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Innovative raw materials to be used in aquaculture fish species feeding and the importance of fish welfare to obtain a high nutritional quality and antibiotic-free product

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SUMMARY

In Europe, rainbow trout is one of the main species of farmed freshwater fish that requires innovative studies to improve fillet quality, environmental sustainability, farm management and fish welfare. The constant expansion of aquaculture must necessarily be associated with continuous innovations in fish feeding formulations and develop together with continuous improvements in feed formulations. The main challenge is to identify new and alternative raw materials to be used in substitution of conventional ones, namely fish meal and fish oil, without lost the benefits to human health associated with the consumption of these products.

A global food strategy that guarantees sustainable and quality food is therefore necessary, with a general approach that aims to an only one global health, in which human health, animal health and environment health are protagonists. In this context, the role of the Veterinary Doctor in protecting food safety throughout the supply chain, from farming breeding to the consumer's table, is crucial. The primary challenge is to identify alternative raw materials complementary to fish meal and fish oil able to guarantee good zootechnical performances and at the same time the already known benefits for human health associated with the consumption of aquaculture products. Moreover, in the last few years the prevention of fish diseases has become a priority in order to avoid the use of antimicrobials in farming fish. In fact, in recent years the topic of antimicrobial resistance has become a priority at the international level, and the Food and Agriculture Organization (FAO) and the World Organization for Animal Health (OIE) adopted resolutions for public health promoting a global action plan, the One Health (OH) approach, to control the pressing problem posed by antimicrobial resistance.

In this context, this PhD project was initially focused on the study of the main factors of fish welfare in fish farm, which are mainly feed quality and water quality, and secondary

innovative techniques to improve fish welfare. The final scope of the project was improving biosafety and management with the purpose of achieving high standards of animal welfare and avoiding the use of antibiotics in the aquaculture production.

The present PhD project was possible thanks to the support and collaboration of one of the main rainbow trout (*Oncorhynchus mykiss*) farm leader in Europe, the Erede Rossi Silvio Trout Company, who participated to finance the project and let the experimental trials possible to realize. The Company production policy were rearing antibiotic free fish and working towards the improvement of rearing techniques, biosafety and the management of environmental parameters, in order to reach high standards of animal welfare, in the hope that this will result in a successful battle against antibiotic resistance.

Due to the Covid-19 pandemic and the restrictions imposed by the Italian Government, activities and application studies were not possible to conduct in presence during the 2020 and part of the 2021 year. The first year of the PhD project was focused on the theme of water quality and aquatic environmental protection, studying the topic by consulting the most recent literature on that subject and conducting the statistical elaboration of data of the fish Company concerning the analysis of farming water, considered as inlet water and outlet water, and the impact of the feed administered to fish on the farming water quality. The final aim was to investigate and evaluate the connection between feeding techniques and growth fish performances, fish fillet quality, process sustainability and water quality environment.

During the three years of the project, alternative raw materials to be utilized in the formulation of feed for the main fish species of interest in aquaculture were discussed both as researcher and with the representatives of the private company involved in the project (Erede Rossi Silvio Trout Company). In fact, in aquaculture the main challenge is to formulate balanced and sustainable diets, especially for protein sources. The increase of the global demand of aquaculture products needs to find new raw materials to be included in fish

feeding, respecting at the same time the circular economy and the environmental sustainability prospective. In aquaculture the use of quality feed affects the quality of the final fillet as well as the characteristics of the aquatic environment in which farming fish themselves live (Thorpe et al., 1995; Welker et al., 2018; Fiordelmondo et al., 2020).

With the end of the presence restrictions, the second year of the project completed the performing feeding trials started in the first year. In the second year a collaboration with the University of Veterinary Medicine of Vienna (VETMEDUNI, Austria) started and continued in the third year (Department for Farm Animals and Veterinary Public Health, Clinical Division of Fish Medicine; under the supervision of the head of the Clinical Division of Fish Medicine, Prof. Monsour El-Matbouli). The third year of the project focused on performing studies on fish welfare in standard farming conditions underling the main factors that must be controlled during a standard farming cycle. In particular, scientific parameters to evaluate the welfare condition of farming fish were performed before the slaughtering phase, in the reason that no studies are available on this specific topic. In order to respect fish welfare, the condition of welfare/stress was evaluated as acute stress and chronic stress in different groups of rainbow trout to investigate the welfare status of the fish during the last part of a conventional farming cycle.

During the course of the PhD triennium most of the activities related to the rainbow trout species, with the support of the Erede Rossi Silvio Trout Company. With regard to the conducted feeding trials, three studies were focused on the zootechnical performances of rainbow trout after a partial substitution of fish meal with an alternative protein source, namely duckweed meal, hydrolysed fish proteins and guar meal. In particular, in the first trial duckweed meal derived from *Lemna minor* was included in the formulation of three experimental feeds (L1, L2, L3) for rainbow trout at 10%, 20%, 28% of the protein source respectively as partial replacement of the two main protein feedstuffs, fish meal and soybean

meal, in order to evaluate an alternative protein source less expensive and more sustainable than conventional ones. The substitution has mostly concerned soybean meal and secondly fish meal. Increasing the duckweed inclusion, the other protein sources were adjusted to get isonitrogenous (41%) and isolipidic (20%) diets, as the control diet (LC). The productive results showed that the final body weight in L1 (340.53 g) and L2 (339.42 g) was not different from LC (348.80 g); L3 trout significantly ($p < 0.05$) exhibited the lowest one (302.16 g). Similar trends were found in final mean length, weight gain, specific growth rate, food conversion rate. Somatic indices were affected by duckweed inclusion. Diets had not significant effects on the proximate composition and fatty acids of the fillet in L1, L2, L3 respect to LC. Based on these results, duckweed meal derived from *Lemna minor* can be included in the feed for rainbow trout without negative effects on the growth performances at 20% of the protein substitution.

In the second trial the rainbow trout species was employed fish by-products to obtain hydrolysed fish proteins. In other papers, fish meal was replaced greater than 29.7% by enzymatically hydrolysed tuna backbone by-products in the diets of juvenile rainbow trout (Bae et al., 2019), or by a mixture of animal by-products (approximately 23%) in the growth of juvenile snapper *L. argentimaculatus* (Khalid et al., 2007). Our trial was performed on gilthead sea bream (*Sparus aurata*) in order to investigate the effects of dietary supplementation with hydrolysed fish proteins, derived by rainbow trout processing as fish by-products, on pre-growing performance in gilthead sea bream (*Sparus aurata*) juveniles. Three groups of 170 gilthead sea bream each (initial body weight 37.8 ± 0.5 g) were employed in triplicate indoor tanks. Two groups (L1, L2) were fed with two different diets including FPH (354 g/kg and 177 g/kg, respectively) whereas the third group received FPH-free diet (LC), having the main protein fraction represented by fish meal and soybean meal. At the end of the trial, good productive and zootechnical parameters were obtained in all the

three groups with similar performances. The final mean weight ranged from 76.6 g to 78.0 g; specific growth rate was from 1.26 to 1.31%; food convertion rate was between 1.24 ± 0.04 and 1.26 ± 0.01. The survival rate was high in all the groups; the three diets exhibited high palatability. Somatic indices were similar in terms of condition index. Fat somatic index was between 7.5 and 8.1 in all the three groups. Liver histology did not differ between groups. Looking to these results, the trial showed that the use of FPH as alternative source of animal proteins is feasible in the feeding of gilthead sea bream juveniles.

Another feeding trial considered the possibility of rearing rainbow trout with a partial substitution of fish meal with guar gum at different level of inclusion. Reducing the amount of fishmeal and fish oil used in fish feed is a priority for the aquaculture sector, stimulated by lower prices for plant-based raw materials. In recent decades, significant progress has been made in identifying alternative ingredients to fishmeal to be used in fish feeds. Considering that guar meal has a higher protein content, better amino acid profile and lower cost per unit of produced protein compared to soybean meal (Hardy, 1999), the effects of the alternative diets with guar gum inclusion at 5% and 15% were evaluated on growth and quality parameters in rainbow trout. In the conducted trial, the addition of guar gum as an alternative feedstuff significantly improved faecal viscosity into farmed water. The productive parameters obtained applying the diet at low level of substitution (5%) were similar to the control group referring to final mean weight, weight gain and specific grow rate, significantly different in comparison with the diet with the 15% of guar meal inclusion. In fact, the final mean weight of trout receiving the three diets showed results similar between D5 (201.00 ± 3.7 g) and CD (198.8 ± 3.8 g), both significantly different in comparison with D15 (171.2 ± 10.1 g) that had the lowest mean weight. The same trend of difference was observed considering weight gain and specific grow rate. The food conversion rate had the most convenient performance in D5 (1.18 ± 0.01) and in CD (1.15 ± 0.02) respect to D15 (1.56 ±

0.17). Survival rate ranged between 98.07% (D5) and 97.3% (CD) without notable differences. Feed palatability resulted very high in CD and D5, differently from D15 that was slightly lower than the other two diets. From a zootechnical point of view, this trial showed that the inclusion of guar in rainbow trout feeding was satisfactory at 5% of inclusion. An additional cost of feedstuffs analysis was considered, showing that the diet with the 5% of guar inclusion was less convenient respect to the other two diets. In this situation, fish farmers have to pay attention and evaluate every decision to take in relation to the productive performances and the cost of production of rainbow trout size-portion.

Concerning the trial based on the by-products, it must be said that this issue has become more pressing worldwide due to the ever-increasing amount of waste and wastewater. Recently, more attention has been focused on the possibility of extracting precious nutrients from rendered fish proteins. In our trial, productive parameters were evaluated and compared between gilthead sea bream which received the experimental diets with hydrolysed fish proteins inclusion and the control group, who instead received a diet free from hydrolysed fish proteins. In addiction, the liver of five sea bream per diet was sampled and standard histological techniques were applied. The results showed that hydrolysed fish proteins from rainbow trout processing still contains valuable nutrients, which could be successfully considered as possible feedstuff, and so that the use of hydrolysed fish proteins as an alternative proteins source is feasible in the feeding of juveniles of gilthead sea bream in the pre-growing phase.

To combine fish growth and environmental sustainability, the research of proteins of vegetable origins involved the aquafeed production. For this topic, the attention was put on duckweed (*Lemna* spp.), a free-floating aquatic plant that provides a good source of protein. Looking to the composition of duckweeds, they provide in particular a good source of protein, and secondary lipid and minerals. Moreover, macronutrients and other compounds,

such as β-carotene and xanthophyll, increase the importance of duckweeds as a potential ingredient to be essayed in aquafeed. Based on these considerations, a trial was performed in order to evaluate the effects of duckweed meal as partial replacement of the main conventional protein sources (fish meal and soybean meal) in three different low fish meal diets on productive performances of rainbow trout reared during the on-growing phase and compared with coetaneous fish receiving a conventional feed. For that trial, the species *Lemna minor* was collected from ponds, proximate composition and amino acid profile were determined, and then the plant was included in the formulation of three experimental diets for rainbow trout at 10%, 20%, 28% of the protein source. Increasing the duckweed inclusion, the other protein sources were adjusted to get isonitrogenous (41%) and isolipidic (20%) diets, as the control diet (LC). After 90 days, fish were weighed and the most important productive performances, fillet quality and fatty acid profile were determined and compared with the control group. Duckweed meal derived from *Lemna minor* looked able to be included in the feed for rainbow trout without negative effects on the growth performances up to 20% of the protein substitution, whereas highest levels affected growth performances and feed conversion rate.

Keywords: rainbow trout, water quality, alternative raw materials, fish welfare.

