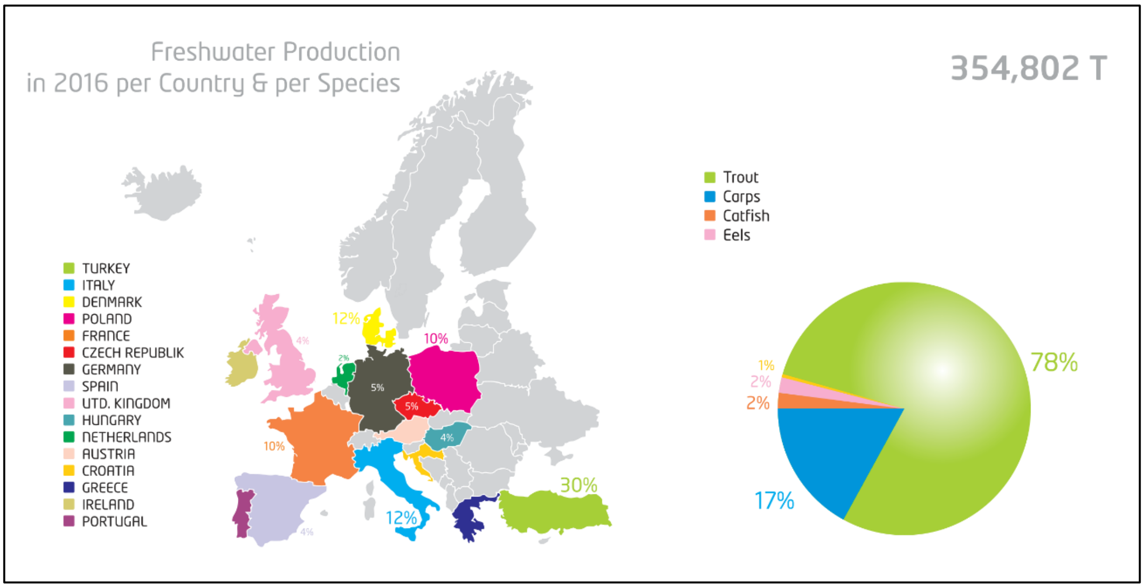


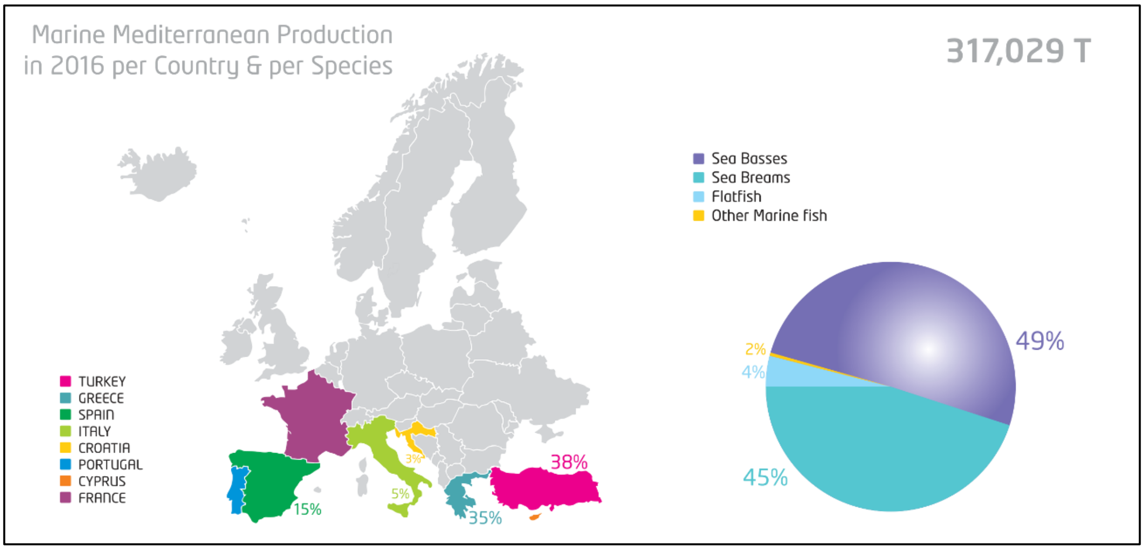
Compared to other producer countries, the European Union (EU-28) covered 3% of the total world production of the catching and aquaculture in 2015, and ranked fourth after India (EUMOFA, 2017).

According to aquaculture production statistics published by the Federation of the European Aquaculture Producers (FEAP) in 2017, European fish farming production reached 2,297,571 tons in 2016. This figure was obtained considering the twenty-one major fish-producing countries, including Norway and Turkey. 70% of the total production is represented by marine coldwater species, 16% by freshwater species and 14% by marine Mediterranean species. The main ones are salmon, trout, sea bream, sea bass and carp which make up 95% of the total European production (Figures 1.2a – 1.2c).

Figures 1.2a – 1.2c: Marine cold water, freshwater and marine Mediterranean productions in 2016 per country and per species (Source: FEAP).







Italy is the seventh largest fish producer in the EU with an estimated total quantity for 2016 of 53,790 tons. This data refers not only to the three most important species produced by aquaculture techniques, such as rainbow trout (*Oncorhynchus mykiss*, Walb.), sea bream (*Sparus aurata*, L.) and sea bass (*Dicentrarchus labrax*, L.), but also to sturgeons (*Acipenser* spp.), common carp (*Cyprinus carpio*, L), European eel (*Anguilla Anguilla*, L.) and meagre (*Argyrosomus regius*, Asso, 1801). Before Italy come Norway (1,307,182 t), Turkey (247,754 t), Great Britain (178,887 t), Greece (108,959 t), the Faroe Islands (77,000 t) and Spain (64,754 t).

Over the last 15 years, Italian finfish production has suffered a decrease in the main freshwater fish species reared (trout, eel, carp, catfish) and recently the production of the eurhyaline species has also declined (Table 1.1).

Based on these considerations, the main features of fish aquaculture in Italy are discussed in this chapter, focalizing attention on the single sectors of farmed species and their trend for the future.

With regard to sea bream and sea bass, at the beginning of the 80s, the farming of eurhyaline species was very limited due to the problems at reproduction and larval phases. However, in the mid-80s these problems had been solved and Mediterranean hatcheries, including those in Italy, started to supply large quantities of good quality fry. This situation led to an increase in its own production up to about 120 million fingerlings in 1998 and 1999. Subsequently, juvenile production decreased for a few years and started to rise again in the following years. In 2016, the production of fingerlings was estimated at around 75 million gilthead sea bream and 26 million sea bass. From the 90s until the new millennium, Italian production of commercial size sea bream increased steadily. Due to the development of mariculture techniques, with floating cages in sheltered areas, the product coming from inshore and offshore facilities converted more than half of the total Italian production. Today Italian offshore fish farms are characterized by medium production capacities with individual production at around 800-1000 t/year. Apart from a few farms, most of the companies use medium-sized circular cages, of about 1000-2000 m^3, equipped with polyethylene collars.

The offshore facilities are characterized by lower production costs compared with land-based farms, where energy and oxygen consumption negatively affect final costs. The offshore facilities further developed when cage technology was able to ensure a minor risk level and when 5-10 g fingerlings began to be available on the market. Moreover, products from open-sea facilities show a higher growth rate and a better flesh quality compared with those reared at high loading rates (30-40 kg/m^3) in land-based farms.

Table 1.1: Italian production (t) of major finfish species: 2000-2016.
Data elaborated from FEAP.

	2000	**2004**	**2010**	**2016**
Rainbow trout	44,500	39,000	39,000	33,800
Sea bass and sea bream	15,440	18,750	19,400	14,400
European eel	2,800	1,500	960	850
Sturgeons	400	400	1,900	1,000
Striped bass hybrids	-	-	400	400
Catfish	600	181	300	350
Meagre	-	696	320	190
Carps	100	222	700	700
Total	63,840	60,749	62,980	51,690

Current Italian production of sea bass and sea bream is stagnant and unable to compete with other Mediterranean producers. It ranks fourth after Turkey (72,342 t sea bass and 67,612 t sea bream), Greece (46,000 t sea bass and 59,000 t sea bream) and Spain (23,445 sea bass and 13,740 t sea bream) (FEAP, 2017). After the economic crisis of recent years, Greece ranks second, whereas Tukey has the dominant role and has undertaken to restructure its plants. At the same time, Spanish production is growing far more than that of Italy.

The strong competition, due to the importation of small size fish (B = 200-300 g), has induced many companies to diversify the commercial size, resulting in an appreciation for the intermediate (A = 300-400 g; AA = 400-600 g) and large categories (AAA = 600-800 g; G or E > 800 g).

On the other hand, with regard to fish consumption, our country is the first in the Mediterranean area and needs to import over 50,000 tons of sea bass and sea bream annually particularly from Greece and Turkey (FAO, 2018). Therefore, Italian companies have had to begin to take into account the need for improvement of the quality of the product, with particular attention to feeding management and to the fishing and slaughtering processes.

Unfortunately, national policies for species diversification were unable to offer new opportunities to farmers, so the production of “new species” is still extremely low. The transfer of the coordination of the research programmes from a national to a regional level has led to numerous small projects that were unable to guarantee continuity in research activities and has very often resulted in the repetition of the same experimentations.

For some species like meagre (*Argyrosomus regius*), unknown to the Italian market until a few years ago, the demand has been increasing, especially in Tuscany. For other species, mainly flatfish and Sparidae such as red porgy (*Pagrus pagrus*, L.) or blackspot sea bream (*Pagellus bogaraveo*, Brünnich, 1768), the quality and quantity of the fry are insufficient to sustain industrial productions. For sharpsnout sea bream (*Diplodus puntazzo*, Walb.) production is limited by the presence of parasitic pathogens.

The only partly successful activity involving a new species, is the fattening in large open sea cages of tuna (*Thunnus thynnus*, L.) caught as adult, but such an activity has low sustainability both for frozen mackerel, pilchard, herring and bogue used as feed, and for its impact due to the use of young tuna coming from the wild.

As regards rainbow trout (*Oncorhynchus mykiss*), it is the major fish species reared in Italy. In 2016, the national production of portion-size trout (350 g) amounted to 33,800 tons (FEAP, 2017). Italy was a leading producer in Europe until the late 90s with over 51,000 tons in 1997 (Melotti and Roncarati, 2009). Subsequently, difficulties due to the significant competitiveness of the Atlantic salmon market, together with the scandal of Belgium dioxin, the incidence of transmissible spongiform encephalopathies (TSEs) and the forbidden use of terrestrial animal origin meals in the feeds (2001) have led to an increase in production costs and a reduction in produce to 39,600 tons in 2005 and 2006. Efforts by farmers to defend the image of trout as rich in beneficial properties resulted fruitless.

Following this, a general awareness of the importance of the traceability and labelling of trout products has grown in agreement with the Codex Alimentarius (FAO/WHO, 2003), the guidelines of the Italian Association of Fish Farmers (API, 2003) and the FEAP code of conduct (FEAP, 2000).

In order to support farmers, two steps have been taken: the reduction of economic costs and the analysis of consumer demand. Concerning the first aspect, the most important feed manufacturers have introduced certain substitutes for fish meal and fish oil in trout feed as cheaper protein and lipid sources in order to reduce feed costs and enhance the sustainability of trout aquaculture (Kaushik et al., 1995; Romarheim et al., 2006). As far as the second step is concerned, surveys carried out in different European countries ascertained that in Italy rainbow trout is perceived as a traditional product with over two thirds of the production being consumed as whole fish (Cookson, 2002).

Nowadays, farmers are adapting trout product to market demand and are supplying the hypermarkets and chain stores and the results appear satisfying. Therefore, size differentiation, packaging and product processing (e.g. smoked trout or gastronomic preparations of the fillet) seem to play an important role in meeting the needs and attitudes of the modern consumer. Furthermore, the red-pink flesh coloration, caused by the deposition in the muscle of relatively large amounts of carotenoids obtained from the diet, such as astaxanthin, is becoming decisive for the diversification of the product. In this case, the fattening productive cycle lasts 3 months more and the final size is around 500 g.

Unfortunately, future trends in the sector indicate that saturation point has been reached, and rainbow trout production remains at 33,800 tons (FEAP, 2017). The trout culture will be forced to work on reducing the environmental impact, acquiring improved efficiency in management and controlling pathologies in order to obtain a high-quality product.

In addition, the prospects are not good for the production of rainbow trout for sport fishing due to fact that these fish can only be introduced in private waters. However, 13,000 tons are destined for recreational ponds and artificial reservoirs for fishing competitions.

An interesting opportunity could be offered by the rearing of other salmonid species such as brown trout (*Salmo trutta morpha fario*, L.) and brook trout (*Salvelinus fontinalis,* Mitchill, 1814), which are appreciated both for sport fishing and for the quality of the meat. In Tuscany, operators are working to create a local brand called "trota dell'Appennino", in order to qualify their product.

As regards eel, the European aquaculture production depended and depends on juveniles ("ragani"), glass eels and elvers. Their availability, formerly used for human consumption or for stocking rivers, has declined markedly; the number of juveniles coming from the Atlantic has steadily fallen and stands now at around 5% of the average level in the 1970s (EIFAC/ICES, 2006).

Moreover, the availability of young eels for aquaculture purposes has shown a significant reduction, resulting in a very high increase in prices. In the 90s, Italian production of eel was estimated at around 4,000 tons. At that time, farming techniques had been greatly improved and feed formulations were satisfactory (Roncarati et al., 1998). However, due to the increasing cost of seeding, the profit margins of intensive farms fell and productions progressively decreased to 850 tons in 2016. This reduction in production was also recorded in other European countries, such as Denmark, whose eel production fell from 3,700 tons in 2008 to 2,885 in 2016.

Considering the decline in catches, eel is now listed in the European Habitats Directive as a species of Community interest and the fishing and exploitation of eel in the wild may be subject to management measures. Besides, the European Commission (EC) has instituted an Eel Recovery Programme, as suggested by the ICES declaration of 1998, which reported that the European eel stocks need protection and the current fishery is not sustainable anymore (EIFAC/ICES, 2006).

Prospects for the eel sector are considered very limited, not only for the scarce availability of seeding, but also due to the demand, which shows a marked decline. In fact, in the last few years, eel farming has suffered a steady decline due to the increase in the price of "ragani" and elvers and a reduction in demand from Germany and the Netherlands, which produce eels themselves or buy them from Denmark. Considered as a whole, the European production of eel has decreased as a consequence of the increase in production costs and the rise in price of the smoked eel.

Only product coming from brackish waters seems to be attractive to the market. This is the case of eels from Valli di Comacchio, located in the province of Ferrara and spreading over 10,800 ha; Valli di Comacchio is known for being the most important area for the production of both fresh and processed eel (Cecchini, 2001).

Unfortunately, environmental modifications of the area and the strong reduction in the migration of elvers from the sea ("montata") has brought about the closure of the marinated plant. In 2004, the building of the old "Azienda Valli Comunali di Comacchio" was completely restructured in order to process eel products following the ancient traditional techniques dating back to 1818 (“Fabbrica Normale” of Body Administration of Comacchio). The recent institution of the Delta Park of the river Po, in which Valli di Comacchio is located, can help promote the marinated eel of Comacchio and, in general, may be an important tool in qualifying the species.

As an alternative fish to eels, some plants have attempted to rear hybrid striped bass (*Morone saxatilis* x *Morone chrysops*), which originates from the United States; currently, there have been favourable results, with production exceeding 400 t/year. The larval phase is carried out in hatcheries applying techniques derived from those of sea bass and sea bream. On-growing takes place in outdoor basins, where the hybrid striped bass are cultured for 16-18 months to a large marketable size (800-900 g). They are sold as whole fish to chains of supermarkets that promote this product especially when sea bass and sea bream are not available or not convenient. The Italian Ministry of Agriculture has recognised the importance of this fish, having included in the updated list of commercialized fish products, assigning “persico-spigola” as its common name (DM 31 January 2008 – Denominazione in lingua italiana delle specie ittiche di interesse commerciale).

Another Italian aquaculture sector is represented by sturgeon farming which topped 1,000 tons in 2016 (FEAP, 2017). In recent years, sturgeon farming has obtained a high economic return orienting the culture to caviar production. One of the most important species reared for roe is the white sturgeon (*Acipenser transmontanus,* Richardson, 1836), since on intensive farms it grows and matures faster than in the wild (Doroshov et al., 2005). In Italy, three companies are specialized in this sector. After determining the gender at 3 years old, they sell the males for meat and rear the females to maturity. The majority of white sturgeon females mature at 7-9 years old at a live weight of 30-40 kg and produce approximately 3-4 kg of roe (weight considered before salting for caviar).

The Italian production of caviar amounts to 21 t/year. Unfortunately, the synchronization of ovarian maturation and the prevention of disease transmission are still crucial factors that may reduce productivity. In this situation, future trends are not encouraging because the long maturation of sturgeons makes it more difficult to attract investors for such business.