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1. INTRODUCTION – ONE HEALTH, CIRCULAR ECONOMY APPROACH AND PRINCIPLES AF FISH NUTRITIONS

In recent decades the aquaculture sector has seen a constant growing development due to the growing demand at the retail level, diversification of farmed species, outsourcing of product processing and synergies between producers, processors and retailers (D'Agaro et al., 2022). World fish consumption is constantly increasing and in 2030 total world fish production is predicted to rich the 202 million tonnes, of which aquaculture accounts for around 106 million tonnes of fish produced (FAO 2022). According to the report of D'Agaro and colleagues (D'Agaro et al., 2022), Italian aquaculture production has grown by 4% in the last five years, ranking in fourth position in Europe. In Italy, the most farmed species is the rainbow trout, the production of which contributes approximately 25.5% of the total value and 69.9% to the volume of farmed fish. In the last ten years, trout production in Italy has increased overall by 8.5% with an average annual growth rate of 2.5% (D'Agaro et al., 2022). Aquaculture is an important source of aquatic food and sector for food security as many wild fisheries around the globe are over exploited or reaching their maximum sustainable potential. As a result, aquaculture has become one of the world's largest growing food production technologies.

An important element that has been developing in recent years at global level is the so called "One Health" perspective. One Health (OH) is an approach that aims to promote human, animal, and environmental health through multidisciplinary and multi-sectoral approaches. It recognizes the intrinsic linkages between animal diseases, public health and ecosystem transformation and address them in a holistic and systemic way in order to achieve more sustainable results. The OH concept means an integrated approach to health focused on interactions between human, animals and environment. OH refers not only to the absence of diseases but more in general to the broadest concept of well-being (for humans and animals),

which is correlated to the health of the environment. Animal health is crucial to improve Food and Nutrition Security on one side and Public Health on the other side.

Based on these considerations, in collaboration with the Erede Rossi Silvio Trout Company, a PhD project was conducted with the title "Research and study of innovative raw materials to be used in feeding the main fish species of interest to aquaculture in order to obtain a product of high nutritional quality and antibiotic free". In recent years the Erede Rossi Silvio Trout Company was in line with the modern global problems related to the exploitation of ocean and sea, production sustainability and high quality of the final fish product, and consequently paid an increasing attention to animal welfare thanks also to the use of high quality feeds and a less intensive and more respectful farming method. Other key point of the Erede Rossi Silvio Trout Company fish production are respecting the aquatic environment and obtaining high quality and healthy fish products. With this perspective, they have already started a production line based on the antibiotic free principle. To reach the aim of the antibiotic free production, fish firstly must be healthy to avoid inflammation or diseases that require the use of drugs during the farming cycle. This goal were the object of the present PhD project. First of all, breeding density must be respected. Fish are social animals that live in shoaling. However, high densities could have negative implications on stress indicators, on the quality of fish fillet, and on the morphology of the reared fish, such as partial atrophy of some fins, stockier body structure with a decrease in the length/height ratio. In fish farm, the possibility of movement plays a crucial role on fish welfare and suitable densities for the considered fish species are able to positively affect the well status of the farming fish. Correct farming densities has an impact also on the immune system thus preventing attacks by aquatic pathogens (Fiordelmondo et al., 2020). In the Erede Rossi Silvio Trout Company the productive cycle starts from young rainbow trout with an average body weight of 90 ± 2 g, which are grown at a stocking density of 20 kg/m^3 until reaching the market size of 350 g. In

particular, at the beginning of the life cycle, the fish density is low, about 8 kg/m^3 of water, in order to limit the stress of young trout and prevent gill diseases or bacterial infections. This density subsequently increases until the end of the cycle, when it is 20 kg/m^3 maximum. Furthermore, the quality of the farming water is crucial, as well as feed quality, which must respect the specific nutritional requirements of the raring species and adopt correct farming processes.

The concept of food quality is more complex that it looks. Nowadays it is strongly connected with the sustainability of the faming process for the raw material used. In fish feeds fish meal and fish oils are partially substituted with other ingredients, namely insect meal, vegetarian ingredients, and fish by-products (Ferraro et al., 2010; Chemello et al., 2020; Parisi et al., 2020; Fiordelmondo et al., 2022). In recent years, feed for the main fish species of interest to aquaculture has made encouraging developments towards environmental sustainability. Two elements have strongly contributed to this breakthrough: the respect of the aquatic environment, protected by specific rules, and the adoption and the optimization of the feed extrusion technique, that allows to rich a conversion index close to the unity and high digestibility of fish feed.

Speaking about feed ingredients, few consequences are related to the correct balance of the protein component of the diet: the optimization of fish growth and conversion rates; the reduction of the environmental impact due to the excretion of nitrogen and phosphorus in the aquatic environment and the production of suspended solids; the reduction of feed costs. From dietetic and nutritional point of view, fish meal is theoretically the ideal protein ingredients, because it is characterized by a high content of protein (>65%) of high biological value, thanks to balanced levels and quality profiles in essential amino acids and for the high digestibility of nutrients and energy. They also provide mineral elements, vitamins, essential fatty acids (PUFA) and contain substances that stimulate appetite (Kissil et al., 2000; Kaushik

et al., 2022). Nowadays the high costs of fish meal and the risk of over-exploitation of the stocks of fish of clupeids (group of fish to which the most important species captured for the production of fish meal and fish oil belongs) make the use of fish meal and fish oil not sustainable anymore for that ecological and economic aspects. The use of expensive fish meal for fish feeding is leaving space to cheaper and less impactful alternative diets, based on protein sources able to replace this ingredient with others (insects, vegetables, algae, by-products from aquatic organisms) (Gasco et al., 2020; Parisi et al., 2020). The soybean meal is the conventional protein source mostly used as a complement to fish meal and consequently the demand for this feedstuff has significantly increased in the last few years, and the properties of other plants have been investigated to evaluate their potential use in fish feed as protein source (Doroty et al., 2018; Ceschin et al., 2020). It is still a current topic due to the urgent problem of finding new protein sources as alternatives to the standard ones to be used in aquafeed. From the point of view of the sustainability of raw materials, using vegetable proteins in fish feeding means pay particular attention on the quality of the protein and therefore on the profile of essential amino acids, the presence of anti-nutritional factors, the content of fibre, carbohydrates, oligosaccharides, mineral elements, and finally on the palatability of vegetable diets (Doroty et al., 2018; Parisi et al., 2020). In the Erede Rossi Silvio Trout Company the inclusion of protein of vegetarian origin is about 25-30% of the total protein, using mainly soy and wheat derivatives. With the administration of specific diets for each stage of fish development, in the optimal breeding conditions of the Sefro farm plant, trout were exposed to a lower risk of disease (Austin et al., 2016). These actions therefore allow to prevent sickness and avoid the use of antibiotics.

Lipids represent a fundamental fraction of fish diet, playing structural and energetic functions. As vertebrates, fish require three long-chain polyunsaturated fatty acids (PUFAs) for their normal growth and development: eicosapentaenoic acid (EPA), docosahexaenoic

acid (DHA), and arachidonic acid (AA). The biochemical, cellular, and physiological functions of these three PUFAs are approximately the same in fish and other vertebrates. In our case, in the feed used by the Erede Rossi Silvio Trout Company the record lipid contents never exceeds the 21%, which corresponds to the maximum content of feed for fish fattening and which guarantee superior quality of fish production. Extreme attention was paid from that Trout Company in the formulation of balanced specific diets to meet all the nutritional requirements of the considered farmed fish species. The organoleptic characteristics and the quality of the fish feed play a crucial role as the administered feed represents the only food available for farming trout, and furthermore is able to directly shape the composition of the fish fillet meat. In this context, it should be remembered that the extruded feed shows a higher absorption capacity of fats then the pellet feed, and its specific weight is easy to control. As consequence, it is possible to control fish feeding in quantity and quality, and consequently it is possible to shape the composition of the fish fillet meat obtained from farmed fish.

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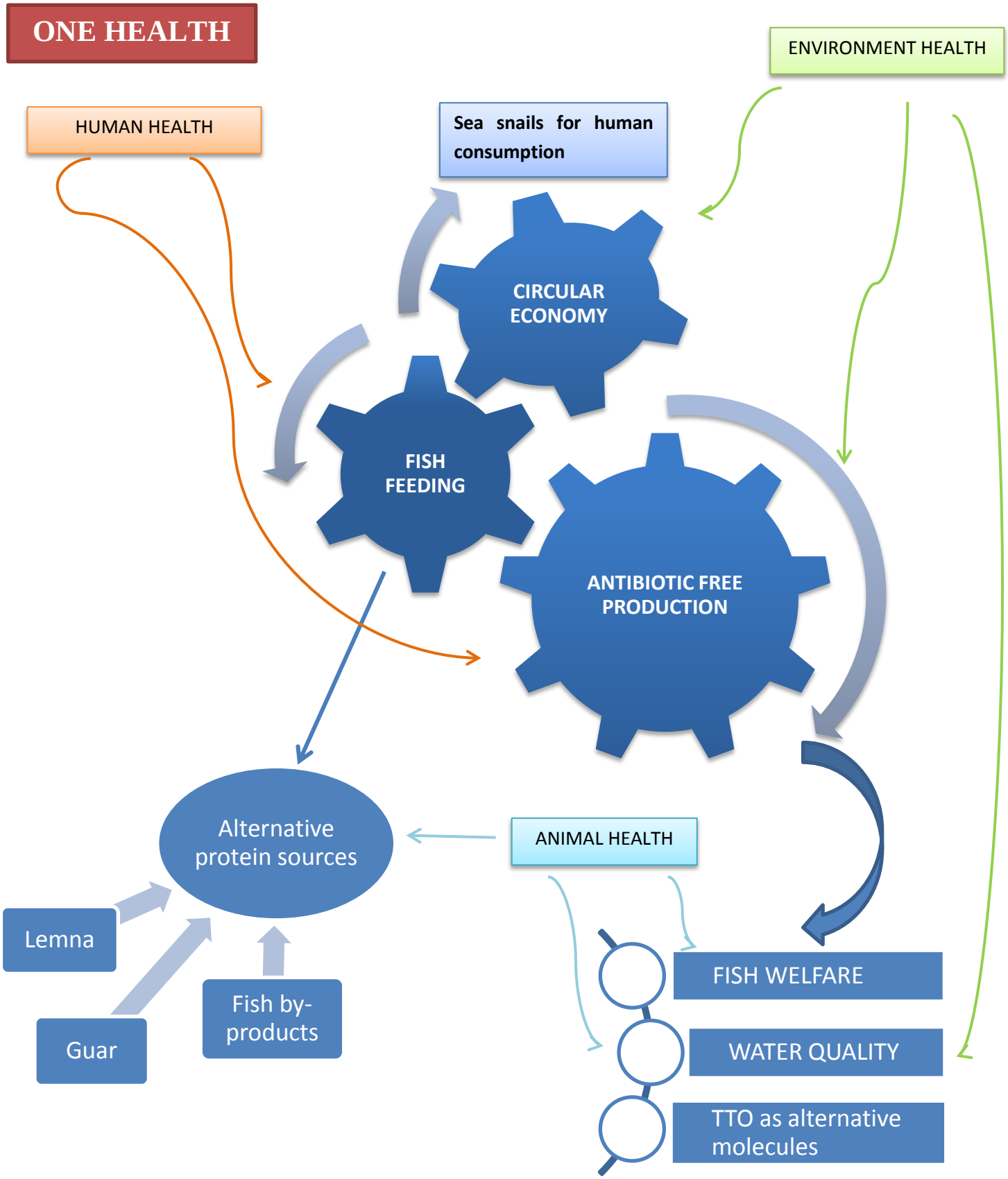
GRAFIC ABSTRACT IN THE NEXT PAGE



The present PhD project had the aim to find strategies to increase the non-specific immune defences of farming fish avoiding the use of drug, in particular antibiotics, in order to obtain a high nutritional quality products as human food. Considering that animal health is crucial to improve food and nutrition security on one side and Public Health on the other side, fish welfare is an essential factor to improve and the constantly interaction between fish surface and aquatic environment must be considered. Improving fish health means also working on fish nutrition, topic strictly connected to aquatic sustainability, based on the characterization of new raw materials to be used as protein sources in partial substitution of fish meal.

2. ROLE OF WATER QUALITY ON PRESERVING ANIMAL WELFARE AND PROTECTING THE AQUATIC ENVIRONMENT

2.1. Introduction

In the last two decades, new strategies for improving feeding techniques for the main fish species have been developing in order to reach global sustainability, in particular in an environmentally responsible manner. At the start of the new millennium, the European Union adopted the Water Framework Directive (2000/60/EC), which introduced a transnational vision of the management of the freshwater environment able to protect, manage and improve the quality of water resources across the EU. In 2018, the European Environment Agency showed the efforts carried out by countries to monitor and assess the general health status of water bodies and stressed the importance of also promoting the sustainable use of available water resources in inland anthropic activities of the primary sector, such as agriculture and aquaculture, performed in strict accordance with natural waters. Many papers (Torphe et al., 1995; Boyd et al., 2001; Aubin et al., 2011; Moraes et al., 2015) have focused on the environmental impact of trout farms when uneaten food, fish catabolites (metabolites, ammonia gill excretion and carbon dioxide) and chemical treatments are not controlled and are discharged into the natural receiving waters. In the case of aquaculture specialized in the growing phase of rainbow trout (*Oncorhynchus mykiss*), the rearing technique is based on the use of flow-through systems which consist of raceways or concrete tanks with water constantly flowing down basins and the removal of waste at the outlet by gravity and the water current (Parisi et al., 2013).

In aquafeed, two elements have mostly contributed to improving aspects of change concerning fish feeding, in particular, in rainbow trout: the use of the feed extrusion technique and the adoption of restrictive environmental rules. First, regarding rainbow trout

feeding, the best technique is based on the fact that the feed distribution must be carried out until reaching a level close to satiety (Bureau et al., 2006). In fact, the feed conversion index and the protein efficiency index improve when rainbow trout are fed with a rationing level equal to 70% of the "ad libitum technique" (Bureau et al., 2006). This technique drastically reduces the amount of food not eaten by trout.

Feed quality also directly affects water quality, because a proportion of the feed intake by fish is returned to the environment as metabolites or soluble by-products of metabolism (Thorpe et al., 1995). In this context, two main levels of measures (legislative and productive) have been adopted. Restrictive environmental rules of EU countries have imposed settling areas to eliminate solid waste, whilst new feeding technologies have also been considered. The evaluation of the impact of rainbow trout farming on the receiving water quality needs to take into consideration the water quality monitoring over a long period, based on a decade (Tahar et al., 2018) or two years, in different flow-through farms (Aubin et al., 2011). Concerning feeding technologies, nowadays, modern systems use extruded feed instead of pellet feed. In particular, the metabolite status of fish depends on the degree of gelatinization of the feeding starch. In the modern aquatic system, the removal of metabolites can be decreased using formulations with ingredients able to help bind metabolite matter, allowing these particles to be more easily and thoroughly removed from the water (Tyapkova et al., 2016; Funk et al., 2019; Welker et al., 2019).

Furthermore, small feed portions not consumed are decreased by a smart diet formulation and processing; nowadays, extruded feed represents the best solution, instead of the pellet one, which was previously the common feeding strategy (Tyapkova et al., 2016; Funk et al., 2019; Welker et al., 2019). The digestibility of the extruded feed increases up to 96% relative to the raw wheat starch, while, in the case of pelleted feed, this raw material is digestible to approximately 54% (Burel et al., 2000). Moreover, the catabolic residues emitted by rainbow

trout, fed with extruded feed with gelatinized starch, are easy to remove from the water, thanks to their sedimentation (Welker et al., 2019). Therefore, extruded feed also enables high environmental sustainability due to the increase of digestibility and stability in water, with a consequent reduction of suspended solids and nutrients. With regard to the rearing environment and its traits, it is appropriate to consider how it influences the conditions of fish welfare. Correct water exchange is essential: water dilutes and removes the catabolites of fish, as well as feed residues, thus reducing the exposure of the farmed subjects to dangerous nitrogen compounds which are also able to negatively influence the state of fish welfare.

To check that the water quality is suitable for the farming of rainbow trout, a multitude of physical and chemical parameters must be considered (Alzieu C., 1990). In the case of suspended solids, their concentration is particularly important, because they directly influence the water turbidity, which can prevent the vision of the fish and finally compromise their life cycle. According to Boyd (Boyd et al., 2001) and Becke (Becke et al., 2019), water turbidity values higher than 400 mg/L can cause thickening and deformation of the gill filaments, with trout consequently suffering. At the same time, dissolved organic substances, including the sedimentable (undigested portion of the ration and food residues) and non-sedimentable (product of endogenous metabolism) solids in suspension, must be taken into consideration. For these reasons, checking the water quality is of primary importance for the welfare of trout. Inappropriate rearing conditions, such as inadequate space, excessive densities and poor feeding, can have strong negative repercussions for farmed fish species. Damaged, eroded or haemorrhagic fins are not only correlated with pathological events but also with inadequate environmental factors, connected to stress-related aspects such as a fish stocking density that is too high with a non-optimal water quality (Alabaster et al., 1982).

Considering the use of diets administered to salmonids, many papers have focused on the emission of catabolites in the external environment. According to European Environment

Agency, 15–25% of the total food energy is lost in ammonia and urea through the gills and is released into the environment.

Most of the papers on this topic have focused their attention on relationships between feeding strategies, animal welfare and environmental sustainability and in the current literature the correlation between water quality and productive performances of farmed rainbow trout is still a key point of discussion.

Based on these considerations, a study focusing on the trend of the most important water quality parameters in rainbow trout farming of central Apennine in terms of long-term activity (2009-2019) was carried out. Before the decade focused on in this study, the first historical monitoring of water quality took place in a trial carried out in 2004, when the owners started to re-think the farming technique adopted until then, based on high stocking densities (40 kg/m^3), in order to produce a higher fish welfare status (Melotti et al., 2004). Considering this, the present study aimed to evaluate the suitability of changes adopted in rearing and feeding techniques to improve growth performance, sustainability and the water quality environment. The raceway water quality was monitored in terms of the Total Suspended Solids (TSS), Biochemical Oxygen Demand (BOD_5), Chemical Oxygen Demand (COD), Total Ammonia Nitrogen (TAN), Nitrites (NO_2-N), Nitrates (NO_3-N), pH and Total Phosphorus (TP). These parameters were investigated over a decade, starting in 2009 and were compared to the respective annual values of the 10 years after, until the 2019 year, in order to show differences in the water quality and feed conversion rate.

2.2. Materials and Methods

2.2.1. Trout farm description and water quality monitoring

A study on the water quality trend was performed on the rainbow trout fattening farm of the Erede Rossi Silvio Trout Company, located in the Apennine area of central Italy (Picture 1, 2, 3), based on raceways in parallel (120 m^3 each). During the time of the study, the genetic line of rainbow trout did not change and it was directly controlled by the farm's owners, who had a hatchery unit (broodstock maintaining and fingerling production) in a different area from which fishes for fattening came and were selected.

On the rainbow trout farm in which the study was conducted, the feeding technique was the same during the entire experimental period (2009-2019) and the feeding rate was the same for fish at the same life cycle stage. The feed was distributed with a semi-moving wagon up to the level close to satiety and at the same time during the day, twice a day.

The water supply system used on the farm allowed at least one complete daily water change. The inlet water came to the farm system with a constant velocity of 0.25 m/s and flowed through four parallel raceways. A water quality sample was obtained monthly during the last decade (2009-2019) in correspondence with the lagoon basin (outlet water) below the raceways and receiving downstream. All samples were collected in early morning before feeding using a polypropylene bottle with a screw cap (ISPRA 2014). In order to monitor water quality, different physicochemical parameters were investigated. The dissolved oxygen, temperature and pH were measured using portable electronic devices (YSI mod. 55 and 60). At the same time, five samples of 500 cm^3 water were collected for laboratory-based determination of the following parameters: total suspended solids (TSS), biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total ammonia nitrogen (TAN), nitrites (NO_2-N), nitrates (NO_3-N) and total phosphorus (TP). Nitrogen (N) compounds and TP were

determined using a spectrophotometer (Hach mod-2005, Hach Company, Loveland, USA), following the American Water Works Association and Water Pollution Control Federation of American Public Health Association (APHA) standard methods (APHA 1995). TSS was recorded following official methods (IRSA 2003). BOD_5 and COD were determined according to IRSA-CNR (IRSA 2003) and ISPRA (ISPRA 2014) methods, respectively. In order to quantify the results of the waste water of the rainbow trout farm, the quality of the inlet water was also assessed by analysing the values of TSS, COD, BOD_5, NO_2-N, NO_3-N and TAN, using the same laboratory methodology as described for the outlet water. Each sample of inlet water was collected using a sampler that conducted an average sampling process over three hours. During each year of the study, inlet water was collected seven times, in the months of January, April, May, June, August, October and December. The inlet water showed constant values for every parameter from 2009 to 2019, with the following averages: TSS, 5 mg/L; COD, 5 mg/L; BOD_5, 5 mg/L; NO_2-N, 0.09 mg/L; NO_3-N, 1.4 mg/L; TAN, 1.5 mg/L. Since these values were always suitable for farming rainbow trout, additional investigations and more frequent water samples were not necessary, allowing us to focus our attention on the analysis of the outlet water.

2.2.2. Experimental design and rearing of the rainbow trout

In order to summarize the methodology that was followed, Figure 1 shows the experimental design step by step. With reference to the first year (2009) and the final year (2019) of the considered decade, fish growth and water quality assessments were also evaluated by considering the balance of nitrogen and phosphorus released in waters. In a fish plant, the productive cycle starts from young rainbow trout, with an average body weight of 90 ± 2 g, which are grown at a stocking density of 20 kg/m^3 until reaching the market size (350 g). In particular, at the beginning of the life cycle, the fish density is low, about 8 kg/m^3 of water, in order to limit the stress of young trout and prevent gill diseases or bacterial infections. This

density then increases with the increasing size of the trout until the end of the cycle, when it reaches 20 kg/m^3 of water.

Picture 1, 2, 3 - Rainbow trout fattening farm of the Erede Rossi Silvio Trout Company. Apennine area of central Italy.