With regard to catfish, until 1990 production was well consolidated with over 3,000 t/year, especially in Emilia Romagna, Lombardia and Veneto, where the common catfish (*Ictalurus melas,* Rafinesque, 1820) had a tradition as food supply and sport fishing. Since 1994, a drastic and progressive reduction took place plummeting to 350 tons in 2016. This fall was brought about by a herpesvirus first isolated in two catfish farms in 1994. After that, mass mortalities were recorded in the largest Italian plants and most of these were forced to give up farming common catfish (Melotti et al., 1999). Nowadays, this pathogen is still particularly dangerous, since vaccines are unavailable and, furthermore, channel catfish (*Ictalurus punctatus*, Rafinesque, 1818) cannot be reared as a substitute since it is not appreciated by the Italian market.

Regarding Cyprinids, the common carp (*Cyprinus carpio*), produced in the past for recreational fishery, has declined to almost zero tons over the last few years due to the strong competition from Eastern European countries, such as Hungary and Croatia (the main exporters to Italy), making it difficult for Italian farmers to stay in the market. Concerning the prospects for the carp, a possible way to sustain the field could be the diversification of the species, taking into consideration Chinese carps or the common tench (*Tinca tinca*, L.). The latter seem to play an important role in valorising local production like, for example, the “Tinca gobba dorata del Pianalto di Poirino” in the region of Piemonte.

With regard to other cyprinids raised in Italy, goldfish (*Carassius auratus*, L.) are produced for ornamental purposes providing about 20 million specimens per year, but this practise is a restricted business. The plants were the goldfish are reared, are often involved in koi carp production, estimated at around 3 million specimens in 2006. Recently, a viral disease (KHV) has compromised its expansion causing high mortality rates in several countries worldwide (EU, United States and Asia) (Ronen et al., 2003). Thus, every international trading and shipment operation of koi stocks requires health condition certifications, which prove very expensive for the Italian farmers.

In recent years, European and Italian aquaculture has shown a markedly different trend from world aquatic production. Asia, and particularly China, has recorded a constant and rapid growth and Latin America has also had a moderate development.

In our continent, Norway has registered a very strong increase in salmon and large size rainbow trout production. For the former species, Norway represents the world leader, whereas it is the main continental producer of trout. This situation has had negative consequences on the production of Salmonids by countries traditionally involved in trout farming (France, Italy, Denmark), which, due to the fierce Norwegian competition, have been obliged to reduce their production.

The Italian situation has become critical since the steps taken in order to diversify the product have not been sufficient to relaunch the Italian trout onto the market.

Italian production of marine fish species (sea bass and sea bream) faces a very similar situation to the trout sector. Greece and Turkey sell their product almost exclusively on the Italian market at prices that are cheaper compared to that of our national product. Therefore, the market available to the Italian farmers is being eroded. Consequently, our producers can only count on the better quality of the Italian product.

2. EFFECTS OF DIFFERENT FEEDS ON PERFORMANCES OF RAINBOW TROUT (O*ncorhynchus mykiss*) BROODSTOCKS

2.1. Introduction

In aquaculture, the feeding of broodstocks plays a key-role because it affects the quality of the gametes, their fecundity, hatching rate, fry survival and incidence of larval deformation, regardless of genetic selection and the strain employed (Bromage et al., 1990; Migaud et al., 2013). In recent years, the research of feedstuffs alternative to fish meal, in order to increase the sustainability of the productive cycle, has also been focalized on the feeding of rainbow trout (*Oncorhynchus mykiss*) broodstocks (Kaushik and Seiliez, 2010; Kwasek et al., 2014). Efforts are being concentrated on new diets able to guarantee health benefits, satisfying the essential requirements of females and males, as well as a good quality of gametes, larvae and fingerlings.

The feeding plan for broodstocks can significantly affect the yolk sac reserves of the eggs, as well as the larval development and survival rate (Fernández-Palacios et al., 2011) and innovative feeding strategies have been adopted, which also include new plant feedstuffs. Considering the response of several metabolism-related and growth-related genes and proteins in trout subjected to different dietary methionine levels (deficient or adequate), it was shown that the diet of the broodstocks can influence the metabolism of the progeny in rainbow trout (Seiliez et al., 2017).

Based on these assumptions, a trial was carried out employing 4-year-old broodstocks of rainbow trout in order to evaluate the effects of two feeds containing feedstuffs of different protein and lipid source on reproductive performances in terms of the quality of eggs, hatching and fingerling survival rate and mean body weight.

2.2. Materials and methods

Four months prior to the spawning season, broodstocks were selected and divided into two experimental groups, with two replicates each (GF1-GF2, GV1-GV2), represented by two raceway sectors (10 m x 1 m x 1 m). Each group contained 20 females/replicate, whereas 10 males/group were reared in two other raceway sectors. Groups GF (females and males) were fed a diet containing fish meal and fish oil as control feed. Groups GV (females and males) received a diet including legume protein feedstuffs and oil of vegetable origin (50% linseed oil and of 50% fish oil). The ingredients of the two feeds are reported in Table 2.1. GF, GV broodstocks were fed at a daily ratio of 0.8% body weight for 120 days until the spawning season. The water temperature, dissolved oxygen and pH were periodically recorded. The proximate composition and amino acid and fatty acid profile of the two feeds were analysed according to international methods (Tables 2.2 – 2.4).

Table 2.1: Ingredients (g/kg) of the two feeds for GF and GV broodstocks.

Ingredients (g/Kg)	GF	GV
Fish meal (65% - anchovy)	340	
Gluten corn meal	290	290
Wheat meal (12 CP)	280	5
Pea meal		200
Gluten (wheat)		100
Soybean meal		195
Peanut meal		100
Fish oil	80	50
Linseed oil		50
Lecithin - Soy (70%)	5	5
Vitamin C	1	1
Mineral-vitamin premix	4	4

Table 2.2: Proximate composition (%) of the two feeds for GF and GV broodstocks.

Proximate composition (%)	GF	GV
Dry Matter %	91.53	91.88
Crude Protein %	44.85	43.65
Lipid %	14.21	14.16
Ash %	6.03	2.98
Fibre %	1.13	3.62
Metabolizable energy (kJ/g)	18.92	18.78

Table 2.3: Amino acid profile (%) of the two feeds for GF and GV broodstocks.

Amino acid profile (%)	GF	GV
Arginine%	2.09	2.26
Histidine%	0.99	0.86
Isoleucine%	2.08	1.80
Leucine%	5.46	5.70
Lysine%	2.19	1.26
Methionine%	1.21	0.68
Phenylalanine%	2.37	2.44
Threonine%	1.73	1.43
Tryptophan%	0.58	0.22
Valine%	2.31	1.98
Ca%	1.52	0.14
Available P%	1.20	0.13

Table 2.4: Fatty acid profile (%) of the two feeds for GF and GV broodstocks.

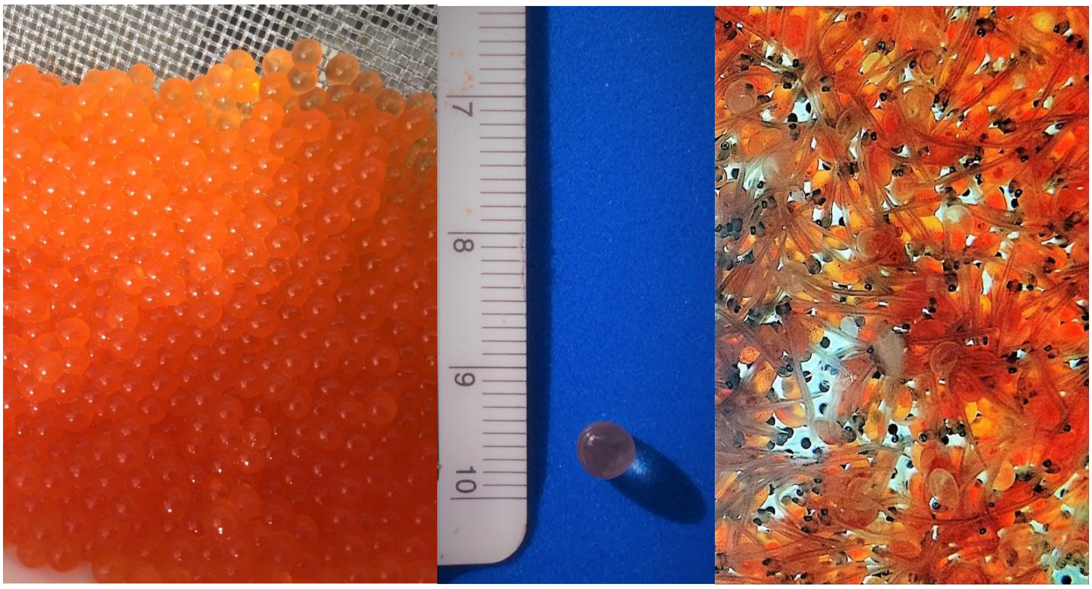
Fatty acid profile (%)	GF	GV
LA (18:2n-6)%	5.51	26.2
LNA (18:3n-3)%	10.23	6.4
ARA (20:4n-6)%	0.59	0.83
EPA (20:5n-3)%	7.43	3.82
DHA (22:6n-3)%	4.96	2.49
Total n-3%	22.62	12.71
Total n-6%	6.1	27.03
n3:n6	3.71	0.47
Total phospholipids%	7.62	3.53

At the beginning of November, the females of the two groups were checked and 4 females/replicate/group were isolated in $2m^3$ tanks, anesthetized and stripped. After stripping, the fish were weighed. The eggs were removed from each mature female by hand-pressure on the abdominal wall (Figure 2.1) and separately dry-fertilized in a plastic bowl with 2.5 ml of sperm per litre of eggs, obtained from a pool of 5 males. 2-3 minutes after fertilization, the eggs were washed 3-4 times and transferred into trays with running water supplied from a natural spring (10.0 ± 0.5°C), maintaining in separated trays the eggs obtained from the different females (GF, GV). Water exchange ranged from half an hour to 1 hour and 45 minutes in each tray. The hatching rate was recorded on the viable eggs.

Figure 2.1: Hand stripping of female rainbow trout broodstocks.

GF and GV larvae and fingerlings of the different groups were monitored daily over 90 days starting from the yolk sac resorption. The stocking density was 5,000 larvae/tray.

Figure 2.2: Rainbow trout eggs and larvae.



The size and the proximate composition of the feed administered to fingerlings of the two groups are reported in Table 2.5.

Table 2.5: Size (mm) and proximate composition (%) of the feed administered to fingerlings of the two groups.

Size (mm)	0.3 → 1.0
Moisture (%)	14.7
Crude protein (%)	57
Lipid (%)	17
Ash (%)	10

The survival rate and mean body weight of the two groups were updated weekly. Data were submitted to one-way ANOVA and the differences between the means were evaluated using the Student-Newman-Keuls (SNK) test (SAS, 2004) and considered significant at $p < 0.01$.

2.3. Results

Gamete and fingerling data differed among the batches, showing a significantly higher performance with regard to the quality of viable eggs in groups GF compared to groups GV. The hatching rate of GF was 92±2% *versus* 71±3% of GV (Table 2.6). The mean body weight of fingerlings at the end of the trial was 4.49 g for the GV and 5.56 g for the GF group (Figure 2.3). The survival rate of fingerlings at 90 days also showed notable differences being 74±4% *versus* 49±3% in GF and GV groups, respectively (Figure 2.4).

Table 2.6: Reproductive performances of the two groups of broodstock females.

	GF	**GV**
Mean body weight (g)	1842 ± 190	1737 ± 148
Mean weight egg (g)	0.053 ± 0.002	0.048 ± 0.003
Total eggs/female (n.)	2467 ± 155	2239 ± 174
Egg diameter (mm)	4.2 ± 0.1	3.9 ± 0.2
Hatching rate (%)*	92.2 ± 2^{a}	71 ± 3^{b}

*Hatching rate was calculated on incubated eggs
Data are expressed as mean ± sd. Different superscripts (a, b) in the same row indicate significant differences between the fish groups ($p < 0.01$).

Figure 2.3: Mean body weight (g) of fingerlings during the 90-day trial.

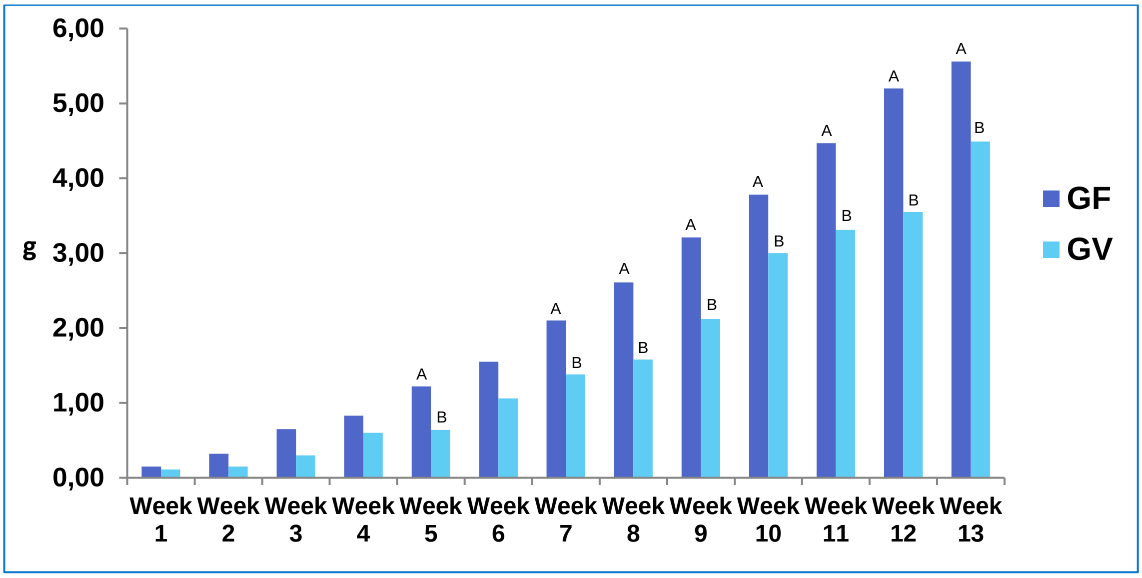
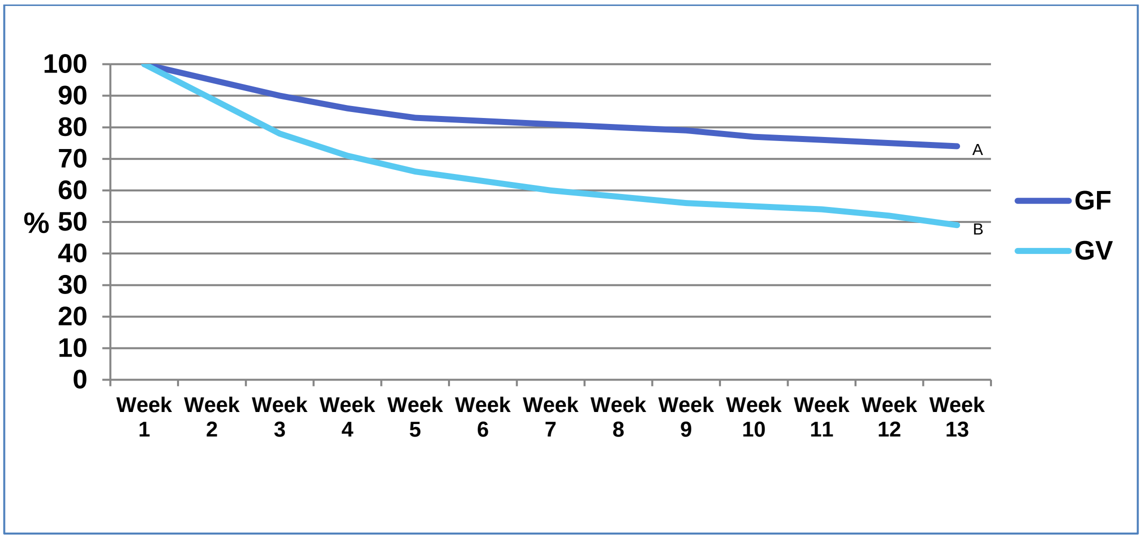


Fig. 2.4: Survival rate (%) of fingerlings during the 90-day trial.



2.4. Discussion

The present trial evaluated the effects of the total substitution of fish meal and the partial replacement of fish oil in broodstock feeds (GV) on the reproductive performances of rainbow trout. The substitution of fish meal and fish oil with vegetable sources in aquaculture feeds is an important step in preserving wild fish stocks (clupeids) and reducing feeding costs. The group of females fed on GV feed exhibited significantly lower reproductive performances than broodstocks fed on the GF diet in terms of hatching rate, fingerling body weight and survival at 90 days (Table 2.6, Figures 2.3 and 2.4).

GF group results, in terms of eggs quality (egg diameter, egg weight, hatching rate), agreed with those reported in literature (Bobe and Labbé, 2010). In the GV feed, some amino acids (Lysine, Methionine, Tryptophan), minerals (Ca, P) and n-3 highly unsaturated fatty acids (HUFA) were imbalanced, whereas the GF feed showed a better amino acid profile and a higher percentage of eicosapentanoic acid (EPA) and decosahexanoic acid (DHA). The low amino acid content, particularly in methionine, may have affected the survival and mean body weight of fingerlings, as reported in recent papers (Fontagné-Dicharry et al., 2017; Seiliez et al., 2017). The fatty acid low content may have affected the egg diameter and hatching rate, although in the GV feed a higher n3/n6 HUFA ratio was used with respect to some other studies. Results reported in literature show that at least 1.1% n-3 HUFA dietary content is necessary to obtain good reproductive performances (Vassallo-Agius et al., 2001; Bransden et al., 2005).