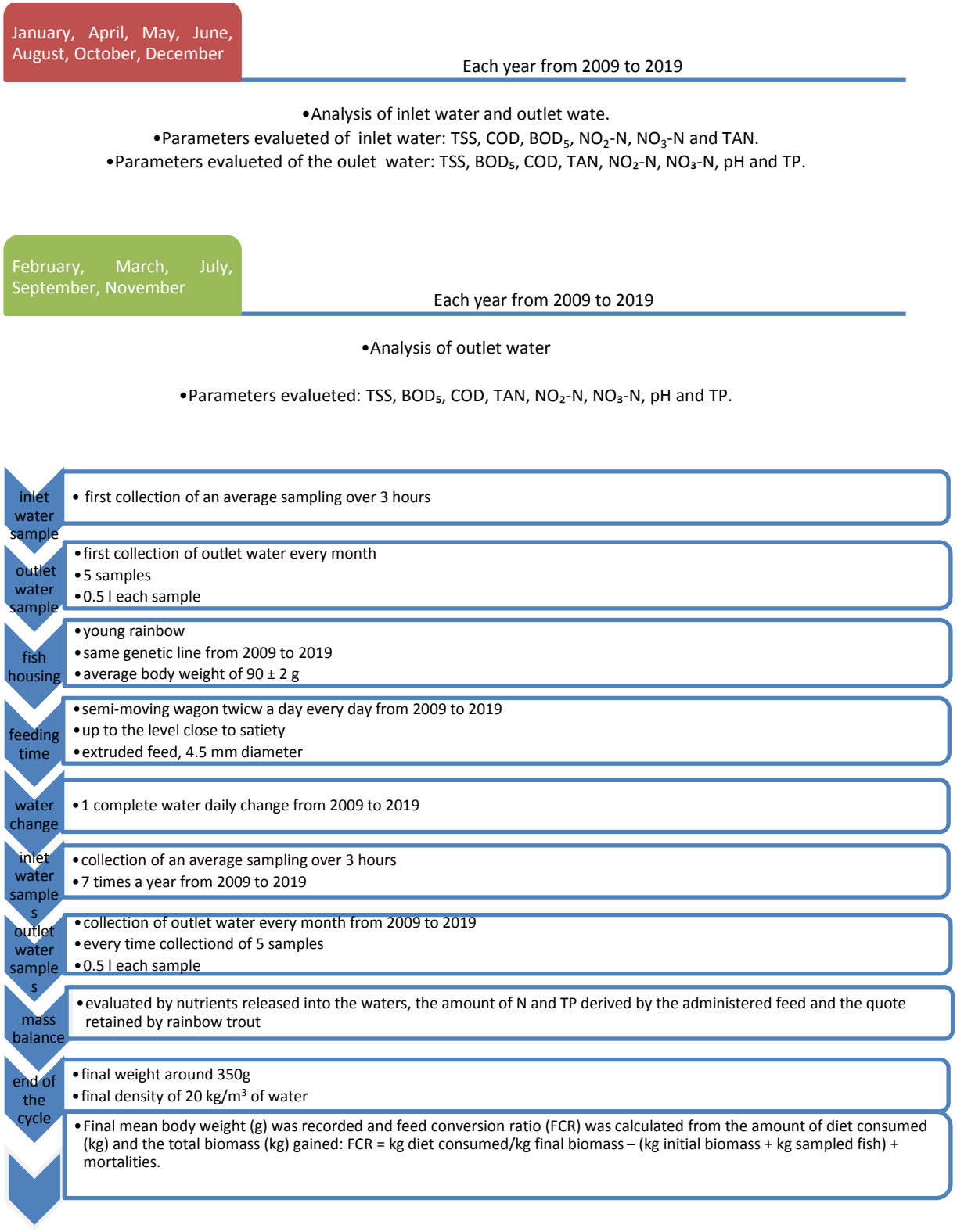


Figure 1 - Diagram showing the experimental design adopted during the 10 years of the study.

Fish received extruded feed (closed formula) that was 4.5 mm in diameter and was manufactured by the Erede Rossi Silvio Trout Company itself that had vegetable feedstuffs available at the land farm. The proximate composition of the two feeding types (pellet, extruded) employed to feed rainbow trout during the decade is reported in Table 1.

Table 1 - Proximate composition of the feeds employed for the rainbow trout growing in the decade object of water quality monitoring (2009-2019).

Feed	**Pellet**	**Extruded**
Chemical composition (%)		
Moisture	6.8	5.5
Crude protein	45.7	44.8
Crude lipid	16.0	21.0
Ash	6.7	8.4
Gross energy (MJ kg)	21.16	18.38

The final mean body weight (g) was recorded and the feed conversion ratio (FCR) was calculated from the amount of food consumed (kg) and the total biomass (kg) gained: FCR = kg food consumed/kg final biomass - (kg initial biomass + kg sampled fish) + mortalities.

In order to evaluate the mass balance of nutrients released into the water, the amount of N and TP derived by the administered feed and the amount retained by rainbow trout were considered, as shown in Table 2, by comparing the budget of these compounds, expressed as

the seasonal mean of the first year (2009) and the last year (2019) of study and applying the coefficients indicated by Bureau et al. (Bureau et al., 1999) and applied by other authors (Aubin et al., 2011).

Table 2 - Total ammonia nitrogen (TAN) and phosphorous (TP) budget in the lagoon basin outlet water.

	TAN (mg/L)	TP (mg/L)
2009:	**0.55**	**0.011**
2019:	**0.46**	**0.009**

2.2.3. Statistical analysis

All collected data on the outlet water quality were analysed to determine whether there were significant differences over the 10 considered years. For this aim, the months of the year were divided to define the four seasons: winter included the months of December, January, February and March; spring was represented by April, May and June; summer included July, August and September; and autumn included October and November.

After all the seasonal data were collected, the mean of each parameter was calculated season by season every year and finally data were finally organized as graphics.

The seasonal mean of the investigated parameters (TSS, BOD_5, COD, NO_2-N, NO_3-N, TAN, TP and pH), recorded per year, was subjected to one-way analysis of variance (ANOVA) using the General Model Procedure of SPSS 25 (IBM Corp., New York, USA) (IBM 2017),

in order to assess if data means were statistically difference within the season of different years. Significance was considered if $P < 0.05$ and the means were compared using the Student-Newman-Keuls (SNK) test.

2.3. Results

The results of the water quality parameters measured in the outlet lagoon basin in the 10 years-study are reported in Figures 2–9, comparing the mean of the same season of the 10 years.

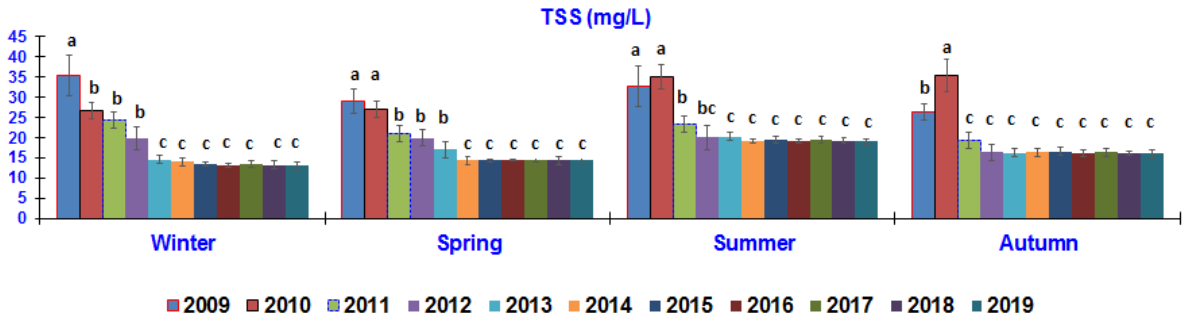


Figure 2 - Trend of outlet water referred to the Total Suspended Solids (TSS) (means ± standard deviation) seasonally determined during the 10 years-study. Different letters (a, b, c) per season show significant differences ($P < 0.05$) among the 10 years of sampling.

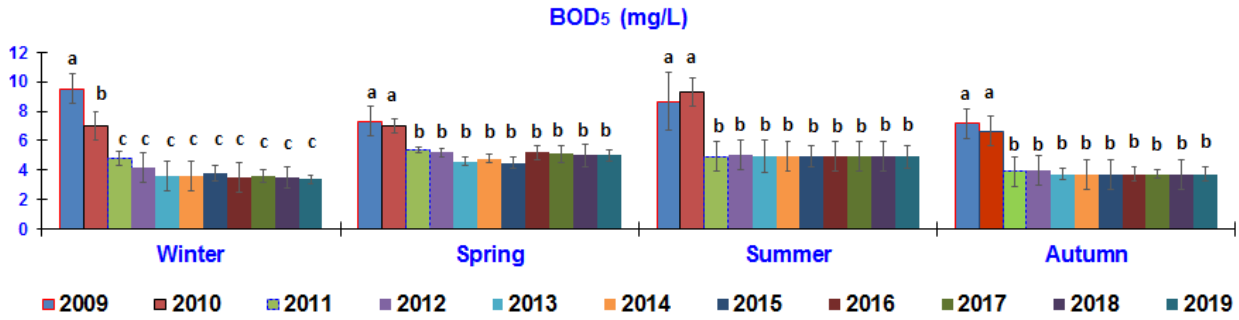


Figure 3 - Trend of outlet water referred to Biochemical Oxygen Demand (BOD_5) (mg/L) (mean ± standard deviation) seasonally determined during the 10 years-study. Different letters (a, b, c) per season show significant differences ($P < 0.05$) among the 10 years of sampling.

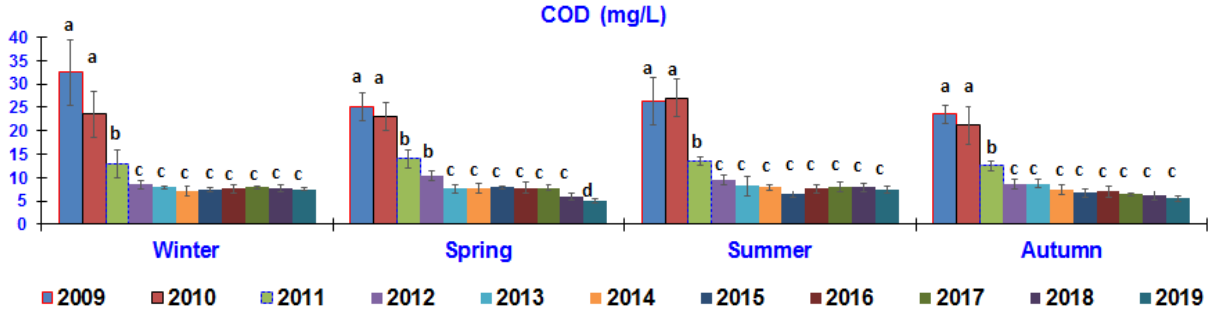


Figure 4 - Trend of outlet water referred to the Chemical Oxygen Demand (COD) (mg/L) (mean ± standard deviation) seasonally determined during the 10 years-study. Different letters (a, b, c, d) per season show significant differences ($P < 0.05$) among the 10 years of sampling.

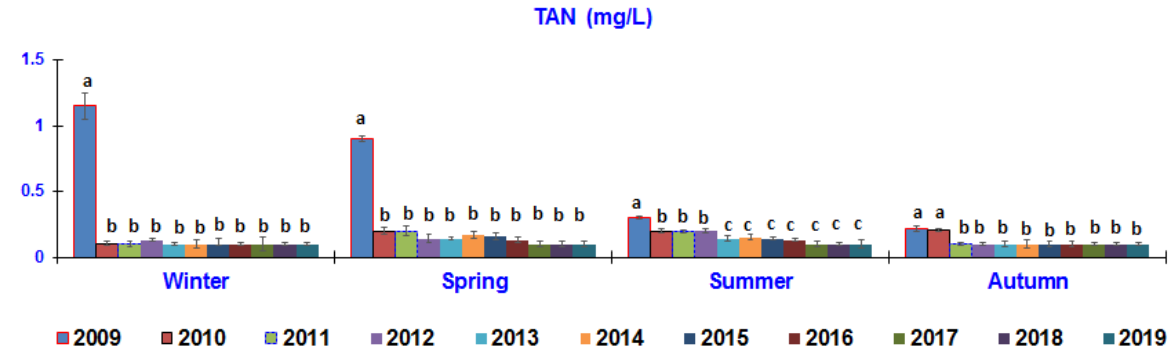


Figure 5 - Trend of outlet water referred to the Total Ammonia Nitrogen (TAN) (mg/L) (mean ± standard deviation) seasonally determined during the 10 years-study. Different letters (a, b, c) per season show significant differences ($P < 0.05$) among the 10 years of sampling.

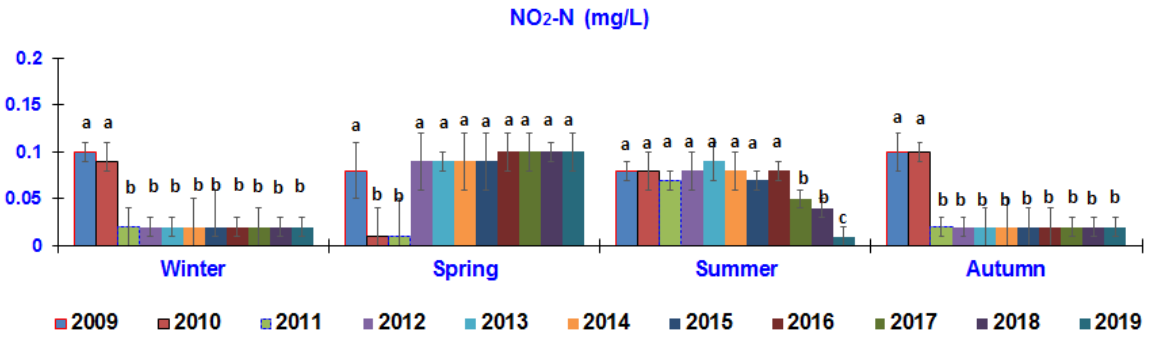


Figure 6 - Trend of outlet water referred to the Nitrites (NO_2-N) (mg/L) (mean ± standard deviation) seasonally determined during the 10 years-study. Different letters (a, b, c) per season show significant differences ($P < 0.05$) among the 10 years of sampling.

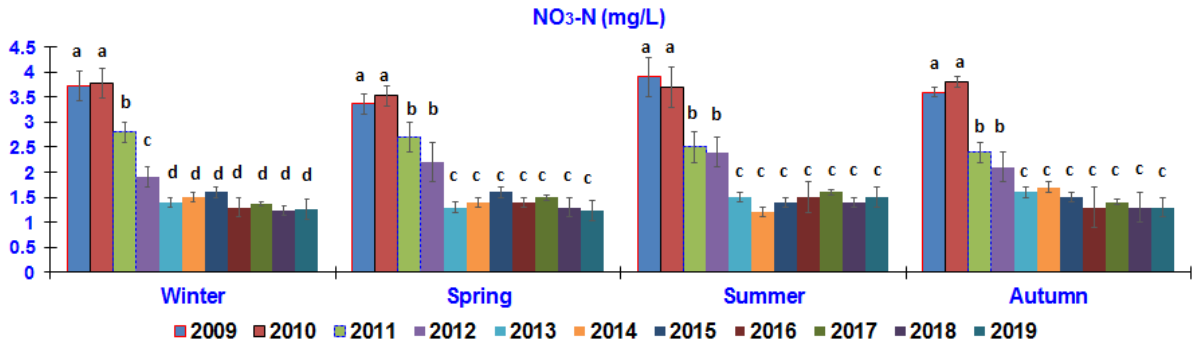


Figure 7 - Trend of outlet water referred to the Nitrates (NO_3-N) (mg/L) (mean ± standard deviation) seasonally determined during the 10 years-study. Different letters (a, b, c, d) per season show significant differences ($P < 0.05$) among the 10 years of sampling.

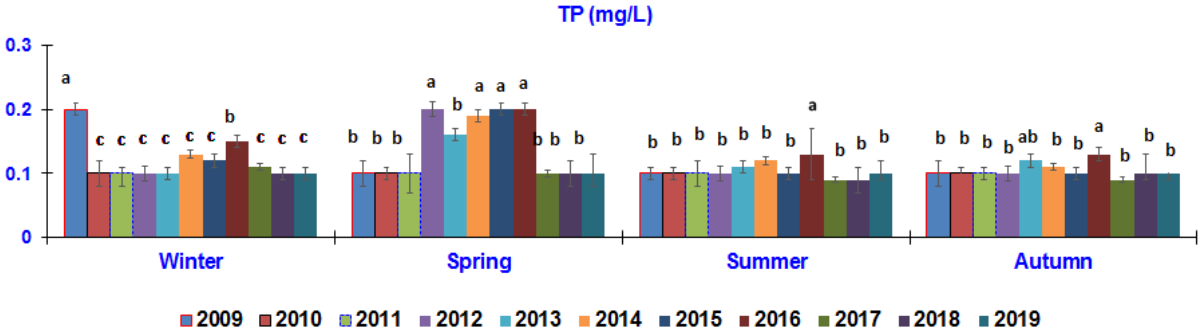


Figure 8 - Trend of outlet water referred to the Total Phosphorus (TP) (mg/L) (mean ± standard deviation) seasonally determined during the 10 years-study. Different letters (a, b, c) per season show significant differences ($P < 0.05$) among the 10 years of sampling.

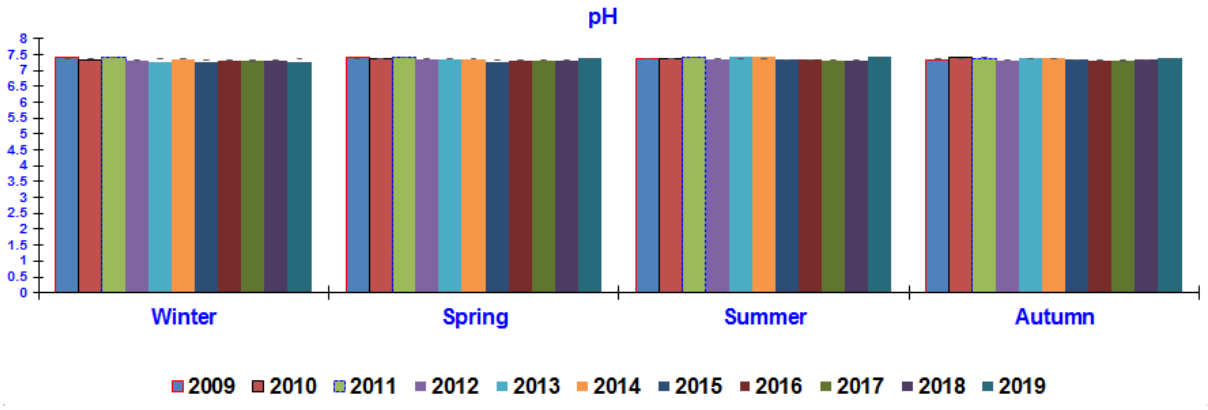


Figure 9 - Trend of outlet water pH (mean ± standard deviation) seasonally determined during the 10 years-study.

The TSS (Figure 2) content showed a significant decrease when passing from the first seasons of sampling to the last years. In winter, TSS exhibited a significantly decreased mean value from 35.5 ± 5 mg/L in 2009 to 13 ± 3 mg/L in 2019, with an intermediate reduction notably shown in 2010-2012 (26.75-19.8 mg/L); in 2013 (14.5 ± 2 mg/L), TSS reached the lowest values until 2019. In spring, the same trend was observed, when the means decreased from 29 ± 3 and 27.3 ± 2.6 mg/L (2009-2010, respectively) to around 14.3 mg/L, which was maintained from 2014 to 2019. In the summer season, the recorded means were 37.20 ± 5 mg/L in 2009 and 35.4 ± 4 mg/L in 2010, without significant differences, whereas a marked reduction was observed from the year after (2011, 23.4 ± 3 mg/L), gradually diminishing from 2012 and remaining at around 16 mg/L until 2019. In autumn, the highest TSS content was recorded in 2010 (35.5 ± 3.9 mg/L), being significantly different from the analyses of the first year of monitoring (2009, 26.5 ± 2 mg/L), but from 2011 the mean value was significantly lower and was maintained at around 16.00 ± 2.79 mg/L until the end of the

study. In particular, considering the seasonal average of TSS detected in autumn, there was a reduction of almost 55% of this parameter.

Considering the trend of BOD_5 (Figure 3), there was a marked improvement in 2019 compared to the 10 years before. In winter, BOD_5 recorded a significant decrease, passing from the mean value of 9.5 ± 0.9 mg/L in 2009 to that of 7 ± 0.8 mg/L in 2010 and further decreased to around 3.7 ± 2 mg/L in the following years, until 2019. In spring, the highest levels were determined in 2009 (7.33 ± 1 mg/L) and 2010 (7.06 ± 0.7 mg/L); after this, the means significantly stayed at around 5.00 ± 0.7 mg/L until 2019. In summer, the highest levels of BOD_5 were observed in 2009-2010 (8.7-9.3 mg/L), which were significantly different from all of the following means, being around 4.9 ± 1 mg/L until the end of the monitoring period. In autumn, a similar trend was also observed, with a notable difference between the first two years of sampling (7.18-6.72 mg/L in 2009-2010) and the other following years, being around 3.7 ± 0.7 mg/L until the end of the study.

In terms of COD determination (Figure 4), all four seasons showed a similar trend, resulting in a sharp reduction through the years. In winter, the highest levels were recorded in the first two years (2009, 32.5 ± 5.1 mg/L; 2010, 23.5 ± 4.4 mg/L), significantly decreasing in 2011 (13 ± 3.1 mg/L) and reaching the lowest mean in 2012, when the average of around 7.5 mg/L was maintained until 2019. In spring, the highest values occurred over the first two years (25 ± 1.3 mg/L) and then decreased in 2012-2013 (25-23.1 mg/L), until reaching the significantly lowest level in the last year (5 ± 0.2 mg/L). In summer, the first two years exhibited the highest levels (26.3-27 ± 1 mg/L). The mean value was decreased in 2011 (13.5 ± 0.3 mg/L) but was significantly higher than that in the following years, being around 7.5 ± 0.6 mg/L until the end of the study. In autumn, the mean of this parameter was 23.52 ± 0.5 mg/L in 2009 and 21.14 ± 0.7 in 2010; then, it decreased to 12.5 ± 0.3 mg/L in 2011 and reached 6.8-5.51 mg/L in the last years, without notable differences.

Regarding the TAN parameter (Figure 5), a clear reduction was observed in the range of the years taken into consideration. In the winter season, the data showed a significant decrease from the first year of study, from 1.15 ± 0.3 mg/L in 2009 to 0.1 ± 0.02 mg/L in 2019. In spring, TAN significantly decreased from 0.90 ± 0.2 mg/L in 2009 to 0.10 ± 0.02 mg/L in 2019. During summer time, a significant reduction was observed; in fact the value was 0.30 ± 0.01 mg/L in 2009 and significantly decreased from 2010-2012 (0.21 ± 0.2 mg/L) and then further decreased to 0.1 ± 0.02 mg/L in 2019. In autumn, the TAN value decreased from 0.21 ± 0.22 mg/L in 2009 and 2010 in the following years (2011-2019), when a mean value of 0.1 ± 0.02 mg/L was constantly recorded.

In terms of the NO_2-N parameter (Figure 6), in winter the mean values (0.1 ± 0.09 mg/L), detected in 2009-2010, significantly decreased to 0.02 mg/L throughout the following years. In spring, an opposite trend was noted, with the lowest concentrations (0.01 mg/L) occurring in 2010-2011 in comparison to all other years (0.1 mg/L). In summer, a similar trend was observed, although the last year of monitoring showed the lowest mean (0.01 ± 0.002 mg/L) recorded among the years. On the contrary, in autumn the TAN level detected in the two first years (0.1 mg/L) significantly decreased to 0.02 ± 0.001 mg/L in 2019.