These variations could be due to differences in feedstuffs: in the GV diet the fish meal was totally replaced by meal obtained from different legumes, and 50% of the fish oil was substituted by linseed oil, with consequent differences in the content of the essential nutrients.

Based on the results of this trial, it was possible to assume that feedstuffs containing vegetable protein and fat administered to broodstocks, affected the quality of gametes and the progeny of rainbow trout negatively.

3. RAINBOW TROUT (*Oncorhynchus mykiss*) GROWING TRIAL USING DIETS WITH DIFFERENT ADDITIVE CONTENTS

3.1. Introduction

The rising interest in fish welfare, sustainability and health management has focalized the attention of research on fish feeding based on feedstuffs alternative to fish meal and fish oil, in order to preserve wild fish stocks (clupeids) and reduce the cost of formulated diets (Matos et al., 2016). These issues are always connected with the production of trout rich in long chain n-3 polyunsaturated fatty acids (PUFA), since they are beneficial to cardiovascular health.

Nowadays, terrestrial plant-based feedstuffs (soybean, wheat, pea, corn, etc.) are increasingly used as substitutes in feeds for salmonids. Studies conducted on diets containing low rates of fish meal and high levels of plant protein sources, have shown lower growth performance in rainbow trout, mainly linked to reduced feed intake and antinutritional factors (Bell et al., 2003; Pierce et al., 2008). Furthermore, in the feeding of rainbow trout broodstocks, the total substitution of fish meal and the partial replacement of fish oil in feeds with vegetable sources (soybean and pea meals) negatively affect the reproductive performances of rainbow trout in terms of egg quality (egg diameter, egg weight, hatching rate), fingerling body weight and survival rate at 90 days (Bobe and Labbé, 2010; Meligrana et al., 2017). In addition, the vegetable sources affect the quality of the lipid content of the fish meat due to the lower rate of PUFAs, compared to fish fed on oil from aquatic origin (Roncarati et al., 2010; Matos et al., 2016). However, thanks to the high availability, low costs and the decreased presence of dioxins and pollutants, the replacement of fish oil with vegetable oils appears to be a sustainable prospect.

The most important obstacle to the substitution of fish oil with other sources, is represented by the need to maintain a balanced supply of essential fatty acids and an optimal health status. The change in the lipid origin and fatty acid composition in the diet may alter the haematology, metabolism, physiology and immunology of the fish, which, in turn, could affect fish health and resistance to stress and disease (Turchini et al., 2009; Oliva-Teles, 2012). The high percentage of inclusion of vegetable oils in the diets has been associated with lipid droplet accumulation in the intestinal tissue of fish (Caballero et al., 2002).

Phospholipids are surface-active substances and can therefore be used in a wide variety of ways: as an emulsifier, absorption enhancer, dispersion agent and wetting agent. Phospholipids play an important role in animal nutrition as emulsifying agents, and assist in the digestion of fat. They also help in fatty acid absorption by the formation of micelle structures.

The beneficial effects of dietary phospholipids in fish include improved growth in both larvae and early juveniles, increased survival rates and decreased incidence of malformation in larvae, and even increased stress resistance (Fontagné et al., 1998).

In fish species, juveniles receiving diets including phospholipids show enhanced growth, high survival rates and stress resistance; in larval fish stages, a reduction of the incidence of morphological alterations has been shown (Cahu et al., 2003; Wold et al., 2007).

In the grow-out phase, phospholipids are necessary as emulsifiers in the formation of mixed micelles in the digestive tract (Olsen and Ringø, 1997) and appear to be crucial for lipid transport (Fontagné et al., 1998; Caballero et al., 2006).

A specific class of phospholipids, the lysophospholipids (LPL) are of particular interest in the absorption of nutrients as they are more hydrophilic than other phospholipids. For animals to utilize fat, they have to digest and absorb it from the gastro-intestinal tract.

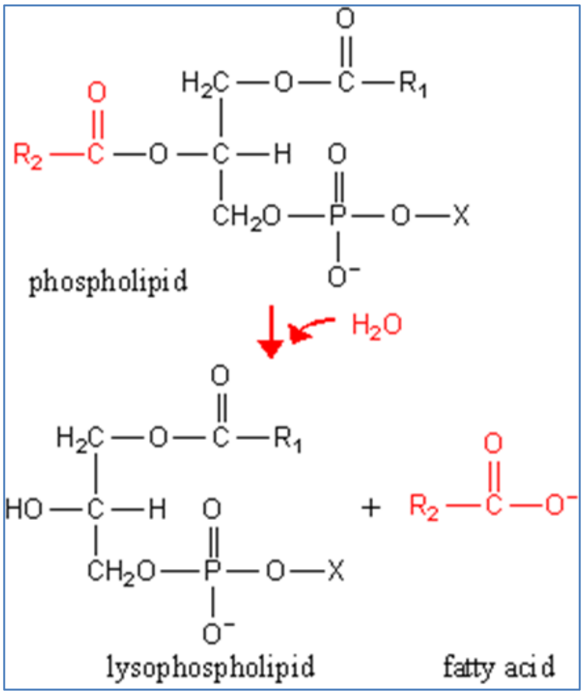
Since fat is insoluble in water and difficult to handle in a water medium, as in the gastrointestinal tract, water-insoluble ingredients like fatty acids, fat–soluble vitamins and certain amino acids rely on emulsification to become water soluble and thus, bioavailable.

LPLs spontaneously form micelles with bile salts, fatty acids and monoglycerides. These micelles are smaller and more stable than those formed with other phospholipids found in, for example, lecithins.

The LPLs are simple phospholipids that have been recognized for decades as components in the biosynthesis of cell membranes. LPL refers to any phospholipid that is missing one of its two O-acyl chains (Figure 3.1). Thus, LPLs have a free alcohol in either the sn-1 or sn-2 position. The LPLs are recognized as important extracellular signalling molecules and lipid mediators. LPLs aid digestion of other lipids, by breaking up fat globules into small micelles.

Lysophosphatidic acid (LPA; receptors LPA1– LPA4) (Figure 3.2) and sphingosine 1-phosphate (S1P; receptors S1P1 – S1P5) have enabled a greater mechanistic understanding of their diverse roles in biological processes. LPA and S1P regulate the development and function of numerous organ systems, including the cardiovascular, nervous, immune, and reproductive systems. Altered LPA signalling has been implicated in the aetiology of disorders, such as inflammation, autoimmune diseases, neuropathic pain, atherosclerosis, cancer and obesity (Ishii et al., 2004).

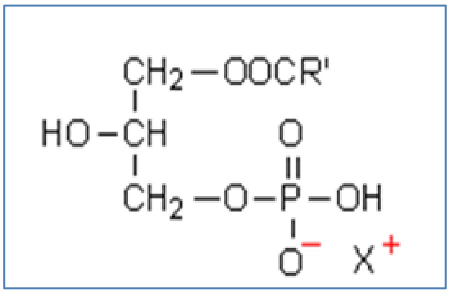
Figure 3.1: Biosynthesis of lysophospholipids.



LPLs modify the fluidity of membranes as a membrane transducer to accelerate diffusion through the cell lipids (Lundbaek and Andersen, 1994).

In pigs, fed on a diet including LPLs during the growing phase, a greater digestibility of nutrients as a consequence of the high rate of emulsification was observed (Kim et al., 2018). However, an increase in the bile salt micelle capacity and the interior capacity of micelles in the intestine to increase long-chain saturated fatty acid solubilisation in the presence of phospholipids, may have also contributed to this effect (Reynier et al., 1985).

Figure 3.2: Lysophosphatidic acid.



With regard to the possibility of providing potential health benefits while satisfying nutritional requirements of the cultured species, the supplementation of functional nutrients is recommended as a promising strategy in order to sustain the growth and the welfare status of the fish.

3.1.1. Aim of the study

A leading company in the ingredient manufacturing industry set out to test a new emulsifier with the aim of optimizing the three steps in lipid digestion (emulsification, hydrolysis with micelle formation and adsorption) in animal species of zootechnical interest.

In the present study, a growing trial investigated the effect of this new emulsifier supplemented into the diet at two different doses, on the growth performances and feed conversion of rainbow trout during the pre-growing phase.

3.2. Materials and methods

3.2.1. Growing trial

The trial was performed at a hatchery located in Colli sul Velino (RI), 100 km from Camerino University (UNICAM), School of Biosciences and Veterinary Medicine. The hatchery was rented from the foremost European company of rainbow trout aquaculture (Eredi Rossi Group with headquarter in Sefro, Macerata) according to an agreement between the ingredient manufacturing company and UNICAM, in which the fish farms of Eredi Rossi Group were involved. Some of the tanks of the Colli sul Velino farm were utilized to carrying out the trial for the manufacturing company.

Figure 3.3: Tanks employed for the trial.

The trial began on 20 April 2018 and lasted until 20 July 2018. The acclimation phase to the new diets lasted 6 days.

For the trial, we employed 12 tanks (3 tanks per group) (length 5.65 m; width 0.77 m; depth 0.50 m) of 2.20 m^3 volume each. During the first week of May, all the tanks were covered with an antifouling net in order to avoid algal development and to keep away ichthyophagous birds.

Figure 3.4: Net covering the tanks used for the trial.

3.2.2. Feeding protocol

The test additive emulsifier was supplied by the manufacturing company in a ready-to-use powder form. The test product was used at doses of 0.05 and 0.075 of complete feed. For the aim of the study, 4 groups with 3 replicates each, were considered:

1) Positive Control (normal energetic level)

2) Negative Control (Positive Control without a fraction of energy added)

3) Negative Control plus additive at 500 ppm

4) Negative Control plus additive at 750 ppm

The Positive Control, Diet 1, was formulated in accordance with the person in charge of the nutrition line of Eredi Rossi Group in order to obtain a feed similar to the diet employed for rainbow trout juveniles of Eredi Rossi Fish Company.

The feeds were manufactured at the feed mill of Eredi Rossi Company located in Cassolnovo, via del Porto 26, province of Pavia, which is dedicated to the production of feeds for all the rainbow trout lines and products (pink and white rainbow trout). Feedstuffs to be included in the feeding plan were provided by Eredi Rossi Group. The ingredients, chemical composition and amino acid content of the experimental diets are reported in Table 3.1.

In the feed mill plant of Eredi Rossi Group, a line of extruders dedicated to the preparation of the experimental diets was used. We planned to use it on a Saturday, in order to give the mill Company time to clean all the equipment and to remove the residuals of other feeds at the end of the working day on the Friday before.

All the ingredients were mixed according to the target formulation. The extrusion process was performed in order to optimize the inclusion of the emulsifier. The feeds were 4.5 mm in size. After the coating, the four diets were stocked in buckets and maintained in an aerated room. Samples of each diet were taken for proximate composition analysis. The feeds were transported to the trout farm and subsequently the growth trial was performed.

3.2.3. Feed analyses

Proximate analyses were conducted using standard methods for both the test diets. Moisture analyses were performed by drying the samples to a constant weight at 105 °C for 24 h in an oven; crude protein was analysed utilizing the Kjeldahl method and crude ash was also analysed by incineration at 525 °C in a muffle furnace for 12 h (AOAC, 1990). Methanol/chloroform extraction method (Folch et al., 1957) was used to analyse crude fat levels of the samples.

Table 3.1: Ingredients, amino acid content and proximate composition of the experimental diets (%).

	Diet 1 Positive Control	**Diet 2 Negative Control**	**Diet 3 NC + Emulsifier 500**	**Diet 4 NC + Emulsifier 750**
Herring meal 70%	31.200	31.200	31.200	31.200
Chicken meal 70%	10.000	10.000	10.000	10.000
Wheat gluten	6.000	6.000	6.000	6.000
Soybean meal 48%	15.000	15.000	15.000	15.000
Decorticated pea	10.000	10.000	10.000	10.000
Wheat meal	8.700	9.700	9.650	9.625
Fish oil	12.000	11.000	11.000	11.00
Soybean oil	4.300	4.300	4.300	4.300
Lysine	0.400	0.400	0.400	0.400
Methionine	0.100	0.100	0.100	0.100
Monosodium Phosphate	0.400	0.400	0.400	0.400
Vitamin Premix	1.500	1.500	1.500	1.500
Choline (liquid, 75%)	0.400	0.400	0.400	0.400
Emulsifier	**0.000**	**0.000**	**0.050**	**0.075**
Proximate composition				
Dry matter (%)	94.5 ± 0.0	94.5 ± 0.0	94.5 ± 0.1	94.5 ± 0.1
Crude protein (%)	47.4 ± 0.0	47.4 ± 0.0	47.4 ± 0.0	47.4 ± 0.0
Crude lipid (%)	22.20 ± 0.1	21.10 ± 0.1	20.81 ± 0.0	20.80 ± 0.1
Crude fibre (%)	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.4 ± 0.0
Crude ash (%)	8.9 ± 0.0	8.9 ± 0.0	8.9 ± 0.0	8.9 ± 0.0
Calcium (%)	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.0	1.6 ± 0.0
Phosphorus (%)	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0	1.2 ± 0.0
Metabolizable energy (MJ/kg)	18.40 ± 0.0	18.00 ± 0.1	18.27 ± 0.0	18.26 ± 0.0

3.2.4. Fish employed

For the trial, we employed 6,000 rainbow trout supplied by the Eredi Rossi Group hatchery, which came from the pre-growing sector where they were reared.

The initial mean weight of the rainbow trout was 47.8±3 g.

The fish were stocked in 12 tanks (3 tanks per diet group) at 4.93 kg/m^3. The diets were administered by hand twice a day, 6 days per week, and the quantity of the daily ratio was recorded.

3.2.5. Morpho-biometric parameters and indices

The mean body weight of the trout was calculated every month in order to update the daily ratio, weighing 100 fish/tank/each group of diet.

Figure 3.5: Trout sampling.

At the end of the trial, the fish were weighed (Ohaus Adventurer SL Precision Balance, Mod: AS8100) and the following zootechnical parameters were determined:

- Mean body weight (g)
- Total length (cm)
- Weight gain (%) = (final weight−initial weight)×100⁄initial weight,
- Specific growth rate (SGR, %⁄day) = {Ln (final weight)−Ln (initial weight)⁄duration}×100
- Feed conversion rate (FCR) = live weight gain (g)/feed administered (g)
- Survival rate (%) = final number of fish/initial number of fish*100

The following indices were also determined: condition index (KI) = (fish weight/fish length^3) x 100, viscerosomatic index (VSI) = (viscera weight/whole body weight) x 100, perivisceral fat index (PFI) = (perivisceral fat/body weight) x 100 and hepatosomatic index (HSI) = (liver weight/body weight) x 100.

Sanitary conditions, such as fin erosion, parasites and bacterial diseases were also investigated.

3.2.6. Histology

At the end of the experiment, the distal intestinal tract of five rainbow trout per diet was sampled and fixed in 10% buffered formalin. This tract was chosen since, at this level, previous studies have observed morphological modifications following the use of vegetable protein in diet (Baeverfjord and Krogdahl, 1996; Urán et al., 2008a; Penn et al., 2011). Standard histological techniques were applied to investigate potential differences in the intestinal structure among the groups, taking into consideration the thickness of the wall (serosa, muscular layer, submucosa and mucosa), the presence of muciparous cells, supranuclear vacuoles and lymphoplasma cells in the lamina propria. A representative portion of each sample was dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Tissues were serially sectioned at 4 μm using a rotary microtome (Leica RM2235, Leica Microsystems, Wetzlar, Germany). The slides were stained with hematoxylin and eosin. Processing of the tissues took place at the Laboratory of Animal Pathology in UNICAM. Histological examinations were performed by means of light microscopy (Nikon Phase Contrast 0.90 Dry, Japan).

3.2.7. Water quality

During the trial, water quality was monitored in order to determine physico-chemical parameters, such as temperature, dissolved oxygen and pH, using a portable electronic device (YSI mod. 55 and 60). 500 cc of water were collected and lab determination of total ammonia nitrogen (TAN), nitrites (NO_2), nitrates (NO_3) and phosphates was carried out using a spectrophotometer (Hach mod-2005), according to APHA methods (1995).

3.2.8. Statistical analysis

Data concerning zootechnical parameters were submitted for analysis of variance to one way (ANOVA) using SPSS 25 (IBM Corp., 2017) and the differences among the means were evaluated utilizing the Student-Newman-Keuls (SNK) test. Probabilities of p <0.05 were considered significant.

3.3. Results

Data on growth performances and feed conversion rate of rainbow trout fed for 90 days with the experimental diets are reported in Tables 3.2 – 3.4. The final mean body weight recorded at the end of the trial is reported in Figure 3.6. The histology of the distal intestinal tract is reported in Figures 3.7a – 3.7d.

Table 3.2: Productive performances recorded at the end of the trial.