In terms of the NO_3-N parameter (Figure 7), a similar trend was observed in all the seasons, with a notable reduction emerging in 2013 and being maintained throughout the last years. In winter, the content dropped from 3.73-3.78 mg/L in 2009-2010 to only 1.22 ± 0.3 mg/L in 2019. In spring, the value significantly changed from 3.37 ± 0.5 mg/L in 2009 to 1.23 ± 0.3 mg/L in 2019. In summer, the value ranged from 3.90 ± 0.5 mg/L in 2009 to 1.50 ± 0.3 mg/L in 2019. In autumn, the concentration ranged from 3.60 ± 0.4 mg/L in 2009 to 1.30 ± 0.2 mg/L in 2019.

Considering the TP parameter (Figure 8), in winter the values significantly decreased from 0.2 ± 0.01 mg/L in 2009 to 0.1 ± 0.002 mg/L in 2019. In spring, the highest values (0.19 ± 0.2 mg/L) were recorded in the central period of the study (2014-2016); then, a reduction was observed, that is, 0.1 mg/L. In summer and winter, although there was a significantly higher level in 2016 (0.13 ± 00.2 mg/L), TP remained at a low value (0.1 mg/L) until 2019.

The pH parameter (Figure 9) did not show significant variation, remaining approximate neutral during the entire studied period and the obtained values were always in accordance with the range considered appropriate for rainbow trout (Melotti et al.,2004; Tahar et al., 2018).

At the end of the study, the FCR was 1.1 and significantly improved in comparison with the FCR obtained in 2009 (1.4). The survival rate was maintained at around 97% from 2012 onwards compared to the survival rate of the years before (94%).

In Table 2 the nutrient budget of outlet water is reported.

In 2009, TAN was approximately 0.55 mg/L, whereas in 2019 the budget had an average value of 0.46 mg/L. The same trend was observed for the TP budget, which ranged from 0.011 mg/L (2009) to 0.009 mg/L over the 10 years.

2.4. Discussion

The aim of the current study was to investigate the impact of a new type of feed on water quality. Based on a comparison of the values of the analysed parameters from 2009 to 2019, a notable decrease was observed in the TSS, BOD_5, COD, TAN, NO_2-N, NO_3-N, pH and TP.

As other studies have shown (Tahar et al., 2018; Dalsgaard et al., 2011; Galezan et al., 2020), BOD_5 is a very important parameter for assessing the water quality because it indicates the consumption of oxygen in the processes of indirect oxidation by the metabolic systems of aerobic microorganisms present in the water. Aside from the TSS level (Galezan et al., 2020), BOD_5 determination is essential for detecting the oxygen demand of the aerobic microbial flora required for the decomposition of organic substances present at a certain temperature in a defined time range. Therefore, it is an indicator that increases as the amount of organic substance to be mineralized increases.

The low values of BOD_5 detected during 2019, together with the decreased of COD levels, indicate that aerobic microorganisms prevailed over anaerobic ones and therefore the self-purifying capacity of the watercourse can be considered good. The reduction of the COD parameter that exceeded 76.5% is proof that the technical and management interventions implemented at the breeding rainbow trout farm effectively resulted in an improvement in the environmental protection. This result is in agreement with data presented by Galezan (Galezan et al., 2020), while Tahar (Tahar et al. 2018) reported lower BOD_5 values.

Based on analysis of the main water quality parameters of the trout farm involved in the trial over the considered decade, a significant improvement in the rearing environment was shown. The significant reduction registered can be attributed to the different type of feed adopted, which changed from pelleted to extruded feed. Together with adequate quantities of oxygen dissolved in the water and through the nitrification process, a decreased amount of TAN can be converted into nitrites and nitrates. Nitrates are less toxic to fish over a long time period throughout the nitrification process (Galezan et al., 2020). A trend of the reduction of undesirable physicochemical traits was clearly observed when the new feeding type was introduced in 2011. Based on a comparison of the pellet feed with the extrusion technology, the digestible level of the diet was shown to have improved. This extruded manufacture

drastically reduces the amount of food not eaten by trout and increases the feed efficiency (Kaushik et al., 2013).

The property of the extruded feed, which floats more than the pellet feed, increased its availability to the trout for a longer time in the water column before falling to the bottom of the raceways (Tyapkova et al., 2016; Welker et al., 2018). Fish can quickly intercept food without its dispersion and waste production, which occurs more frequently when using pellet feed because it sinks and degrades more easily and quickly in the water column. In particular, concerning the two different feed techniques, which are the pellet feed and the extruded feed, the processing style changed from the first to the second in 2011 but the raw materials remained the same. The fish meal and fish oil feedstuff were the same and both were of a high quality. The owner of the farm where the study was conducted monitored the feed quality through periodic laboratory analysis to ensure that the composition of the feed remained unchanged. During the decade of the study, no differences in feed quality were shown and for the most important nutrients no raw material changes were reported. For this reason, in the current study, the specific chemical analysis of the feed was not taken into consideration in order to stress the topic of water quality.

NO_2-N represents an important stage in the oxidation of organic substances containing nitrogen. It is therefore a transitory form of nitrogen which is transformed into nitrates due to the bacterial activity in the presence of optimal quantities of dissolved oxygen in the water tanks.

Nitrates represent the final indicator of the degradation protein processes; the main sources are correlated with anthropic activity, and in surface waters the trend is usually seasonal.

Taking into consideration the TSS parameters detected in 2009 and comparing them with those of 2019, a significant reduction was clear, especially the trend observed during winter and autumn.

According to a number of studies that have focused their attention on the relationship between feeding management and water quality (Thorpe et al., 1995; Boyd et al., 2001; Dalsgaard et al., 2011; Welker et al., 2019; Galezan et al., 2020), the significant improvement in water quality at this farm was due to the adoption of the modern type of feed based on the extrusion technique. In fact, compared to the pellet food, the new extruded feeding technique showed a greater stability in water and it allowed the food to be available to trout for a longer period of time. There are other positive elements to consider. The extruded feed had a higher fat absorption capacity and it was possible to know its specific weight. Therefore, it was easy to control the fish feeding and, as a consequence, the composition of the meat obtained from this fish. Our study supported the idea that extrusion is nowadays the best processing feed technique in aquaculture, as previously shown by other studies. Welker (Welker et al., 2018) compared the extrusion technique with the pellet feed by analysing the water stability, faecal durability and digestibility and found the best results with the use of extruded feed. Similar results were reported by Tyapkova (Tyapkova et al., 2016). Moreover, in aquaculture, the extrusion technique positively affected the water quality: food waste due to dust, breaks and "leaking/leaching" was decreased, improving the availability of nutrients for fish and minimizing the environmental impact. Another element of the extruded feed is that it is characterized by a low sedimentation rate. To ensure feed suitable for trout, a low sedimentation rate is one of the main physical properties to consider because it means that the extruded feed is available to the fish for a long time; consequently, the fish can rapidly intercept the food without its dispersion and waste production. On the other hand, the use of pellet food means that feed is crumbled, which involves the fragmentation of food cylinders.

As a consequence, this could lead to a loss of food (even if decreased). The feed was pulverized and this waste could remain at the bottom of the raceway water, becoming a possible source of water degradation, as well as microbial contamination.

Based on data concerning the monitoring of the rearing waters and considering the loads of N and TP of extruded feed, which took place in the last 10 years, it is possible to say that the improvement of the feed administered to rainbow trout is proof of the excellent quality of the ingredients used in fish diets (Islam MS, 2005; Jia et al., 2015).

Our data are in accordance with previous studies (Aubin et al., 2011; Moraes et al., 2015; Tahar et al., 2018; Boyd et al., 2021) that estimated ammonia and phosphorus emissions in water tanks from dietary analysis. In particular, we found lower TAN values than those reported by Moraes (Moraes et al., 2015) and Aubin (Aubin et al., 2011), which is a sign of the high feed quality used on the farm involved in our study. Concerning the P values, our results are in line with those reported by Moraes (Moraes et al., 2015) and are lower than the values reported by Aubin (Aubin et al., 2011) and Dalsgaard (Dalsgaard et al., 2011).

In this study, the amount of TAN and TP present in the tanks that in the past had hosted trout that received pelleted feed was compared to current tanks that host trout fed with extruded feed. The significantly decreased TAN and TP loads are proof of an improvement in diet quality, with a consequent benefit for the environment and the health of the trout.

The present study provides insights into the connection between fish feed and water quality, that is the fish habitat, which has to be suitable for producing healthy fish and as waste water, which returns to natural water bodies, taking into consideration specific parameters to investigate the water quality. In particular, in our study, an improvement trend was mostly demonstrated by the TSS, confirming that feed manufactured by the extrusion technique was of high quality. In particular, a notable positive change in the general trend was demonstrated

after the new extruded food processing style was introduced, which occurred in 2011. In fact, all parameters showed an important decrease after the adoption of the extruded feed. More specifically, in the last years of the analysed decade, a more favourable feed conversion rate was shown with respect to when pellet feed was administrated, resulting in better growing performances exhibited by trout whilst saving approximately 40% of the feed. In fact, the feed conversion index and the protein efficiency index improve when rainbow trout are fed with a rationing level equal to 70% of the "ad libitum technique" (Bureau et al., 2006).

Another important change, which could have contributed to improving the efficiency of feed conversion, could be justified by the fact that, on the farm, only trout larger than 90 g are reared, which do not require meal or crumbled feed. The fragmentation of crumbled diets leads to a more pulverized feed. In this case, the uneaten extruded feed, over 4 mm in size, is easier to remove than the pelleted diet.

Regarding the feedstuffs of the extruded feed, the inclusion of vegetable sources (soybean, wheat and pea) from the owners' farm, located close to the fish plant, can be reported as an example of sustainability with positive effects on the production cost. On the fish farm, the fish stocking density was decreased (20 kg/m^3) with respect to the first years of the decade, when rainbow trout were stocked at double the biomass. The choice to limit the culture density is the basis of the application of the "multisite" rearing technique, which aims to increase biosecurity, preventing diseases due to a vertical transmission of pathogens (Delabbio et al., 2005). The decision of this plant to rear only rainbow trout starting from pre-fattened fish showed more advantages in terms of the survival rate, which was more satisfactory in the last years.

Concerning, more specifically, the fish farming density, before the decade focused on in this study and in 2004, the owners decreased the fish density in their raceways to improve the

welfare of the reared rainbow trout. From 2009 to 2019, the fish density remained the same due to the effective improvement in animal welfare.

As previously shown by Welker (Welker et al., 2019), the correct management of tanks is essential to ensure good water conditions for trout and the appropriate use of water oxygenation systems contributes to maintaining optimal living conditions for these fish. This aspect could be connected to the improvement of the outlet basin receiving water from the raceways before being introduced into the stream.

Similar to what was observed by Becke (Becke et al., 2019), the pH was always close to neutrality and it was kept as stable as possible. This helped to minimize environmental stress and allowed nitrifying bacteria to effectively remove the nitrogen that accumulated in the sediments. Conversely, in other studies, the pH values were closer to 6 (Moraes et al., 2015) or between 6.5 and 7.5 (Tahar et al., 2018), which means that the effluent water was more acidic than the water considered here.

On the farm, the workers carried out normal system control every day, in order to ensure an oxygen level in the water that never dropped below 85% of the saturation level and its concentration was never less than 5 mg/L. The water supply system used on the farm allowed at least one complete daily water change, which directly affected the water chemicophysical parameters. Furthermore, the receiving water body has benefitted from these modified techniques, in particular the outlet basin, which was populated by floating macrophyte duckweed (*Lemna* spp.) that are known to be suitable for wastewater treatment and for food for pigs and poultry (FAO, 2010; Chakrabarti et al., 2018; Stadtlander et al., 2019). The duckweed filters nutrients, with a reduction of the eutrophic load of the water; it is an excellent purifying plant and absorbs the nitrogen in the water by eliminating nitrates (FAO, 2010; Stadtlander et al., 2019).

Another aspect to consider is that, by comparing the parameters of the inlet water with those of the outlet water, which are the focus of the current study and due to the fact that the inlet water showed the same values over the 10 years of the study, it was possible to affirm that there was an improvement in the farming water quality. In fact, as shown before, the values of TSS, BOD_5, COD, TAN, NO_2-N and NO_3-N were lower in 2019 than 10 years before.

Many papers have focused on the environmental impact of trout farms on the natural receiving waters in terms of uneaten food, fish metabolites and chemical treatments. For example, in a study carried out by Tahar et al., in 2018 (Tahar et al., 2018), inlet and outlet concentrations of water parameters for four consecutive flow-through rainbow trout farms over a ten-year period were analysed in Ireland to characterize the impact of each fish farm on the water quality as a function of their production and to identify any seasonal variability.

2.5. Conclusions

In the present trial, water parameters were investigated to determine whether the new feeding strategy based on extruded feed changed the water composition. The analysed parameters (TSS, BOD_5, COD, NO_2-N, NO_3-N, TAN and TP) showed an important improvement from 2009 to 2019; the pH parameter did not show important variation during the studied period.

Water is effectively the habitat where rainbow trout live so it must be monitored and preserved as best as possible in terms of temperature and physicochemical parameters and quality. All these aspects are the basis of ensuring high-quality rainbow trout farming. Sustainable management, together with a genetic program of rainbow trout specimens employed, based on selected fish showing the best performance in feed efficiency, will be the next challenge to further improve the fish performance and environment.

Considering what has been discussed, the feedback of these results should be considered a significant index of improvement in the quality of the environment and the adoption of the modern formulation certainly contributed to obtaining this result.

2.6. Water quality control at Erede Rossi Silvio Trout Company

The Erede Rossi Silvio Trout Company takes care on the global economic and productive realities, and took choices production in line with the principles of environmental sustainability, taking in mind that the control of water quality is crucial for trout welfare. Inadequate breeding conditions, such as insufficient space, high farming density or low diet quality, could have a negative impact on fish health. Just considering that damaged, eroded or haemorrhagic fins are not only related to pathological events, but also to inadequate farming environment caused by stress conditions, high stocking density, or not optimal water quality. To check the quality of farming water, the Erede Rossi Silvio Trout Company carries out physicochemical analysis of water at least every 6 months determining all the necessary parameters for an opportune farm management. The Company performs water quality checks availing a certified laboratory centre specialized in water analysis. The check of oxygen, temperature, pH, ammonia, nitrites and nitrates levels occurs daily. Furthermore, concerning the correlation between farming water quality and food management, the constant bibliographic updating showed how the adoption of a modern type of feeding based on the extrusion technique led to a significant improvement on water quality. For this reason, the Erede Rossi Silvio Trout Company decided to leave the use of pellet feed in favour of the extruded one. Indeed, the extrusion technique favours a greater stability in water than the pellet one, with various advantages, listed below:

- high stability in water, allowing to feed to stay more time available to fish;

- low sedimentation time;

- better feed quality, thanks to the extrusion process itself;

- high absorption capacity of fats;

- specific weight control;

- absence of dust and fragmentations, reducing waste, improving the availability of nutrients, and minimizing the environmental impact.

Showing these characteristics, the cylinders of the extruded feed stay longer than the pellet in the water column and fall down to the bottom of farming tanks more slowly, increasing the interception time by trout. In this way, the extruded technique drastically reduces the amount of feed not ingested by fish, which instead settles on the bottom of farming tanks, with a consequent economic loss and pollution of farming waters. Table 3 shows the values of the main water quality indexes in tanks hosting fish fed with pelleted feed, used in the Erede Rossi Silvio Trout Company trout farms to 2009, and tanks hosting fish fed with the extruded feed, whose use started later.

Table 3 - Average values of TTS, COD, BOD, nitrites, nitrates, ammonia nitrogen and total phosphorus referring to the year 2009 and to the year 2019 expressed in mg/L (mean ± standard deviation).

	2009	**2019**
TTS (mg/L)	33.00 ± 5.53	13.50 ± 2.08
COD (mg/L)	28.88 ± 6.08	10.00 ± 0.00

BOD (mg/L)	8.75 ± 2.12	3.33 ± 2.17
Ammonia nitrogen (mg/L)	0.68 ± 1.01	0.15 ± 0.01
Nitrites (mg/L)	0.09 ± 0.03	0.17 ± 0.10
Nitrates (mg/L)	3.64 ± 0.43	1.24 ± 0.56
Total Phosphorus (mg/L)	0.16 ± 0.07	0.10 ± 0.00

Furthermore, the feed quality affects directly the water quality because a part of the feed ingested by fish returns to the aquatic environment as metabolites or as soluble metabolic by-products. Table 4 shows the balance of nitrogen (N) and phosphorus (P) between water tanks in which it is used a pellet feed and water tanks that host trout are fed with extruded feed. Note the significant difference amount of nitrogen and phosphorus.

Table 4 - Balance for nitrogen (N) and phosphorus (P) in raceways where pellet feed is used (1) and in those where extruded feed is used (2).

Raceways	**Type of feed**	**N in feed (kg)**	**N in trout (kg)**	**P in feed (kg)**	**P in trout (kg)**	**Amount of P (kg)**	**N and P in trout (%)**
1	PELLET	8.79	4.01	1.30	0.39	0.91	45.6 N
							30.0 P

2	EXTRUTED	8.79	6.25	1.08	0.53	0.66	71.1 N
							49.0 P

Another factor to consider is that the extruded feed shows a higher fat absorption capacity, and so it is possible to know the specific weight. Cause it is possible to control fish feeding, consequently it is also possible modifying the composition of the fish fillet obtained.

2.7. Notes

Publication

The study on the importance of water quality in rainbow trout farm for the animal welfare and the environment respect was objected to a research article published in the Animal journal on September 2020 in the Special Issue " Feeding Strategies to Improve Sustainability and Welfare in Animal Production" (Fiordelmondo E, Magi GE, Mariotti F, Bakiu R, Roncarati A. Improvement of the Water Quality in Rainbow Trout Farming by Means of the Feeding Type and Management over 10 Years (2009-2019). Animals (Basel). 2020 Sep 1;10(9):1541. doi: 10.3390/ani10091541.).

3. THE IMPORTANCE OF FISH WELFARE TO AVOID THE USE OF ANTIBIOTIC

In fish farming the first step to avoid the use of antibiotic is improving fish welfare and therefore avoiding any stress condition that could negatively affect the health status of farming fish. The modern literature show data on fish anatomy, fish pharmacology (Burka JF and Johnson G, 2002) and fish environment (Toni et al., 2018; Saraiva et al., 2019; Ojelade et al., 2022) which support the idea that fish have the innate ability to live negative experiences such as pain, fear or stress, similar to what happens in vertebrate organisms (Chandroo et al., 2004; Sneddon LU, 2019). This implies that fish have the capacity to suffer, and to respecting fish welfare it is necessary to take this aspect into consideration. Considering that, it is necessary to mark the main factors that induce a reduction of welfare in farming fish:

- The correct management of on-growing ponds is essential as it reduces the exposure to not sufficient water quality. In particular in the Erede Rossi Silvio Trout Company during all the breeding phases, water physicohemical analyses were carried out at least every 6 months (Fiordelmondo et al., 2020);
- High breeding densities favour the establishment and spread of infectious diseases, water pollution, cannibalism and the inset of skin lesions (Hoseini et al., 2020). At the Erede Rossi Silvio Trout Company the breeding density is always commensurate with the physiological and behavioural needs of rainbow trout in all the farming phases;
- The quality of the raw materials used in the formulation of the fish feeding, in order to satisfy all the nutritional requirements of the specific fish species considered (Johnsen PB and Dupree HK, 1991; Tyapkova et al., 2016; Welker et al., 2018). The Erede Rossi Silvio Trout Company put attention on the quality of fish feed and on the use of diets easily assimilated by trout and formulated with raw materials of their own production;

- Fish catching operations, i.e. grading, must be conducted avoiding any suffering manipulation and using the same method every single time in order to make animals comfortable with the handling procedures (Melotti et al., 1992);
- The temperature and the quality of the farming water, as well as adequate water exchanges, are important factors to be controlled in respect of animal welfare during the phases of movement and transport (Mu G., 2014; Fiordelmondo et al., 2020);
- Before being slaughtered, fish need time to adapt them-selves to the new environment, represented by the pre-slaughter tank (Salati et al., 2016).

In the present PhD project the mechanisms that regulate the physiological response to stress conditions before slaughtering in the last phases of farming rainbow trout (*Oncorhynchus mykiss*) were investigated by conducting plasma analyses during the last phase of a standard fattening cycle and by studies on the gene expression in skin mucus. In fact chemical analysis of blood plasma can indicate the health status of fish by assessing changes caused by stressful conditions related to pollutants, pathogens, heavy metals, etc. (Maita M, 2007). Furthermore, stress conditions could modify the production of skin mucus and its composition, negatively affecting fish health (Reverter et al., 2018; Carbajal-González et al., 2011). The skin mucus is the outermost organ of the fish body and the first line of defence from external aggressions, and knowledge about mucus composition or its changes could be used for monitoring the health status of fish (Kiron V, 2012; Omid et al., 2018). Considering that, the effects of stress conditions on plasma parameters and gene expression were evaluated in the skin mucus of rainbow trout (*Oncorhynchus mykiss*) in the last phase of farming before slaughtering. Blood chemistry analyses were conducted with the aim to evaluate the acute stress, instead samples of skin mucus were collected and analysed with the aim to evaluate the chronic stress. Additionally on skin mucus samples bacteria populations and parameters of mucosal immunity were recorded.

3.1. THE IMPACT OF STRESS ON SKIN MUCUS BACTERIA POPULATION IN FARMING RAINBOW TROUT (*Oncorhynchus mykiss*)

3.1.1. Introduction

For its multiple functions, the study of skin mucus in fish species has become relevant in the last few years. Skin mucus is one of the main natural barriers that protects fish from possible microbiological attack coming from the aquatic environment in which they live (Takeuchi et al., 2021). In healthy fish, skin mucus is a thin layer that covers the body of the fish completely and uniformly, with lubricating and protective functions (Esteban MA, 2012; Tasleem et al., 2020). Its production is carried out by mucin cells and consists of a mix of immunoglobulins, proteins and lipids (Pearson J and Brownlee IA, 2005; Kumari et al., 2019). These nutrients appear to have a nutritional function for the resident bacteria community (Minniti et al., 2019). Proteins are one of the main mucus components involved in the interaction with aquatic microorganism such as bacteria, fungus and protozoan (Dash et al., 2018). For these reasons, the composition of the skin mucus should be taken into consideration to preserve the appropriate balance of the skin mucosal bacterial population. As other authors showed, skin mucus can be considered the first barrier of defence from possible biologic attack (Benhamed et al., 2014; Cordero et al., 2015). In fact, to preserve fish welfare and prevent illness the constant production of mucus by mucin cells is required to guarantee a continuous turnover of its constituents and to prevent the adhesion of pathogens and parasites (Dash et al., 2018; Tasleem et al., 2020). In recent years, zootechnical industries have focused their attention on the prevention of disease to avoid the use of drugs during the different farm phases, in order to obtain high quality productions and healthy foods. For a successful aquaculture, infectious diseases must be prevented by appropriate biosecurity measures and health precautions (Terech-Majewska et al., 2016; Assefa A and Abunna F, 2018; Kulczykowska et al., 2019; Kumari et al., 2019). In particular, nowadays the perspective of