	Initial mean body weight (g)	**Final mean body weight (g)**	**Final mean body length (cm)**	**Survival rate (%)**
Diet 1 Positive Control	47.8±3	133.29±41.0^{c}	21±1	97.9±2
Diet 2 Negative Control	47.8±3	127.54±22.8^{d}	21±2	97.6±3
Diet 3 NC + Emulsifier 500	47.8±3	165.94±37.8^{a}	22.5±1	98.1±2
Diet 4 NC + Emulsifier 750	47.8±3	154.32±34.8^{b}	22.5±1	98.2±3

Data are expressed as mean ± sd. Different superscripts (a, b, c, d) in the same column indicate significant differences between the fish groups (p < 0.05).

Table 3.3: Weight gain and feeding performances recorded at the end of the trial.

	Weight gain (g)	Specific growth rate (% per day)	Feed conversion ratio
Diet 1 Positive Control	85.49±7.5[b]	1.14±0.6[b]	1.23[a]
Diet 2 Negative Control	79.74±8.4[b]	1.09±0.9[b]	1.29[a]
Diet 3 NC + Emulsifier 500	118.14±7.3[a]	1.38±0.9[a]	1.14[b]
Diet 4 NC + Emulsifier 750	106.52±9.5[a]	1.30±0.8[a]	1.15[b]

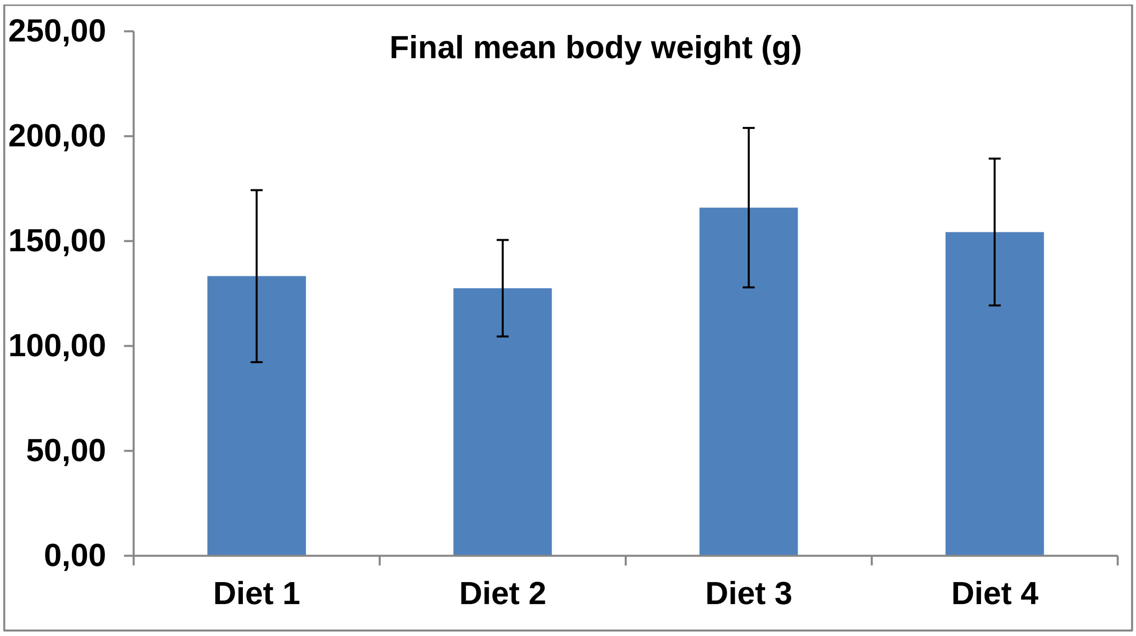
Data are expressed as mean ± sd. Different superscripts (a, b) in the same column indicate significant differences between the fish groups ($p < 0.05$).

Table 3.4: Somatic indices recorded at the end of the trial.

	KI	VSI	PFI	HSI
Diet 1 Positive Control	1.25±0.9[b]	7.82±1.1	1.65±0.3	2.82±0.8
Diet 2 Negative Control	1.18±0.8[b]	8.02±1.2	2.51±0.6	3.28±1.06
Diet 3 NC + Emulsifier 500	1.46±0.8[a]	6.13±1.0	1.81±04	2.88±1.03
Diet 4 NC + Emulsifier 750	1.45±0.9[a]	6.51±1.3	1.94±0.5	2.79±0.8

Data are expressed as mean ± sd. Different superscripts (a, b) in the same column indicate significant differences between the fish groups ($p < 0.05$).

Figure 3.6: Final mean body weight (g) of trout fed the experimental diets.



Data are expressed as mean ± sd.
Diet 1= Positive Control; Diet 2= Negative Control; Diet 3= NC + Emulsifier 500; Diet 4= NC + Emulsifier 750.

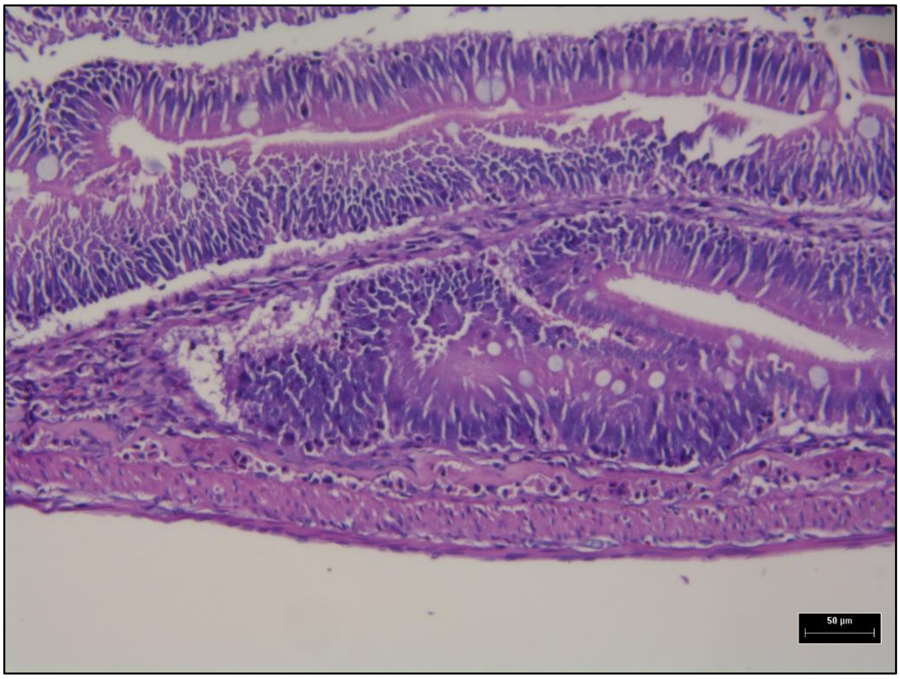
With regard to the final mean body weight, the results proved to be significantly different among the four groups: the fish fed on Diet 3 were the heaviest, followed by those of Diet 4, and finally those fed on Diet 1 and 2 respectively, which weighed far less. On the other hand, no significant differences were observed with respect to either the final mean body length or the survival rate among the groups.

The two diets containing the emulsifier allowed for a significantly greater weight gain and specific growth rate with respect to the Positive and Negative Control diets.

The Diet 3 and Diet 4 groups showed a significantly lower and, therefore, more favourable feed conversion ratio than the other two Control groups.

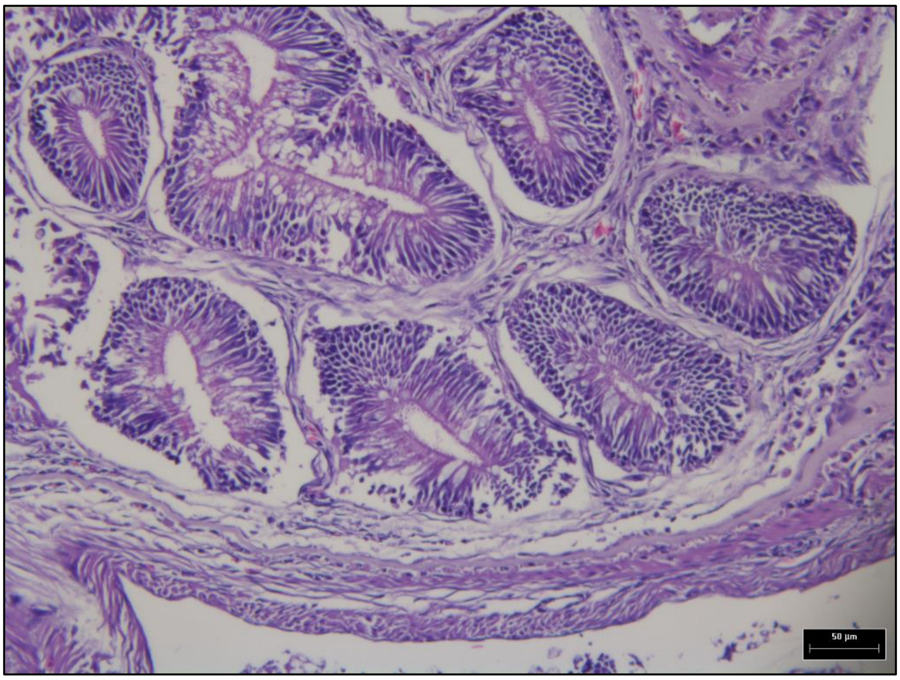
As far as the somatic indices are concerned, no significant differences were noted among the four groups in the VSI, PFI, and HIS, while the KI was significantly higher in the fish fed on two diets containing the emulsifier.

Figure 3.7a: Diet 3. Rainbow trout. Posterior intestine.



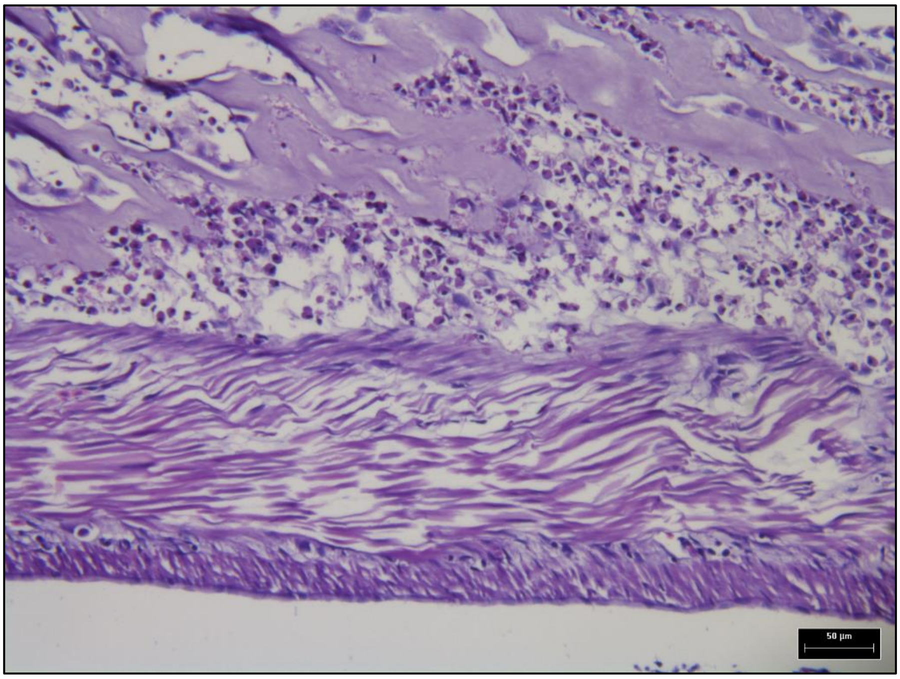
Mucosa with normal epithelium, submucosa and muscular layers (H&E, bar=50 µm).

Figure 3.7b: Diet 4. Rainbow trout. Posterior intestine.



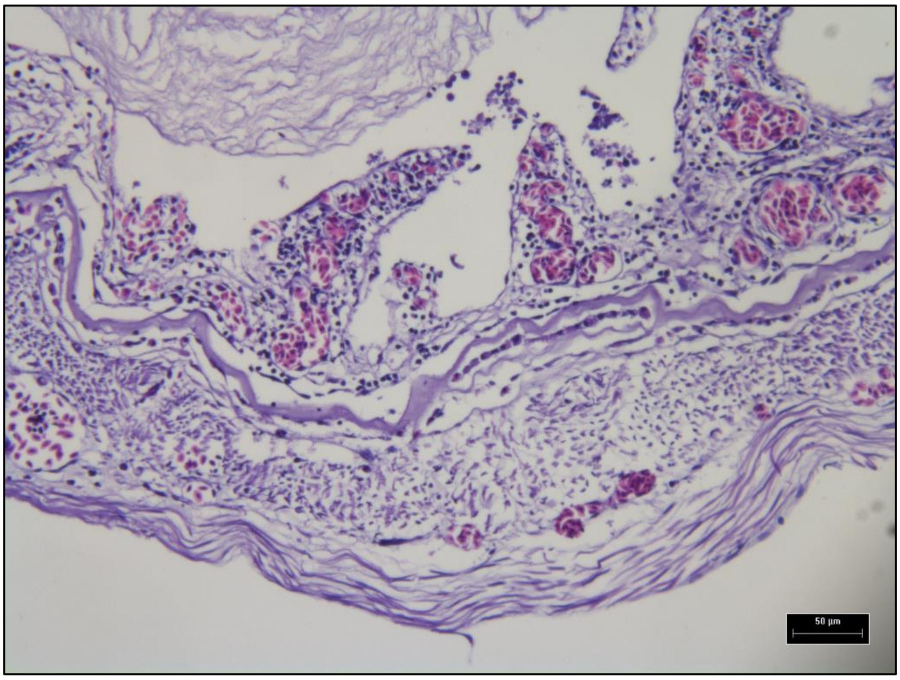
Normal mucosa folds with slight edema (H&E, bar=50 µm).

Figure 3.7c: Diet 2. Rainbow trout. Posterior intestine.



Lamina propria expanded by numerous plasma cells (H&E, bar=50 µm).

Figure 3.7d: Diet 1. Rainbow trout. Posterior intestine.



Mucosa with severe loss of the epithelium. The lamina propria is expanded by lymphocytes, plasma cells and microhemorrhages. The submucosa and muscular layers are edematous and hyperemic. The entire wall is thinned (H&E, bar=50 µm).

3.4. Discussion

The present trial assessed the effect of two supplemental doses of the emulsifier on the growth performance and feed conversion rate in rainbow trout juveniles fed practical growing diets. Water quality remained within a range considered suitable for the species although the water of tanks 1 and 2 showed a minimum level of ammonia nitrogen compared to tanks 3 and 4 which received the feeds with the emulsifier.

After 90 days of experimental feeding, the overall results of rainbow trout fed on Diet 3 and Diet 4 can be considered satisfactory and within the normal range for the species size. A significant difference concerning the histology of the intestinal tract (Figures 3.7a – 3.7d) was observed among the groups.

A good general morphological condition was noted in the fish fed on Diet 3 and 4: the architecture of the intestinal wall was generally normal in terms of thickness and development of the villi, at the level of the lamina propria only a mild lymphoplasma cellular inflammatory infiltrate was detected together with a slight increase in muciparous cells.

On the other hand, the Diet 1 and Diet 2 groups frequently presented an alteration in the structure of the intestinal wall: it showed a severe thinning and the mucosa appeared de-epithelialised while the lamina propria was often highly expanded due to the infiltration of numerous plasma cells and lymphocytes.

The presence of a constant and consistent lymphoplasma cellular infiltrate in the groups appertaining to Diet 1 and 2, characterized, above all, by the presence of plasma cells, is indicative of a local chronic antigenic stimulation, probably due to damage of the epithelium covering the villi. This leads to a secondary stimulation and activation of the lymphoplasma cellular component.

Accordingly, a high level of irritation of the mucosae and a prevalence of hyperplasia of Goblet cells were detected in the fish receiving the Positive Control and the Negative Control diets, whereas a lower degree of irritation was shown in the trout fed on Diet 3 and Diet 4.

This condition is presumably due to the presence of soybean meal at 15% of all the ingredients of the diets. This percentage of protein source of vegetable origin has been included on the basis of the conventional formulation adopted by Eredi Rossi Group in their feeding plants and following the trend of sustainability of feeding in aquaculture. It is well known that sustainable growth of aquaculture will require the development of highly nutritive and functional raw materials to replace fish meal efficiently. However, many studies conducted on fish species to evaluate the performance of plant-based meal, have shown that the inclusion of more than 12% vegetable protein leads to enteritis, as well as depressed immunity (Baeverfjord and Krogdahl, 1996; Bonaldo et al., 2008; Urán et al., 2008b).

It has been ascertained that, in both fish and other vertebrate species, lipid droplets are considered a temporary storage at intestinal level, normally occurring after a meal high in lipids. However, if the lipid accumulation is high, it can destroy the cells and cause damage to the intestinal tissue of fish (D'Aquila et al., 2016). This excessive deposit can be reduced by the addition of phospholipids in the feed (Fontagné et al., 1998; Olsen et al., 2003).

In our trial, in Diet 1 and Diet 2 without the additive, there could presumably have been a delay in the transportation of lipids due to an insufficient production of lipoprotein particles, which is the main cause of severe lipid accumulation.

The emulsifier supplemented in Diet 3 and Diet 4 has resulted in beneficial effects together with a better welfare status of the rainbow trout.

4. QUALITATIVE TRAITS OF BY-PRODUCTS OBTAINED BY RAINBOW TROUT (*Oncorhynchus mykiss*) PROCESSING

4.1. Introduction

Fish processing can provide precious raw materials, which can be applied in a range of products and markets (Ramírez, 2013). When rainbow trout are processed for fillets, the recovery yields are roughly 50% of the fillets. The remaining 50% of the weight of the whole fish is represented by the by-products. For finfish, by-products typically include trimmings, skins, heads, frames (bones with attached flesh), viscera (guts) and blood (Stevens et al., 2018). One of the main problems from a production point of view is the handling of low value materials, which are highly perishable and are, therefore, often treated as waste. In fact, these under-utilised parts are often ground and discarded without any attempt to recover nutrients. However, rendered fish should be considered for reutilization. The use of aquaculture by-products is now increasingly considered to be important for improving economic and environmental efficiency, as well as food security (FAO, 2014). New and more efficient recovery technologies are recommended to increase the yields of aquatic food products and to reduce the amount of processing of the by-products (Gasco et al., 2015). Another challenge could be to optimize applications for the recovered materials so that the processing can be economically sustainable. Hence, in aquaculture, as in other food production sectors, innovation in the utilisation of by-products becomes a key factor for remaining competitive and maintaining long-term profitability (Stevens et al., 2018).

Fish by-products are considered suitable for the possible extraction of valuable compounds including oils, proteins, pigments and minerals that could be used in a variety of industries such as fertilisers, nutraceuticals, and ingredients for foods, aquaculture and agriculture (Rustad et al., 2011; Ramirez, 2013).

In this study, qualitative traits of rendered rainbow trout during processing – including muscle meat and skin – were considered. The proximate composition and the fatty acid profile were evaluated and compared with the traits of the trimmed fillet.

4.2. Materials and methods

The trimmed fillets of twenty rainbow trout, and the rendered portions obtained after the filleting process, were taken from the slaughterhouse of the fish farm which supported the study (Sefro, MC). These samples were then analysed. In order to obtain the trimmed fillets, the industrial filleting was further redefined by a manual trimming, which eliminated any possible residue (Figure 4.1).

Figure 4.1: Manual trimming of rainbow trout fillets.

The proximate analysis (moisture, protein, lipid and ash content) and fatty acid profile were assessed. The moisture percentage was determined in duplicate, according to the procedure of the Association of Official Analytical Chemists (AOAC, 1990); the protein content was measured using the standard Kjeldahl copper catalyst method (AOAC, 1990); the ash content was quantified using the procedure described by the AOAC (1990); the total lipid content was measured using the procedure described by Folch et al. (1957). After determining the total lipid content, fatty acids were converted to methyl esters following the method described by Christopherson and Glass (1969).

4.3. Results

The proximate analysis of the flesh of rendered rainbow trout and trimmed fillet submitted to processing is shown in Figure 4.2. Concentrations of moisture and protein in the fillet of trout were found to be significantly higher than those of the rendered portion, whereas the lipid content prevailed in the rendered rainbow trout. The ash content appeared similar in both parts. Fatty acids, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), ω3 acids, ω6 acids and the ω6/ω3 ratio of the rendered and fillet parts are reported in Figure 4.3. SFAs and total ω6 fatty acids were similar, whereas total MUFAs were higher in the rendered portion. Total PUFAs and ω3 fatty acids were predominant in the fillet.

Figure 4.2: Proximate analysis (% as it is) of the flesh of rendered rainbow trout and trimmed fillet

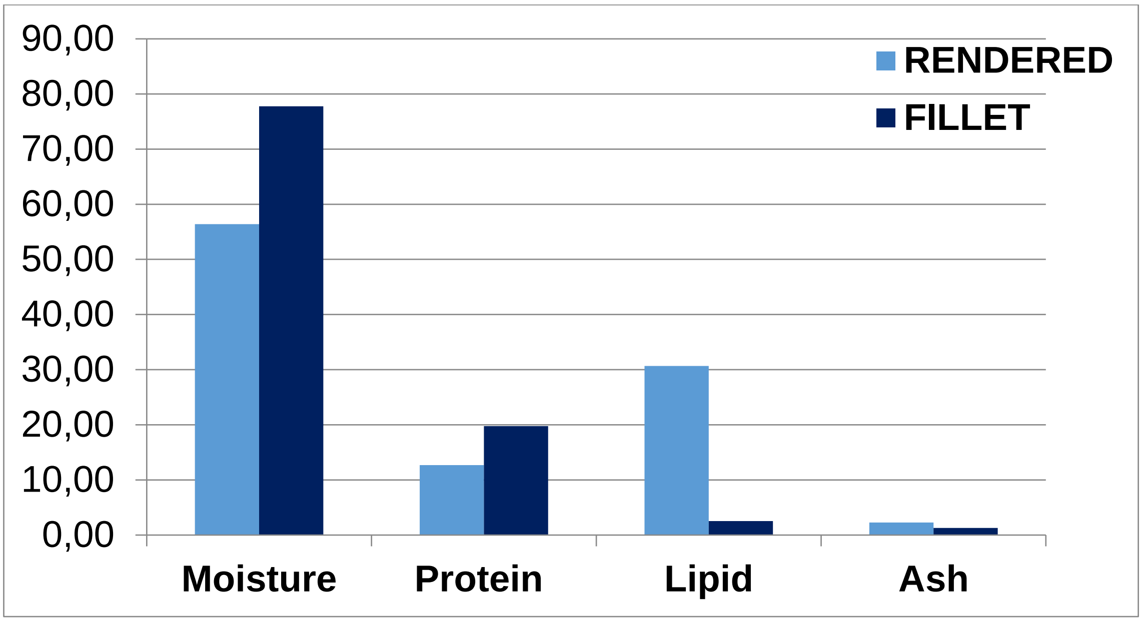
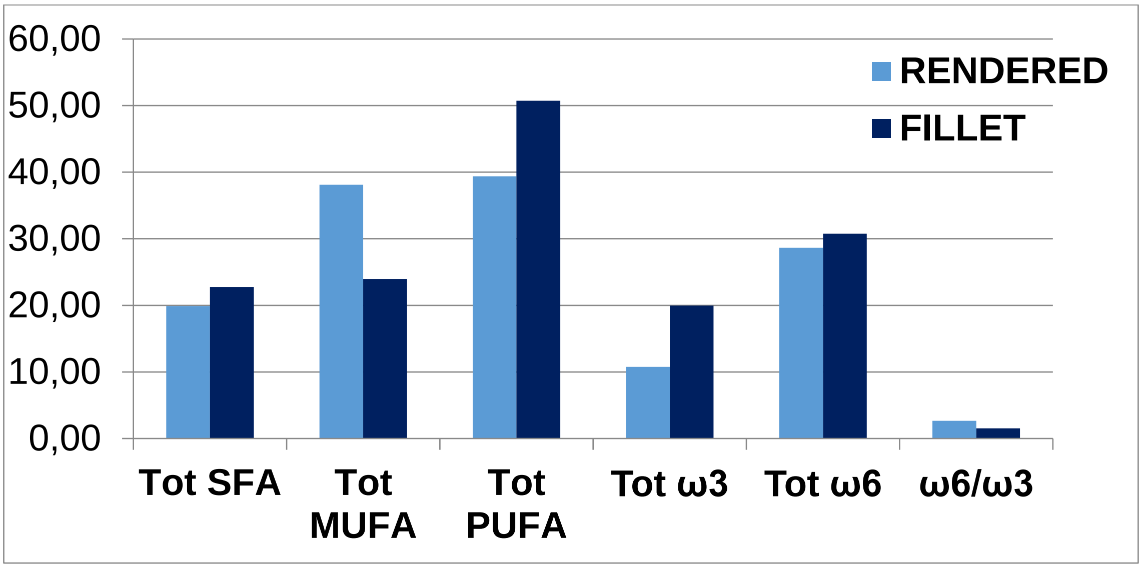


Figure 4.3: SFA, MUFA, PUFA, ω3 acids, ω6 acids (% of the total fatty acids) and the ω6/ω3 ratio of the rendered rainbow trout and trimmed fillet



4.4. Discussion

Approximately one-third of the edible parts of food produced (about 1.3 billion t/year) gets lost or wasted globally (Gustavsson et al., 2011). An estimated 130 million tons of the waste produced in fisheries and aquaculture are a result of poor management of seafood resources (Ghosh et al., 2016). 30%-70% of all fish ends up as a by-product, since fish processing generates materials that may not be used for direct human consumption. These, therefore, can be used for non-food purposes (EUMOFA, 2018). It is reported that fish filleting, salting and smoking generate the major amount of solid waste and by-products (50–75% of the fish), with a total of 3.17 million tons per year. Fish canning represents the second source of solid waste and by-products (30–65% of the fish), totalling 1.5 million tons per year. Crustaceans and mollusc processing also produce a significant quantity of solid residues (20–50%), ca. 0.5 million tons per year (EUMOFA, 2016). In the EU, discards represent a total of around 5.2 million tons per year. The annual discards of fisheries worldwide are estimated to be ca. 20 million tons, which represent 25% of the catch and include “non-target” species, processing waste and by-products (Vidarsson, 2016). Waste and by-products discharged by fisheries are currently on the increase, due to both a net rise in fishery product consumption and to the trend towards ready-to-use products. In fact, in recent years, the habits of consumers have been changing and many people are stimulated to buy ready-to-eat seafood generated from processing. This situation has brought about a significant increase in the amount of waste and wastewater.

Over the last few years, there has been a steady growth in seafood consumption, as consumers have become more aware of its importance as a prime source of nutrients for human health.

Seafood by-products and waste require proper management because their high perishability fosters the development of microorganisms in non-appropriate conditions, constituting a serious environmental hazard. Therefore, applying more stringent quality and hygiene standards, would result in an important step forward for the seafood industry. The results of the study have shown that rendered fish from rainbow trout processing still contains valuable nutrients, which could be considered as possible feedstuff and also successfully employed in various sectors and in innovative ways as, for example, in the production of finger food and fish burgers.

5. GROWING TRIAL OF GILTHEAD SEA BREAM (*Sparus aurata*) JUVENILES FED ON CHIRONOMID MEAL AS A PARTIAL SUBSTITUTION FOR FISH MEAL

5.1. Introduction

In the last few years, insect meal has become one of the most studied sources of protein feedstuffs as an alternative to fish meal for aquaculture feed. Since the International Conference, “Insects to Feed the World”, held in Europe (Wageningen, Netherlands) and jointly organized by the Food and Agriculture Organization of the United Nations (FAO) and the Wageningen University and Research Centre (Wageningen UR) took place in May 2014 (van Huis et al., 2015), larvae, pupae and adult insects are increasingly subjected to testing with the aim of including variably processed meals from these invertebrates in feed formulations for aquaculture species (Finke, 2013; Charlton et al., 2015). FAO recommends employing insects since they assure sustainable production, considering the high nutritional content of many edible insects and their minimal ecological impact (van Huis et al., 2013). In 2015, the European Food Safety Authority (EFSA) published a scientific opinion presenting the biological and chemical hazards associated with the production and consumption of insects as food and feed (EFSA, 2015). Some species are shown to be compatible with use in animal feed in aquaculture (Commission Regulation (EU) 2017/893 of 24 May 2017) and exhibit a great potential as feed ingredients due to their good nutritional quality (Makkar et al., 2014; Tran et al., 2015).

Insects with aquatic larval stages have been studied less than mealworms and flies. Some studies have investigated aquatic invertebrates that could represent an important source of nutrients for both humans and livestock and, in particular, insects with aquatic larval stages have a good chemical composition (van Huis et al., 2013; Henry et al., 2015). Among lake flies, chironomids are common in aquatic environments at different latitudes and are a prey to many fish species. Larvae of this insect can live in freshwater, eurhyaline or marine waters. Although they have a seasonal availability, in their natural habitat, they can reach very high densities, creating swarms of flying insects in summer (Failla et al., 2015). Insects are naturally consumed as feed source in natural water environment by salmonids (De la Noüe and Choubert, 1985; Melotti et al., 1987) and other carnivorous fish species in general. Eggs deposited on the surface of the water after each swarming event, soon develop into larvae. In these situations, chironomid larvae can be captured in large quantities and employed after dehydration for ornamental fish feeding. The freshwater species can be used as natural food to improve growth in fish juveniles (Kamler et al., 2008), since they show a more favourable proportion of long-chain PUFAs compared to terrestrial insects (Fontaneto et al., 2011). In juvenile lake trout, fed commercially available frozen chironomids over a 14-week period, a better growth was observed in comparison with lake trout fed on natural prey (Happel et al., 2016).

In the last few years, chironomids have often been in overabundance in certain seasons of the year and have caused problems in different areas. In recent times, flights in the airports close to the North Adriatic coast have been grounded due to swarms of chironomid flies. Another case has been observed in the Trasimeno Lake, where the densities of chironomids are very high in the summer season, and a great effort is being made to control or reduce them (Pallottini et al., 2014).

Recently a study was performed on chironomid larvae in the area between the Emilia Romagna and Marche regions, in canals where large quantities of larvae were captured and dehydrated. Chemical composition analyses showed good nutritional profiles in the chironomid larvae meal, making it suitable for fish feeding (Meligrana et al., 2016).

5.1.1. Aim of the study

Continuing the studies on the possible inclusion of chironomids in feeds for aquaculture species, a growing trial was performed to test meal obtained from chironomid species collected from aquatic environments (Figure 5.1), on gilthead sea bream (*Sparus aurata*). To this aim, samples of midges at larval stage were converted into meal, analyzed from a qualitative point of view and included in feed for gilthead sea bream juveniles.

Figure 5.1: Chironomid larvae collected from aquatic environments.

5.2. Materials and methods

5.2.1. Chironomid sampling, meal processing, feed preparation and chemical analyses

During the spring and summer of 2017, around 100 kilogrammes of chironomid larvae were collected from ponds and freshwater aquatic environments using nylon net traps. The larvae were washed and kept at a temperature of 4 °C for 24 h. Subsequently, larvae were pooled and dried at a temperature of 60 – 70 °C and milled, following the same procedure adopted in a previous experiment (Meligrana et al., 2016). Samples of meal were analysed for proximate composition (moisture, protein, lipid and ash content), amino acid and fatty acid profile. The same analyses were performed on fish meal to be included in the experimental feeds (Tables 5.1 – 5.2).

Three feeds, one control (L1) and two experimental diets (L2, L3), were formulated in order to be isonitrogenous (around 45%) and isolipidic (around 13%). In the L1 feed, the chironomid meal was absent, and the protein source was mainly represented by soybean meal (32%), followed by fish meal (20%), wheat meal (20%), gluten corn (17%) and haemoglobin (11%). In L2, the percentage of protein source represented by the soybean meal was increased (33.5%), followed by gluten corn (21%), wheat meal (14%), haemoglobin (11%), whereas the source of fish meal was reduced (15%) in order to include the chironomid meal (5%). In the L3 feed, the fish meal was further reduced (8%) and the chironomid meal was increased to 10% of the protein source.

To manufacture the three different diets, the meal of each one was mixed with the other ingredients. The dough of each three was pelleted by pressing it through a sieve (2.2 mm mesh) in a small-scale laboratory pellet mill (Zhenghou Pasen Machinery, Co Ltd). The pellets were then dried in a thermostatic drying oven at 40 °C until the moisture level decreased below 8% and stored at 4 °C in black bags.

The chemical analyses of three samples of each feed were performed according to the Association of Official Analytical Chemists' procedure (AOAC, 1990). The total lipid content was determined using the procedure described by Folch et al. (1957). The amino acid profile of the three feeds was determined by acid hydrolysis (6 N HCl for 24 hr at 110 °C) followed by an ion exchange chromatography utilizing an amino acid analyzer (L-8800 Auto-analyzer, HITACHI, Japan). After determining the total lipid content, the fatty acids were converted to methyl esters following the method described by Christopherson and Glass (1969). The separation of fatty acids was carried out using a GC 3800 gas chromatograph (Varian Strumentazione, Cernusco sul Naviglio, Italy) with a WP-4 Shimadzu integration system (Shimadzu Corporation, Tokyo, Japan), which was equipped with a Supelco SPTM – 2340 capillary column (30 m x 0.25 mm i.d.; 0.25 μm film thickness; Supelco, Bellefonte, Pennsylvania, USA) and a flame ionisation detector.

5.2.2. Fish employed and growing trial

For the trial, 460 sea bream fingerlings, weighing 80.43 ± 1.06 g, were randomly distributed at a density of 8 kg/m^3 in nine 2 m^3 indoor tanks which were supplied by three separate recirculating aquaculture systems at 21.0 ± 0.5°C (Figure 5.2). To avoid possible differences associated with different recirculating water systems, each feed was administered in one of the three tanks of each water circuit.

Figure 5.2: Tanks employed for the trial.

Each feed was assigned to three tanks and distributed twice a day ad libitum over 6 days per week. The feeding trial lasted 90 days and the fish were weighed individually at the beginning of the trial and subsequently on monthly basis. The palatability of the feeds was assayed according to the formula: (ingested feed/administered feed) x 100.

5.2.3. Morpho-biometric parameters and indices

Body weight was measured using an electronic scale (Ohaus Adventurer SL Precision Balance, Mod: AS8100) and body length by a metric scale (Figure 5.3). The following indices were also determined: condition index (KI) = (fish weight fish/$length^3$) x 100, viscerosomatic index (VSI) = (viscera weight/whole body weight) x 100, perivisceral fat index (PFI) = (perivisceral fat/body weight) x 100 and hepatosomatic index (HSI) = (liver weight/body weight) x 100. In order to measure the PFI and the VSI, the fat adherent to the digestive tract was accurately separated and individually weighed.

Figure 5.3: Assessment of morpho-biometric parameters.

5.2.4. Water quality

During the trial, the main water physicochemical parameters (temperature, dissolved oxygen and pH) of the three recirculating systems were recorded on a weekly basis. Total ammonia nitrogen (TAN), nitrites (NO_2) and nitrates (NO_3) were analysed following APHA standard methods (APHA, 1995).

5.2.5. Statistical analysis

Data were submitted to one-way analysis of variance (ANOVA) using the General Model procedure of SAS (SAS, 2004). Differences were considered significant at $p < 0.05$ and means were compared using the Student-Newman-Keuls (SNK) test.

5.3. Results

Throughout the trial, the main water physico-chemical parameters were within the range considered optimal for the species (Bernabè, 1990): pH >7; dissolved oxygen > 6mg/l; salinity 37 ppt ± 1; ammonia nitrogen absent; nitrites absent; nitrates < 60 mg/l.

The chemical composition and amino acid profile of both the chironomid and the fish meal are reported in Table 5.1 and the fatty acid profile in Table 5.2. The chemical composition of the chironomid meal showed a lower protein and ash content and a higher lipid content with respect to the fish meal. The amino acid composition of the chironomid meal had a higher content of valine, threonine and phenylalanine with respect to the fish meal, whereas arginine, histidine, isoleucine, leucine, lysine and methionine were higher in the fish meal. With respect to the fatty acid profile, the saturated fraction in the chironomid meal was lower (34.75%) than in the fish meal (43.79%); the monounsaturated fraction was higher in the chironomid meal (37.83%) than in the fish meal (30.2 %), as were the PUFAs n-6 (21.86%) compared to the fish meal (4.1%); whereas, the PUFAs n-3 were higher in the fish meal (21.91%) with respect to the chironomid meal (5.56%).

Table 5.1: Chemical composition and amino acid content of chironomid meal (CM) and fish meal (FM) used in the feeding trial.

	CM	**FM**
Dry matter (g/kg)	910.4	920.0
Crude protein	585.6	710.0
Crude lipid	145.0	124.0
Ash	107.9	167.0
Aminoacid content (g/kg dry matter):		
Arginine	43.0	49.5
Histidine	19.0	21.0
Isoleucine	22.0	32.0
Leucine	58.7	62.0
Lysine	51.6	56.0
Methionine	22.8	27.0
Phenylalanine	31.6	28.0
Threonine	37.4	32.0
Tryptophan	7.9	8.3
Valine	38.6	33.0

Table 5.2: Fatty acid profile (% of the total fatty acids) of CM and FM used in the trial.

	CM	**FM**
14:0	1.99	10.6
15:0	0.94	0.49
16:0	19.92	26.64
17:0	1.74	0.98
18:0	8.93	4.61
20:0	0.24	0.47
21:0	0.84	0.00
24:0	0.15	0.00
Total SFA	34.75	43.79
14:1	0.01	0.16
15:1	0.35	0.00
16:1	12.82	7.44
17:1	0.45	0.49
18:1	21.92	18.03
20:1	1.80	3.20
22:1	0.00	0.00
24:1	0.48	0.88
Total MUFA	37.83	30.2
18:2 n-6	15.96	2.65
18:3 n-6	0.46	0.00
20:2 n-6	0.00	0.00
20:3 n-6	0.52	0.00
20:4 n-6	4.92	1.45
Total PUFA n-6	21.86	4.1
18:3 n-3	2.95	0.85
18:4 n-3	0.26	0.00
20:3 n-3	0.00	0.00
20:5 n-3	1.76	10.08
22:5 n-3	0.05	1.97
22:6 n-3	0.54	9.01
Total PUFA n-3	5.56	21.91
n-6/n-3	3.93	0.19

The ingredients, chemical composition, energy, minerals and amino acid profile of the three diets are reported in Table 5.3 and the fatty acid profile in Table 5.4. The chemical composition of the three diets was very similar. With regard to the amino acid profile, arginine, histidine, isoleucine, leucine, lysine and methionine showed a decreasing trend from L1 to L3. The opposite tendency was observed for phenylalanine, threonine, tryptophan and valine, which were higher in L3 compared to L1 and L2.

The calcium level was higher in L3 (1.17%) than in L2 (1.09%) and L1 (0.93%). Phosphorus was also higher in the two feeds containing the chironomid meal – L3 (0.95%) and L2 (0.93%) – compared to the feed with the fish meal, L1 (0.85%).

Concerning the fatty acid profile, differences were observed in the MUFAs, which were higher in L3 (27.87%) and L2 (26.6%) in comparison with L1 (24.16%). Total PUFAs n-6 were higher in L3 (14.42%) and L2 (11.41%) with respect to L1 (7.04 %), while the total PUFAs n-3 were lower in L3 (13.72%) compared with L2 (17.06%) and L1 (23.99%) due to EPA variations in L1 (10.16%), L2 (7.04%) and L3 (6.26%) as well as DHA variations in L1 (9.75%), L2 (5.86%) and L3 (3.1%).

Table 5.3: Formulation, proximate composition, energy, minerals and amino acid profile of the three diets used in the trial.

	L1	**L2**	**L3**
Feedstuffs (g/kg):			
Fish meal	180	135	75
Chironomid meal	0	45	90
Soybean meal	285	300	300
Wheat meal	180	125	125
Haemoglobin	100	100	100
Gluten corn	150	190	205
Fish oil	90	90	90
Vitamin and mineral premix	15	15	15
Chemical composition (%):			
Dry matter	91.19	91.25	91.08
Crude protein	45.47	45.62	45.83
Crude lipid	13.50	13.60	13.94
Ash	5.36	5.14	4.73
Crude fibre	2.12	2.27	2.24
Gross energy (MJ/kg)	16.58	16.61	16.73
Amino acid profile (g/kg dry matter):			
Arginine	23.5	21.0	19.7
Histidine	11.5	10.0	9.5
Isoleucine	24.3	22.3	21.5
Leucine	34.0	33.0	30.2
Lysine	28.5	27.0	26.5
Methionine	13.0	11.6	10.2
Phenylalanine	19.0	24.0	27.5
Threonine	19.7	24.0	27.3
Tryptophan	4.0	4.2	4.6
Valine	23.0	26.2	31.4
Minerals (%)			
Calcium	0.93	1.09	1.17
Phosphorus	0.85	0.93	0.95

Table 5.4: Fatty acid profile of the three diets (% of the total fatty acids).

	L1	**L2**	**L3**
14:0	9.89	7.24	5.16
15:0	0.47	0.61	0.53
16:0	24.23	26.72	27.25
17:0	0.54	0.58	0.64
18:0	5.58	5.73	6.28
20:0	0.25	0.13	0.16
Total SFA	40.96	41.01	40.02
14:1	0.11	0.10	0.12
16:1	8.05	10.24	12.12
17:1	0.48	0.45	0.46
18:1	14.23	14.72	14.23
20:1	0.47	0.29	0.26
24:1	0.82	0.80	0.68
Total MUFA	24.16	26.60	27.87
18:2 n-6	5.92	10.04	13.02
20:4 n-6	1.12	1.37	1.40
Total PUFA n-6	7.04	11.41	14.42
18:3 n-3	0.59	0.68	0.69
18:4 n-3	2.14	2.17	2.45
20:5 n-3	10.16	7.04	6.26
22:5 n-3	1.35	1.31	1.22
22:6 n-3	9.75	5.86	3.10
Total PUFA n-3	23.99	17.06	13.72
n-6/n-3	0.29	0.67	1.05
Others	3.85	3.92	3.97

Growth performances, morpho-biometric parameters and somatic indices of gilthead sea bream juveniles fed on the three diets are reported in Table. 5.5.

The L1 and L3 groups showed a better growth performance with respect to the L2 group, with greater final mean body weights and specific growth rates. Feed conversion rates were also more favourable in the L1 and L3 groups, when compared to the L2 group. The survival rate was high in all three groups with no notable differences, ranging from between 94.63% (L2) and 91.60% (L3).

Feed palatability was not affected by the diet, being identical in each group. With regard to the morpho-biometric parameters and somatic indices, no significant differences were observed among the groups in the KI, PFI, VSI and HSI.

Table 5.5: Growth performances, morpho-biometric parameters and somatic indices of gilthead sea bream (*S. aurata*) fed the three diets used in the trial.

	L1	**L2**	**L3**
Initial mean body weight (g)	80.5 ± 1.06	80.5 ± 1.06	80.5 ± 1.06
Final mean body weight (g)	170.64 ± 2.5 ^{a}	161.36 ± 2.7 ^{b}	168.42 ± 2.5 ^{a}
Specific growth rate (%)	0.83 ± 0.1 ^{a}	0.77 ± 0.2 ^{b}	0.82 ± 0.1 ^{a}
Survival rate (%)	94.28 ± 5.36	94.63 ± 4.46	91.60 ± 6.39
Feed conversion rate	1.19 ± 0.02 ^{a}	1.25 ± 0.03 ^{b}	1.2 ± 0.01 ^{a}
Palatability	100.0 ± 0.00	100.0 ± 0.00	100.0 ± 0.00
KI	1.69 ± 0.01	2.02 ± 0.02	1.74 ± 0.01
VSI	6.56 ± 0.03	6.62 ± 0.05	6.57 ± 0.03
PFI	1.06 ± 0.14	1.10 ± 0.12	1.05 ± 0.11
HSI	1.45 ± 0.11	1.43 ± 0.14	1.45 ± 0.12

Data are expressed as mean ± sd. Different superscripts (a, b) in the same row indicate significant differences between the fish groups ($p < 0.05$).

5.4. Discussion

For this trial a diet formulation similar to that adopted by the aquafeed companies, in which fish meal is low and compensated by protein sources of vegetable and terrestrial origin, was utilized. Soybean was employed as the main protein source (32-33%), followed by gluten and wheat meals which are considered more sustainable than fish meal. Haemoglobin was also included to guarantee the presence of high digestible protein due to its high lysine and leucine content (Hertrampf and Piedad-Pascual, 2000). The inclusion of chironomid meal in the feeds represented only 5% and 10% of the protein source of the two experimental feeds, respectively. However, in the two diets in which chironomid meal was included, the replacement of the fish meal reached 25% and 50% respectively, and the gilthead sea bream showed satisfactory growth performances. Many studies have focused on the search for the most suitable insect species to replace the more expensive and unsustainable fish meal in aquaculture diets, and the results are generally encouraging. However, common catfish fingerlings fed on a diet in which *Tenebrio molitor* replaced 50% of the fish meal, had poorer growth performances than those fed on a 100% fish meal diet. Notwithstanding this, the diet containing the insect meal was, in any case, able to sustain growth (Roncarati et al., 2015).

In European sea bass (*Dicentrarchus labrax*) juveniles, the inclusion of *Tenebrio molitor* at 50% of the fish meal resulted in reduced growth performances when compared to the control diet, whereas whole-body crude protein and ether extract were not significantly influenced by the use of insect meal (Gasco et al., 2016).

In juvenile Jian carp (*Cyprinus carpio*) fed diets with different levels of substitution of soybean oil with black soldier fly, no differences in growth performances were reported (Li et al., 2016).

Other authors (Piccolo et al., 2017) recently observed improved growth performances with respect to a control diet where gilthead sea bream juveniles were fed a formulation including *Tenebrio molitor*. In this work, *Tenebrio molitor* larvae meal substituted 25% of the fish meal in the formulation. These authors employed sea bream weighing 105.1 g, which reached market size after 163 days, and attributed the positive results to the inclusion of a moderate percentage of insect meal and to the effect of chitin on protein digestibility, since it confers a higher protein binding capacity (De Marco et al., 2015).

In this study, smaller juveniles (80.43 g) than those used by Piccolo et al. (2017) were employed, and the positive results relating to growth performance can be attributed to the fact that an insect species characterized by an aquatic life cycle was utilized.

The limited amino acid availability of the fish meal employed in the present study, was balanced by the good amino acid profile of the chironomid meal, which contained a good level of tryptophan, leucine, lysine, phenylalanine, threonine and valine. The level of these aminoacids represents a difference from almost all other insect meals, which are considered low in lysine, histidine and tryptophan for fish (Barroso et al., 2014; Magalhaes et al., 2017), whereas *Hermetia illucens* meal is also limited in threonine and sulphur amino acids (Makkar et al., 2014). In the chironomid meal used in the present study, the essential amino acid profile was in line with the estimated requirements for gilthead sea bream (Marcouli et al., 2005; Peres and Oliva-Teles, 2009).

With regard to the somatic indices, the similarity of the results indicated the efficient assimilation of the L2 and L3 diets.

Concerning diet acceptability, the excellent palatability of the experimental diets is presumably due to the fact that the chironomids represent a natural prey for many freshwater and eurhyaline fish species. As such, the chironomid meal may have functioned as a dietary attractant in the two experimental feeds.

The chironomids used in the current study were harvested from wild freshwater environments. The FAO recommends that insects be obtained through production activities in order to guarantee security of the product and to avoid threatening existing populations (van Huis et al., 2013; Failla et al., 2015). However, as mentioned previously, chironomids are often in over-abundance in certain seasons of the year and can be a nuisance in many areas. Such is the case in Trasimeno Lake, Italy, where much effort has been made to control or reduce the density of chironomids (Pallottini et al., 2014).

6. AN ANTIBIOTIC FREE APPROACH IN GILTHEAD SEA BREAM (*Sparus aurata*) PRODUCTION

6.1. Introduction

In recent years, the topic of antimicrobial resistance has become a priority at international level. The prudent use of antimicrobials in human and veterinary medicine is, therefore, a key element of the action plan to contain resistance for the benefit of both animal and human health (EMA/EFSA, 2017). The possibility of rearing antibiotic free fish is one of the most important goals to be achieved in the near future.

In the past, antimicrobials were used in food-producing animals to treat and control bacterial infections in the presence of disease, and for disease prevention and growth promotion in the absence of disease. In the last decade, it has been ascertained that the antimicrobial use in food-producing animals can lead to selection and dissemination of antimicrobial-resistant bacteria in these animals, which can then be transmitted to humans via food. The increase in prevalence of antimicrobial resistance is a worldwide problem. Nowadays, we all recognize the importance of the threat to public health posed by antimicrobial resistance.

Recent papers have shown that healthy animal production systems attempt to reduce the risk of disease outbreaks by implementing agricultural and rearing techniques to improve food security as well as human health. The risks related to animal disease, contamination or survival of biological hazards during processing can be controlled applying “Good Manufacturing Practice” (GMP), “Good Hygiene Practice” (GHP) and “Hazard Analysis and Critical Control Point” (HACCP) programmes.

In response to the urgent need for cross-sectoral action to address antimicrobial resistance, the FAO and the World Organization for Animal Health (OIE) are adopting resolutions supporting the global action plan that emphasizes the need for a “One Health” approach to control this pressing problem. This involves a concerted effort on the part of individuals from many disciplines, including human and veterinary medicine. Moreover, the WHO has developed guidelines on the use of medically important antimicrobials in food-producing animals in order to preserve the effectiveness of these drugs, particularly those judged to be critically important also to human medicine (Figure 6.1).

Figure 6.1: One Health approach towards antibiotic resistance (Source:WHO, FAO, OIE).

The European Medicines Agency (EMA) and the EFSA joint scientific opinion (2017) on the use of antimicrobials claims that antibiotics are mostly used as metaphylactic treatment in aquaculture. The following critical phases of the production cycle requiring most antimicrobial use are:

• salmon: fry in the fresh-water phase (florfenicol and flumequine);

• sea bass and sea bream: juvenile early life stages for tenacibaculosis, photobacteriosis and vibriosis;

• trout: fry (early life stage) for rainbow trout fry syndrome (florfenicol, oxytetracycline), enteric redmouth disease caused by Yersinia ruckeri, furunculosis (sulfadiazine-trimethoprim, florfenicol, oxytetracycline, 1st- and 2nd-generation quinolones).

At European level, the European Platform for the Responsible Use of Medicines in Animals (EPRUMA) has published in 2008 a best-practice framework for the use of antibiotics in food-producing animals, which is based on the adage: “As little as possible and as much as necessary”. Recently, this has been followed up by a “next level” document that lists the building blocks which can be used to form a farm-specific health plan (EPRUMA, 2018).

In the UK, the Responsible Use of Medicines in Agriculture (RUMA) Alliance – an alliance of organisations from across the livestock sector – has developed sector-specific guidelines for responsible use of antimicrobials in poultry, pigs, cattle, sheep and fish. These guidelines place emphasis on the need for disease control, and reinforce the message that antimicrobials should not be used to support failing management systems. They provide advice on strategies to reduce the use of antimicrobials and on their correct administration, storage and recording of use. The study by Jones et al. (2015) found that around 53% of UK dairy farmers were aware of the RUMA guidelines, and 75% of these followed the guidelines either fully or partially. Denmark, similarly, has Guidelines on Good Antibiotic Practice aimed at farmers, with a focus on the prevention of diarrhoea in pigs. In Belgium, an agreement among farmers has developed recommendations on good farming practices and on prudent use of antimicrobials.

In this situation, researchers and farmers are beginning to consider the use of feed additives, such as natural pre- and probiotics, vegetable sources and other supplements that can enhance growth performances of aquatic organisms in farming conditions. In many cases, these compounds are approved for food use by international Organisms, such as the American Food and Drug Administration (FDA).

An example of one of the most studied compounds among these phytogenic categories, is represented by Geranium essential oil, obtained from the leaves of rose-scented geranium. This is known for its diverse biological and pharmacological properties, such as antimicrobial, anti-inflammatory, hypoglycaemic and antioxidant. In general, essential oils are known for their immunostimulant and anti-inflammatory properties (Brum et al., 2017; Hassoun and Çoban, 2017; Al-Sagheer et al., 2018). Moreover, also other compounds produced by organic materials of plants and animal tissues through microbial activity are effective antioxidants due to their significant free radical scavenger properties and are also known to have antimicrobial, anti-inflammatory and immunostimulatory effects; they can, therefore, be considered valuable dietary additives in fish feed (Yilmaz et al., 2018).

In this context, the fish company involved in this Eureka project, has decided to work towards the improvement of rearing techniques, biosafety and the management of environmental parameters, in order to reach high standards of animal welfare, from breeding to transportation and subsequent stages, in the belief that these actions will result in an effective fight against antibiotic-resistance. In 2018, this company obtained the "ABF" certification for sea bream and sea bass rearing in the Adriatic Sea. It is now in the process of going through the same procedure to obtain the "ABF" certification also for rainbow trout farming (Il Pesce, 2018).

6.1.1. Aim of the study

The aim of this study was to compare the growth performance, health status and flesh quality of sea bream farmed adopting an antibiotic free protocol (ABF) in an offshore cage plant, with conspecific wild fish (WLD) of a similar, but slightly larger, size.

For this aim, a trial was performed monitoring the production cycle of gilthead sea bream (*Sparus aurata*) in a marine cage plant located in the Adriatic Sea, in the Gulf of Valona

(Albania). The proximate composition and fatty acid profile of the fillet were evaluated and compared to wild conspecifics.

6.2. Materials and Methods

6.2.1. Description of cages and rearing technique

The plant is composed of 20 circular floating High-Density Polyethylene (HDPE) cages (diameter: 30 m; depth: 15 m) arranged in two parallel lines of 10 cages each. The useful capacity of each net is 10,600 m^3, with a total production volume of 214,600 m^3 (Figures 6.2 and 6.3).

Figure 6.2: HDPE rearing cages adopted for the trial.

Figure 6.3: Marine cage plant in the Gulf of Valona.

No industrial or agricultural installations are present, apart from a small military naval base with very low activity. In this area there are strong freshwater currents which rise up from the bed, and flow into the sea waters. This gives the water its particular chemico-physical characteristics which, combined with the significant depth, make the area ideal for the development of marine life. The area of water inside which the system is laid out is equipped with video surveillance and delimited by the following geographical coordinates:

1) 40° 20’ 37,35” N – 19° 25’ 08,69” E

2) 40° 20’ 31,64” N – 19° 25’ 18,05” E

3) 40° 20’ 38,50” N – 19° 25’ 25,96” E

4) 40° 20’ 32,32” N – 19° 25’ 35,50” E

The sea bream farming begins at the fingerling stage, with a unit weight ranging from between 30-40 g, complying with high quality standards, guaranteed by wholesalers supplying the following requirements:

- the fingerlings must be obtained from broodstocks coming from Mediterranean strains, genetically selected and non-hybrids;

- absence of diseases;

- production cycle must respect animal welfare (reduced densities, elevated water changes, natural food);

- total traceability of the lots;

- the hatcheries must be certified GLOBALG.A.P. (Good Agricultural Practices) which obliges them to totally respect the requirements of this international standard.

In order to avoid stress due to inadequate manipulation, the transfer of the fry takes place without removing them from the water, via pipelines that release the fish directly from the transport tanks, set up on the boat, to the breeding cages.

In order to achieve the market size of 400 g, the production cycle of sea bream takes 16 months.

Low stocking densities (around 15 kg/m^3) are adopted. In these conditions, and throughout the production cycle, the fish are not submitted to grading or manipulation.

The cleaning of the nets is regularly performed using an innovative system consisting of high-pressure pumps, which activate disks arranged vertically in two overlapping rows and move across the whole area of the mesh. This hydro-cleaning system uses only high-pressure sea water.

The personnel involved in fish farming and surveillance activities are trained to apply the practical guidelines contemplated in the animal welfare legislation. Divers are constantly involved in monitoring the submerged parts of the cages: from the checking of the cages to the collection of the dead fish. As for the checking of the nets, the integrity of the meshes is inspected every other day. If necessary, the underwater staff use an innovative device to remove the fouling.

Feed plays an important role in fish farming, since the reduction of farm stress and fish welfare also depend on the quality of the diet used. In fact, the health status of sea bream and sea bass is determined by the type of feed administered and the quality and digestibility of the raw materials used.

The administration of the feed takes place semi-automatically by means of a self-propelled device that distributes the food directly onto the cage several times a day in order to allow all the fish to feed and avoid competition and/or non-homogeneous growth. The feed is transported from the production plant to the farm using only the company's big bags, in order to avoid any contamination. The antibiotic free feeds are stored in well-ventilated warehouses and have to be used up within 6 months of the production date.

6.2.2. Fish employed and sampling

At the beginning of the fattening phase, the plants used fingerlings weighing 30-40 g which reached market size (350-450 g) in about 16 months. The fish received a finisher diet (extruded feed) administered in the last 120 days of the rearing cycle. The feeds had maximum contents of 20% lipids and 45% proteins, as wet weight basis, and were administered at a daily ratio of between 0.8 and 1.2% of body weight, also depending on the water temperature. The farm applied the “Antibiotic free Code of prescription” to guarantee the quality of the product and respect the antibiotic free line.

Monthly sampling sessions of 12 randomly selected fish were carried out from May to July in order to analyse fillet fish quality.

Over the same period of time, a total of 20 wild sea bream were caught using hooks and gill nets in the central Adriatic Sea over 6 fishing sessions. The fish were immediately sacrificed in water and ice, placed in portable coolers containing ice, and transferred to the laboratory.

6.2.3. Morpho-biometric parameters and indices

Body weight was measured using an electronic scale (Mettler 5000 Toledo, Thornton Inc., USA). The total body length and maximum circumference were recorded to the nearest millimetre using a tape measure. The condition index (KI) = (fish weight/length^3) × 100 was calculated. The fish were eviscerated and the perivisceral fat index (PFI) = (perivisceral fat weight/body weight) × 100, viscerosomatic index (VSI) = (viscera weight/whole body weight) × 100) and hepatosomatic index (HSI) = (liver weight/body weight) × 100 were also calculated. Finally, the fish were filleted and the fillets were stored at -40 °C.

6.2.4. Chemical composition and fatty acid profile of the fish fillet

From the fillets, a portion of about 50 g of skinless left dorsal muscle was homogenized and submitted to proximate analysis (moisture, protein, lipid and ash content). The percentage of moisture was determined in duplicate according to the AOAC procedure. The protein content was determined using the standard Kjeldahl copper catalyst method. The ash content was determined using the procedure described by the AOAC (1990). Total lipids were measured using a modification of the chloroform:methanol procedure described by Folch et al. (1957). After determining the total lipid content, fatty acids were converted to methyl esters following the method described by Christopherson and Glass (1969). The separation of fatty acids was performed using a Carlo Erba HRGC 5160 gas chromatograph (Carlo Erba Strumentazione, Rodano, MI, Italy) with a WP-4 Shimadzu integration system (Shimadzu Corporation, Tokyo, Japan) equipped with a Supelco SPTM – 2340 capillary column (30 m × 0.32 mm i.d.; 0.20 μm film thickness; Supelco, Bellefonte, PA, USA) and a flame ionization detector. The operating conditions of the gas chromatograph were as follows: the oven temperature was set at 170°C for 15 min and subsequently increased to 190°C at a rate of 1°C/min, then increased to 220°C at a rate of 5°C/min and held at this temperature for 17 min. The concentration of individual fatty acid was calculated based on the relative proportion of each fatty acid compared with a known amount of the internal standard (17:0) added. The fatty acids were expressed as g/100 g of fillet.

6.2.5. Water quality

During the trial, water quality was monitored in order to determine the main physico-chemical parameters (Table 6.1).

Table 6.1: Water quality parameters monitored during the production cycle.

Salinity (ppt)	37
Dissolved oxygen (mg/l)	7.30
pH	7.00
Nitrogen ammonia (mg/l)	0.13
Nitrites (mg/l)	0.057
Nitrates (mg/l)	1.50
Phosphates (mg/l)	0.095

6.2.6. Statistical analysis

Data were submitted for analysis of variance to one way (ANOVA) using SPSS 25 (IBM Corp., 2017). Differences were considered significant at $p < 0.05$ and means were compared using the Student-Newman-Keuls (SNK) test.

6.3. Results

After 16 months, all the sea bream reached market size.

Mean body weight, body length, and morphometric indices are reported in Table 6.2. The mean size, in terms of body weight and total length, were very similar between the two groups of different origin (Figure 6.4). With regard to the KI, PFI, VSI and HSI no significant differences were observed, nor were there any notable differences in the morphometric parameters between the ABF sea bream and the WLD conspecific.

Figure 6.4: Assessment of morpho-biometric parameters.

Table 6.2: Body weight, total length and morphometric indices of the sea bream of the ABF and WLD groups

	ABF May	**ABF June**	**ABF July**	**WLD**
Body weight (g)	374.25	431.64	359.99	580
Total length (cm)	28.8	29.71	28.56	31
KI	1.57	1.65	1.55	1.95
PFI	0.5	0.4	0.4	0.3
VSI	8.93	8.96	8.86	8.93
HSI	1.93	1.93	1.92	1.89

The proximate composition and fatty acid profile of the ABF and WLD sea bream fillets are summarized in Table 6.3.

Table 6.3: Proximate composition (%) and fatty acid profile (g/100 g) of the ABF and WLD sea bream groups.

	ABF May 2018	ABF June 2018	ABF July 2018	WLD
Moisture (%)	74.5±0.1	75.1±0.2	74.7±0.1	77.4±0.1
Protein (% as it is)	21.0±0.1	20.9±0.1	21.2±0.2	20.7±0.1
Lipids (% as it is)	0.9±0.1^{c}	1.4±0.1^{b}	2.0±0.1^{a}	0.7±0.1^{c}
Ash (% as it is)	1.4±0.1^{a}	1.37±0.2^{a}	1.36±0.1^{b}	1.33±0.2^{b}
Fatty acids (g/100 g meat):				
C14:0	0.024±0.001	0.032±0.002	0.051±0.001	0.000±0.000
C15:0	0.003±0.001	0.004±0.001	0.005±0.001	0.000±0.000
C16:0	0.158±0.001^{d}	0.235±0.001^{c}	0.339±0.001^{b}	0.413±0.003^{a}
C17:0	0.002±0.001	0.000±0.001	0.005±0.001	0.000±0.000
C18:0	0.042±0.002^{c}	0.067±0.001^{b}	0.088±0.002^{a}	0.001±0.000^{d}
C20:0	0.000±0.000	0.004±0.000	0.006±0.000	0.000±0.000
C22:0	0.002±0.000	0.000±0.000	0.003±0.000	0.000±0.000
Total SFA	**0.231**	**0.342**	**0.497**	**0.414**
C14:1	0.001±0.000	0.001±0.000	0.001±0.000	0.000±0.000
C16:1 n-7	0.036±0.001^{c}	0.047±0.001^{b}	0.077±0.001^{a}	0.035±0.001^{c}
C17:1 n-7	0.000±0.000	0.002±0.000	0.000±0.000	0.000±0.000
C18:1	0.026±0.000^{c}	0.039±0.000^{b}	0.052±0.000^{a}	0.004±0.000^{b}
C18:1 cis n-9	0.307±0.001^{d}	0.445±0.001^{c}	0.631±0.001^{a}	0.531±0.003^{b}
C20:1 n-9	0.021±0.000	0.031±0.000	0.044±0.000	0.000±0.000
C22:1 n-9	0.005±0.000	0.007±0.000	0.010±0.000	0.003±0.001
C24:1 n-9	0.000±0.000	0.011±0.000	0.014±0.000	0.002±0.000
Total MUFA	**0.396**	**0.583**	**0.829**	**0.575**
C18:2 cis n-6	0.145±0.001	0.233±0.002	0.347±0.001	0.003±0.001
C18:3 n-6	0.002±0.000	0.003±0.000	0.004±0.000	0.000±0.000
C20:2 n-6	0.005±0.000	0.010±0.000	0.012±0.000	0.000±0.000
C20:3 n-6	0.002±0.000	0.004±0.000	0.005±0.000	0.000±0.000
C20:4 n-6	0.000±0.000^{c}	0.009±0.001^{b}	0.011±0.001^{b}	0.208±0.001^{a}
C22:2 n-6	0.000±0.000	0.002±0.000	0.002±0.000	0.001±0.000
Total PUFA n-6	**0.154**	**0.261**	**0.381**	**0.212**
C18:3 n-3	0.022±0.001^{d}	0.033±0.001^{b}	0.049±0.002^{a}	0.026±0.001^{c}
C20:3 n-3	0.002±0.000	0.003±0.001	0.004±0.001	0.013±0.002
C20:5 n-3 (**EPA**)	0.029±0.001^{d}	0.047±0.001^{c}	0.072±0.002^{b}	0.251±0.003^{a}
C22:6 n-3 (**DHA**)	0.065±0.002^{d}	0.120±0.001^{c}	0.158±0.000^{b}	0.184±0.002^{a}
Total PUFA n-3	**0.118**	**0.203**	**0.283**	**0.474**
EPA + DHA	**0.094**	**0.167**	**0.230**	**0.435**
n-6/n-3	**1.31**	**1.29**	**1.35**	**0.45**

Data are expressed as mean ± sd. Different superscripts (a, b, c, d) in the same row indicate significant differences between the fish groups ($p < 0.05$).

The protein content ranged from 20.7% in the WLD fish to 21.2% in the July sample of the ABF fish. The lipid content ranged from 0.7% in the WLD sea bream to 2% in the July sample of the ABF sea bream. The ash content exhibited no differences between the ABF fish and the WLD fish.

Some differences between the ABF and WLD sea bream were found in the fatty acid profile of the fillets. With regard to the SFAs, the most important fatty acid was represented by the palmitic acid in all the samples. The highest content was recorded in the WLD group (0.413 g/100 g), followed by the ABF July sample (0.339 g/100g), the ABF June sample (0.235 g/100g) and that of May (0.158 g/100g). The total SFAs were high in the WLD (0.414 g/100g) and ABF July groups (0.497 g/100g). The MUFAs showed the same trend with a high level in all the groups. The n-6 PUFAs were more contained in the ABF June group (0.381 g/100g) compared to all the others. The n-3 PUFAs were mainly represented by the WLD seabream (0.474 g/100g) followed by the other three samples. Among the fatty acids of this category, the most important were EPA and DHA in the WLD group, followed at a distance by all the other ABF fish, although very high contents were also present in all the farmed fish.

Throughout the trial, the water of the plant showed no toxic compounds that could compromise the survival rate of the fish.

6.4. Discussion

The off-shore cage plants guaranteed a better product quality due to the greater movement of the fish kept in marine areas characterized by strong currents, the optimal oxygen values and thanks to the chemical parameters that remained within the range suitable for gilthead sea bream (Bernabè, 1990).

The modern and innovative fibre composing the nets of the rearing cages considerably reduced the net damage, avoiding the risk of fish escaping.

The proper size of the meshes and their regular replacement according to the size of the fish, promoted the water circulation inside the cages themselves. Moreover, this type of net was designed to limit and control the development of fouling and the deposit of concretions, algae and microorganisms. In this way, there was no use of antifouling substances and, therefore, polluting elements, such as copper and zinc, were not released into the marine environment.

The sea bream were raised in high-volume cages, where there was plenty of space available and where densities were continuously monitored in relation to size.

Throughout the production cycle, the fish were not submitted to grading or manipulation in order to avoid potential stress that might have led to the onset of bacterial diseases. Therefore, these precautions adopted in the rearing technique contributed to maintaining the fish in good health, as confirmed by the high survival rate.

The large volume of the cages employed and the low stocking densities adopted had a positive effect on the welfare status of the fish. In fact, the volume of water available to swim in, plays an important role in the welfare of the fish, on their morphological traits and on the quality of the product itself.

With regard to the morphometric parameters and somatic indices, the mean length and KI of the ABF fish showed no notable differences with respect to the WLD conspecific of the same mean body weight. In a study by Rodríguez and collaborators (2004), captive black sea bream (*Spondyliosoma cantharus*, L.) achieved significant mesenteric and visceral lipid deposition compared to the wild fish. In addition, farmed gilthead sea bream fed diets including vegetable oil (soybean oil and linseed oil) in substitution of fish oil, exhibited lower HSI than the control fish (Menoyo et al., 2004).

On the contrary, no differences emerged in sea bass fed diets containing freeze-dried microalgae as a partial substitution of protein and lipid from fish derivatives (Tibaldi et al., 2015). Recent studies have shown that the substitution of fish oil up to 60% with vegetable oil did not affect the growth and health status of European sea bass and gilthead sea bream (Nasopoulou and Zabetakis, 2012).

In the present trial, the ABF sea bream displayed a very low lipid fraction similar to that of the WLD captured in the Adriatic Sea, which classified them in the category of lean fish. This result differed significantly from that reported in a trial performed in the Tyrrhenian Sea, which compared sea bream, reared in four circular 3,800 m^3 floating sea cages at a maximum final stocking density of 15 kg/m^3, fed on organic or conventional commercial feeds. In fact, the conventional and organic sea bream showed a much higher total lipid content (8.37 ± 1.0 and 9.52 ± 1.8 g/100 g w.w., respectively) compared to the ABF and WLD fish in our study. However, the organic fish displayed an overall state of well-being and had good quality features despite a lower EPA and DHA content with respect to the conventional fish (Di Marco et al., 2017).

It is well known that in fish, as in other animals of zootechnical interest, the feed affects the quality of the meat. The ABF sea bream were reared using feed containing raw materials of aquatic origin (fish meals and oils, dehydrated algae, etc.), which constituted the main source of essential fatty acids of the omega 3 series, and also raw materials of vegetable origin in full respect of environmental sustainability.

Data concerning the omega 3 content, demonstrated that the ABF samples collected in June and July can be defined as “Rich in omega-3”, in full compliance with the provisions of the Annex to the Regulation (EC) n. 1924/2006 on nutrition and health claims made on foods. On the other hand, the ABF samples collected in May can be identified as “Omega-3 source”.

7. CONCLUDING REMARKS

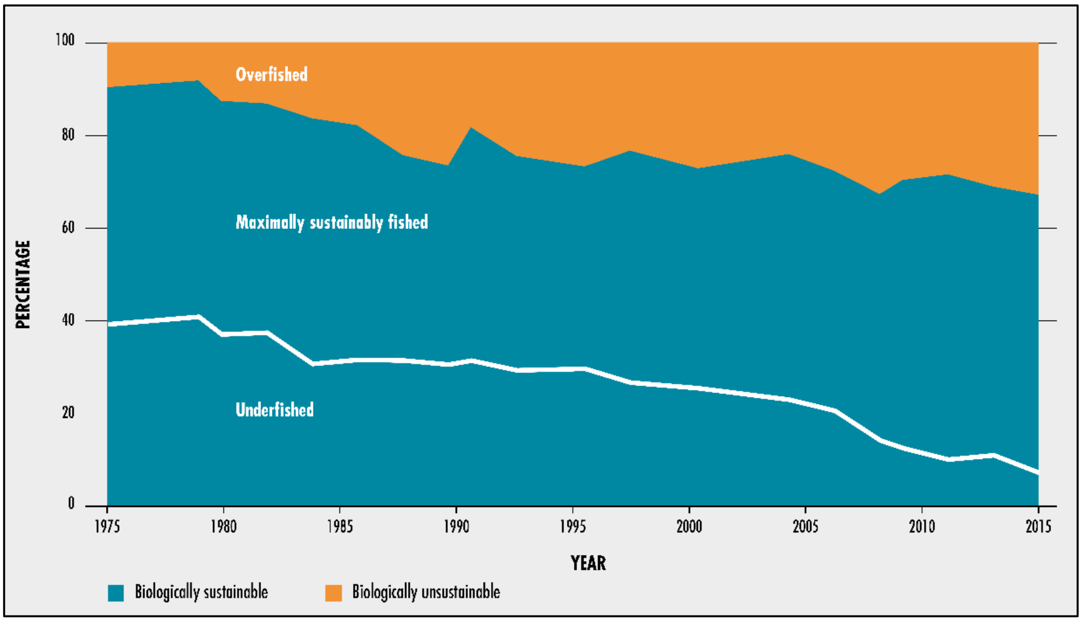
The world population continues to grow exponentially and it is expected to top more than 9 billion by the middle of the twenty-first century. Thus, it is imperative to find a way to provide food for such a number.

While the per capita fish consumption has been rising along with the world population growth, the capture fishery production has remained relatively static since the late 1980s. In this context, aquaculture has been responsible for the continuing impressive growth in the supply of fish for human consumption (FAO, 2018).

The production of fish meal and fish oil for industrial fish feeds is mainly dependent on targeted fisheries of small pelagic fish. However, marine overexploitation has drastically reduced their abundance and, therefore, the production of fish meal and fish oil is unable to support current market trends (Shepherd and Jackson, 2013).

Therefore, the fisheries and aquaculture sector needs to lower the stocks fished beyond biological sustainability (Figure 7.1).

Figure 7.1: Global trends in the state of the world's marine fish stocks, 1975-2015 (Source: FAO, 2018).



In order to be able to preserve natural resources, the sustainability and productivity of livestock and aquaculture systems must be improved and alternative sources are urgently needed. This scenario requires a multi-disciplinary approach towards sustainability. According to the World Committee for the Environment and Development, the term "sustainability" is defined as "a development which satisfies the needs of the present without compromising the resources for the future".

All the studies carried out during the PhD triennium, were performed with the idea of a sustainable approach to aquaculture and with the aim of supporting the fish company, which is a partner in this PhD Eureka project, in its process of innovation. The work was focused on rainbow trout and gilthead sea bream, both of which are highly sensitive to the environment in which they are farmed and account for a large share of Italian aquaculture production.

Over the last few years, the principle of sustainability and the search for a high-quality product has been put at the centre of the aquaculture production system. In fact, farmers have come to understand that it is essential to pay particular attention to the quality of the product and to protect environmental resources. This evolution of the social and productive scenario highlights the need for new paradigms to emergence. These must be focused on the eco-compatibility of the production processes and must safeguard human health and biodiversity.

One of the aspects of sustainability concerns the characterization of the raw materials to be included in feeds, and their effects on growth performance, animal welfare and health status. Furthermore, the quality of feed supplied to fish notoriously affects the quality of the product obtained, determining the choices of consumers who have become more aware that fish products are a source of precious nutrients for human health, such as omega 3 fatty acids. On the other hand, the research is oriented towards the partial reduction of fish meal and fish oil in favour of feedstuffs of plant origin. Moreover, in order to improve the productivity of aquaculture one of the most important problems is to guarantee high quality larvae and juveniles.

To this aim, one of the works of this PhD project focused on more sustainable plant-based feeds for rainbow trout broodstocks and evaluated their reproductive performances. Results showed that feedstuffs containing vegetable protein and fat administered to broodstocks, negatively affected the quality of gametes and the progeny of rainbow trout. However, other trials carried out recently seems to be more encouraging. Reynolds and Caton concentrated their attention on genetic traits and the environment, considering the effects of low water temperature during the first days after hatching: during this period, the metabolism and physiology can be permanently influenced, and this process has been termed "fetal programming" or more recently "developmental programming" (Reynolds and Caton, 2012). Another study demonstrated that rainbow trout fry fed on plant-based diet for a short period during early life, improved the future acceptance and utilization of the same diet when given at later life stages (Geurden et al., 2013). Although these results look promising, alternative raw materials of vegetable origin in aquafeed for fish and crustaceans give rise to ethical concerns when utilized in diets for carnivorous fish (Krogdahl et al., 2003).

As mentioned before, it is imperative to find a more sustainable and economically viable replacement for fish meal and fish oil in aquaculture feeds and, to this aim, many studies have been performed to determine the most suitable insect species. In our research, the suitability of chironomid meal was tested: it was included in the feeds of gilthead sea bream resulting in excellent palatability and satisfactory growth performances.

Nowadays, there is a growing concern related to the problem of food waste production during the supply chain and the consequent environmental burden and economic losses.

Aiming for a “zero-waste” society, where waste from one sector is reutilised in another, the European Commission has strictly recommended improving resource efficiency by converting biomasses into a range of high-quality foods (EC, 2018).

To this end, during the PhD period the discards of the rainbow trout filleting process were analysed from a qualitative point of view; in particular the ventral part of the fillet was taken into consideration. This kind of by-product was shown to be rich in precious nutrients that could be reused for different purposes, such as in animal feed and in innovative foods.

In addition to good breeding practices, a significant role can be played by additives and emulsifiers, which have a preventive function in the onset and spread of diseases. Particular attention should be payed to micronutrients and/or antioxidants, which can positively influence the nutritional and organoleptic quality of the farmed fish. The use of these substances, if of natural origin, represents a further element of innovation compared to conventional formulations.

This research studied the use of a compound added to the feed of rainbow trout, aimed at improving the absorption of nutrients. The supplementation of the emulsifier resulted in beneficial effects together with a better welfare status, particularly of the intestinal tract.

The management of the health status of farmed fish is a fundamental issue in aquaculture. The transmission of pathogens, often associated with overcrowding, which increases the risk of infection, is often the cause of huge economic loss. Antibiotics and various other health care products can no longer be the solution. The increase in prevalence of antibiotic resistance is a worldwide problem. Furthermore, certain antimicrobials are now forbidden for animal use and are, in any case, incompatible with an organic production system. Improving production strategies by means of good breeding protocols can mitigate stress factors and reduce the impact on the ecosystem.

To this purpose, our study also focused on the optimization of the farming technique for gilthead sea bream without the use of antibiotics, in the assumption that a condition of well-being implies an optimal feed conversion rate, a higher survival rate and an enhanced immune capacity, with subsequent benefits, not only in terms of product quality, but also for the environment.

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ABBREVIATIONS AND ACRONYMS

ABF	Antibiotic Free
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
APHA	American Public Health Association
API	Associazione Piscicoltori Italiani
ARA	Arachidonic Acid
ASPA	Associazione per la Scienza e le Produzioni Animali
CCEXEC	Executive Committee of the Codex Alimentarius Commission
CM	Chironomid Meal
DHA	Decosahexanoic Acid
DM	Decreto Ministeriale
EC	European Commission
EFSA	European Food Safety Authority
EIFAC	European Inland Fisheries Advisory Commission
EMA	European Medicines Agency
EPA	Eicosapentanoic Acid
EPRUMA	European Platform for the Responsible Use of Medicines in Animals
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed Conversion Rate
FDA	Food and Drug Administration
FEAP	Federation of the European Aquaculture Producers

FM	Fish Meal
GAP	Good Agricultural Practices
GHP	Good Hygiene Practice
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis and Critical Control Point
HDPE	High-Density Polyethylene
HSI	Hepatosomatic Index
HUFA	Highly Unsaturated Fatty Acids
ICES	International Council for the Exploration of the Sea
KHV	Koi herpesvirus
KI	Condition Index
LA	Linoleic Acid
LNA	Linolenic Acid
LPA	Lysophosphatidic acid
LPS	Lysophospholipids
MUFA	Monounsaturated Fatty Acids
NC	Negative Control
NO_2	Nitrite
NO_3	Nitrate
OIE	World Organization for Animal Health
PFI	Perivisceral Fat Index
PUFA	Polyunsaturated Fatty Acids
RUMA	Responsible Use of Medicines in Agriculture
S1P	Sphingosine 1-phosphate
SAS	Statistical Analysis System

SD	Standard Deviation
SFA	Saturated Fatty Acids
SNK	Student-Newman-Keuls
SPSS	Statistical Package for Social Science
TAN	Total Ammonia Nitrogen
TSE	Transmissible Spongiform Encephalopathies
UNICAM	University of Camerino
VSI	Viscerosomatic Index
WLD	Wild
WHO	World Health Organization

LIST OF PUBLICATIONS DURING THE PhD TRIENNIUM

Catone G, Maranesi M, Petrucci L, Parillo F, Vullo C, Meligrana M, Capezzone C, Gobbetti A, Boiti C, Zerani M: Presence and function of kisspeptin/kisspeptin receptor system in corpora lutea of pseudopregnant rabbits (*Oryctolagus cuniculus*): in vitro studies. *Reproduction in Domestic Animals*, 2018; 53(S2): 119.

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Meligrana M, Roncarati A, Vullo C, Catone G, Melotti P: Qualitative traits of trout and comparisons with the main farmed fish species. In: *Alimenti e nutraceutici: qualità e salute del consumatore*, Camerino, 4 July 2017; 58-59.

Vullo C, Palumbo Piccionello A, Tambella AM, Meligrana M, Bonacucina E, Catone G: Post-operative analgesic effects either of metadone or a constant rate infusion of lidocaine, or the combination lidocaine-metadone in dogs undergoing ovariectomy. In: *Proceedings of the LXXI SISVet Congress*, Naples, 28 June-1 July 2017; 140.

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Meligrana M, Roncarati A, Vullo C, Bucchi V, Melotti P: Developing research into quality traits of farmed fish: rainbow trout (*Oncorhynchus mykiss*) as functional food. In: *Alimenti funzionali e nutraceutici per la salute*, Camerino, 28 June 2016; 66-67.

Vullo C, Meligrana M, Petrucci L, Laus F, Carluccio R, Parrillo S, Marini C, Catone G: Guaifenesin-ketamine-xylazine infusion to maintain anesthesia in mules undergoing field castration. In: *Proceedings of the LXX SISVet Congress*, Palermo, 13-16 June 2016; 184-185.

Petrucci L, Magi GE, Maranesi M, Vullo C, Bastianelli S, Meligrana M, Zerani M, Boiti C, Catone G: Possible presence of an AMH-immuno-like protein in the interstitial cells of eutopic scrotal testis of prepubertal unilateral cryptorchid swine. In: *Proceedings of the LXX SISVet Congress*, Palermo, 13-16 June 2016; 284-285.

Roncarati A, Mariotti F, Felici A, Meligrana M, Melotti P: Suitability of artisanal fishery discards as feed for juvenile tub gurnard (*Chelidonichthys lucerna* L.) reared in sea bottom cages in the mid Adriatic Sea. *Mediterranean Marine Science*, 2016; 17(3): 644-650.

Roncarati A, Meligrana M: Quality traits and fatty acid profile of large-size farmed and wild marine fish. *Journal of Food Processing & Thecnology*, 2016; 7(6Suppl): 60.

LIST OF SCIENTIFIC ACTIVITIES AND TRAINING DURING THE PhD TRIENNIUM

30 October 2018 - Transnational networking event: Interreg ADRION - Ariel Project, Podgorica, Montenegro.

10 October - 10 November 2018 - Research training: Agricultural University of Tirana and Almarina OR Sh.p.k., Albania.

October 2018 - Diploma Nazionale SIUMB di Competenza in Ecografia Veterinaria - Società Italiana di Ultrasonologia in Medicina e Chirurgia.

24 July 2018 - Nominated: "Cultore della materia in Zoocolture e Zootecnia Speciale".

9 - 13 July 2018 - Summer School: Nutrition in Aquaculture, Porto Conte Ricerche, Alghero, Italy.

13 May 2018 - Oral scientific presentation: L'allevamento della trota: esempio di sostenibilitá e qualitá, Sefro, Italy.

7 February 2018 - Oral scientific presentation: Feeding trials using sustainable raw materials for fish species of interest in aquaculture. School of Biosciences and Veterinary Medicine - University of Camerino, Italy.

17 - 20 October 2017 - Presentation of poster: Reproductive performance of rainbow trout (*O. mykiss*) broodstocks fed on different feeds. Aquacuture Europe 2017 - Cooperation for Growth Conference, Dubrovnik, Croatia.

4 July 2017 - Presentation of poster: Qualitative traits of trout and comparisons with the main farmed fish species, Camerino, Italy.

16 June 2017 - Oral scientific presentation: Effects of different feeds on performances of rainbow trout (*Oncorhynchus mykiss*) broodstocks. 22nd Congress of the Animal Science and Production Association, Perugia, Italy.

13 May 2017 - Oral scientific presentation: Caratteristiche qualitative della trota e comparazione con le principali specie ittiche allevate, Sefro, Italy.

24 April - 5 May 2017 - Research training: Royal Veterinary College - Queen Mother Hospital for Animals, University of London, UK.

16 February 2017 - Oral scientific presentation: Research and study of alternative raw materials to feed rainbow trout (*Oncorhyncus mykiss*): sustainable production, aimed at obtaining a quality product. School of Biosciences and Veterinary Medicine - University of Camerino, Italy.

5 - 7 December 2016 - Research training: 18th Practical Short Course: Trends and Markets in Aquaculture Feed Ingredients, Nutrition, Formulation, Optimized Feed Production, Quality Management, Ghent, Belgium.

20 - 23 September 2016 - Presentation of poster: Qualitative traits of by-products obtained by rainbow trout (*Oncorhynchus mykiss*) processing. Aquacuture Europe 2016 - Food for Thought, Edinburgh, Scotland.

12 September 2016 - Presentation of poster: Proximate composition and fatty acid profile of Chironomidae (Diptera) larvae collected in ponds. International Symposium on Insects as Feed, Food and Non-Food - INSECTA 2016, Magdeburg, Germany.

28 June 2016 - Presentation of poster: Developing research into quality traits of farmed fish: rainbow trout (*Oncorhynchus mykiss)* as functional food. Camerino, Italy.

13 - 15 January 2016 - Research training: ARRAINA Course: Long Term Effects of Low Fishmeal and Fish Oil Diets across Life Stages. Universidad de Las Palmas de Gran Canaria, Spain.