one global health is an approach adopted all over the world and the problem of antibiotic resistance is relevant (Kalanxhi et al., 2021; Sridhar et al., 2021) because the use of antibiotics in fish farms can directly impact human health and aquatic environment. To prevent the use of antibiotics in farming animals, it is necessary to improve the natural immune system of animals through respecting animal wellness and avoiding any condition of stress (Assefa A and Abunna F, 2018), that can lead to pathologies by altering the composition of natural barriers. In reared fish many studies have investigated the antibacterial properties of skin mucus, which are mainly the prevention of the colonization of the skin by pathogens (Balasubramanian et al., 2012; Kumari et al., 2019) and antimicrobial properties (Balasubramanian et al., 2012; Minniti et al., 2019). The physiological composition of the bacteria population in fish skin mucus must be respected and preserved for its immune functions, but it should be modified by stress conditions (De Marcado et al., 2017). According to current knowledge, the skin mucus composition is strongly connected to the skin mucus microbiota. The study of the physiological skin bacterial community in fish is really important because in the aquatic environment opportunistic pathogens can easily cause disease by penetrating the natural barrier of skin (Benhamed et al., 2014). In fact, skin microbiota could change in response to external stimuli and stress (Tacchi et al., 2015; Krasnov et al., 2015; Benhamed et al., 2014), facilitating the entry of pathogens. Furthermore, based on the current literature (Esteban MA, 2012; Kelly C and Salinas I, 2017; Reverter et al., 2018; Ruiz et al., 2021) the correct balance of the skin microbiota looks able to prevent the adhesion of pathogens through the production of specific active compounds that could prevent the development of pathologies, playing a crucial role in preserving the health status of reared fish (Gonzalez et al., 2011; Reverter et al., 2018). The specific mechanism involved is not yet completely known, and so more investigations are required.

In this trial microbiota alterations were analysed in response to stress factors in a fattening rainbow trout farm, one of the main fish species reared in aquaculture. This trial aims to provide a contribution to understand the complex system on which the skin mucus is based.

3.1.2. Materials and Methods

3.1.2.1. Sampling

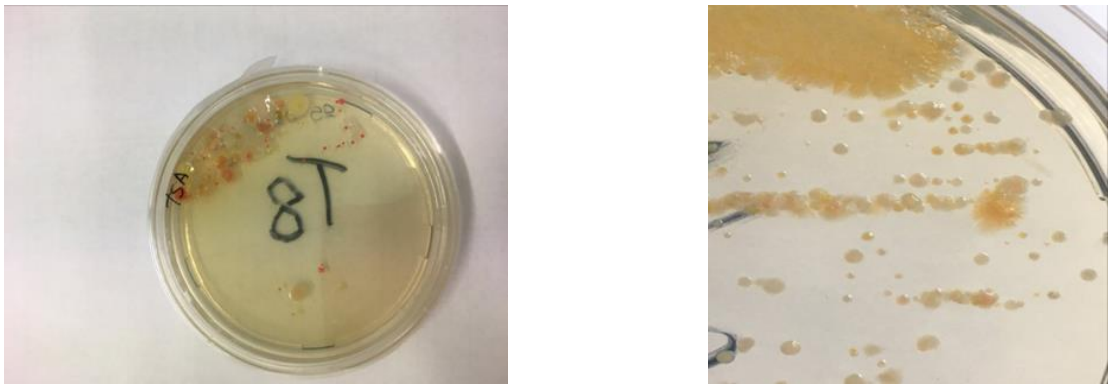
Rainbow trout (*Oncorhynchus mykiss*) were sampled from the fattening farm of the Erede Rossi Silvio Trout Company located in the Apennine area of central Italy, based on raceways in parallel (120 m^3 each). The inlet water came to the farm system with a constant velocity of 0.25 m/s and flowed through four parallel raceways. Water monitoring took place daily to check the levels of oxygen, temperature, pH, ammonia nitrogen and nitrates. Fish were fed with extruded feed distributed with a semi-moving wagon up to the level close to satiety at the same time every day, twice a day. For the trial, two groups of rainbow trout were considered. One group (H group or "healthy" group as control group) was composed by 10 fish, randomly captured with a net from the last tank of this standard fattening farm using the same net and the same method of capture daily adopted in the involved farm for standard procedures. The second group (S group or "stressed group" as the experimental group) is represented by 10 animals subjected to stress and collected at the end of the production line soon after slaughtering. The stress condition was represented by procedures that normally occur in a rearing farm: capture, transfer to the pre-slaughtering tank where trout staid for 4-6 hours, fasting for overall 6-12 hours, suction with a fish and water system and finally transfer to the stunning tank, where fish lost consciousness before be slaughtered. For every fish of both groups, the body surface was swabbed from head to tail using a sterile plastic spatula from the antero-dorsal to the posterior surface, without including the ventral portion to avoid

intestinal and urogenital contaminations. Before the skin mucus sampling, an examination of a general health status of the fish was done for every sampled fish of both groups thought a visual inspection of the entire surface of the body and the observation of the shape and the state of fins, gills, oral cavity, eyes, and urogenital opening. The visual inspection was conducted avoiding any type of contamination of fish skin, using single use plastic gloves and a clean plastic table, and reducing handling and execution time as much as possible. After the visual inspection, for every fish pure mucus was sampled and immediately spread onto tryptose soya agar (TSA) plates and sent to the Department of Fish Clinic of the University of Vienna (VETMEDUNI) in dry ice bags for bacteriology analysis. Only for trout of S group was possible to conduct the internal visual inspection of the abdomen, during the slaughter phase that occurred soon after the skin mucus sampling. This additional inspection was done to detect possible alterations in abdomen cavities and abdominal organs of the stressed fish (S group).

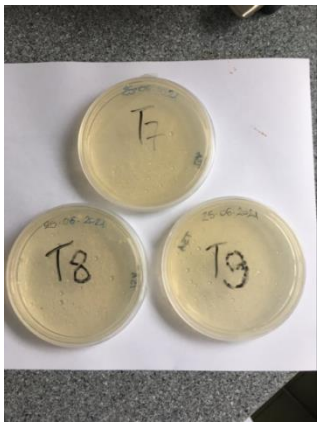
3.1.2.2. Identification of bacteria

In the Microbiology Laboratory of the Department of Fish Clinic in VETMEDUNI, TSA agar plates sent from the Italian farm involved in the trial were subjected to a preliminary incubation at 15 °C for 4 days. From each plate (total number = 20), three colonies of the three most numerous bacterial types were removed: one colony was sub-cultured on the same medium (TSA agar) until pure cultures were obtained (Picture 4, 5). In order to assess whether TSA was effectively a good medium for culturing skin mucus bacteria, the ability of the isolated bacteria to grow was checked also in Columbia Blood Agar at two different conditions of incubation, one at 22 °C for 24 h, and one at 15 °C for 4 days. Sub-cultured were carried forward until three most numerous bacterial population were identified in both group (H group and S group). After having identified the three most represented bacteria colonies in H group in TSA agar (confirmed in a triplicate) (Picture 6) and in Columbia agar,

and similarly the three most represented bacteria colonies in S group in TSA agar (confirmed in a triplicate) and Columbia agar, their identification were conducted. So, the pure cultured colonies were biochemically characterized with API 20NE (Biomerieux, Marcy l'Etoile, France) following the instructions of the protocol given with the API 20NE kit. When the sensibility of the test was not clear, bacteria populations were identify using the MALDI-TOF technique. After their identification, the purified bacterial populations were stored frozen at – 80 °C in tryptose soya broth (TSB, Cultimed) for further investigations.



Picture 4, 5 – Bacterial population in TSA agar plates.



Picture 6 – Growth of bacterial colonies in TSA agar.

3.1.3. Results

3.1.3.1. Fish body conditions

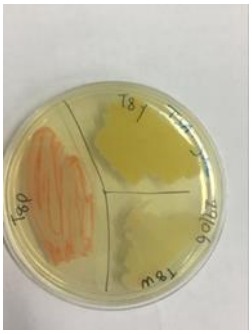
During the inspection examination that was conducted for every fish, any injuries at the fins or skin were found. Any clinical signs of disease or alteration was recorded, and fish from both group showed a good health status. The state of gills, oral cavity, eyes, and urogenital opening showed good condition in all sampled fish, referred to H group as well as S group.

3.1.3.2. Water quality

During all the time of the trial the level of every parameters related to water quality were appropriate. In fact, the levels of oxygen had a mean of 10.64 ± 0.22 mg/L, the temperature a mean of 11.02 ± 0.99 °C, the pH a mean of 7.39 ± 0.07, the ammonia nitrogen a mean of 0.18 ± 0.06 mg/L and the nitrates a mean of 1.16 ± 0.09 mg/L.

3.1.3.3. Identification of bacteria

The observation of different bacterial colonies led to detect the prevalence of three different colours of the bacterial colonies. In fact, in both groups the bacterial colonies showed three predominant colours: white, yellow, and pink, every time well separated from other minor colonies (contaminants or secondary). Concerning the growing colonies phase, authors observed that some colonies did not grow in Columbia Blood Agar at the incubation conditions of 22 °C for 24 h, in which the growth of colonies was generally slower than colonies growing in the same medium at 15 °C for 4 days. Remains that the best growing medium for these bacteria looked to be the TSA agar, which gave every time colonies well separated from the others and with a better defined shape. The different colour of bacteria population was observed in both media (TSA and Columbia), naturally more clearly in TSA agar because the base colour of the medium did not disturb the colour of the colonies. In H group (corresponding to the healthy group) the three most numerous bacterial colonies were *Pseudomonas korensees* in white colonies, *Glutamicibacter protophormiae* in yellow colonies and *Mannheimia haemolytica* in pink colonies (Picture 7). For S group (corresponding to the stressed group) the three most numerous bacterial colonies were *Aeromonas salmonicida* in white colonies, *Pseudomonas fluorescent* in yellow colonies and *Shewanella baltica* in pink colonies. The results of the bacteria identification were the same for both media, TSA agar and Columbia Blood Agar.



Picture 7 - TSA agar plates with bacteria colonies referred to H group: *Mannheimia haemolytica* for pink colonies (on left), *Glutamicibacter protophormiae* in yellow ones on the top; *Pseudomonas korensees* white ones on right.

3.1.4. Discussion

3.1.4.1. Rearing water quality

Considering the aim of the present study, the control of an high level of quality of the farming water was crucial. Only if fish lived in clean water, free from pathogens, environmental contaminants and parasites, it is possible to appropriately conduct the study and correlate the alteration of skin microbiota to stress conditions, and not to a biological attack or contamination. For this aspect, the manuscript published in 2020 by Fiordelmondo et al. was taken into consideration as the referrer to analyse the quality of the rearing water. The same lab analysis were conducted and the obtained results compared to published data. All parameters were conformed every time to the reared species (Fiordelmondo et al. 2020). Considering the correlation between the bacteria population of the rearing water and the bacterial population of fish skin, according to the results found by other authors, there is not a direct correspondence of bacteria populations between aquatic environment and fish skin

(Caballero et al., 2020). For this reason, authors should consider that the obtained results on bacteria population were not necessary directly determined by the bacteria population of the inlet water.

3.1.4.2. Bacteria populations

In order to understand the meaning of the obtained results, every identified bacterium was studied in literature for a comparison to what other authors previously obtained.

Pseudomonas korensees and Pseudomonas fluorescent

Pseudomonas spp. is a group of Gram-negative bacilli, which are opportunistic pathogens. They are motile, rode shaped, oxidase-positive and catalase-positive, non-fermentative bacteria with an oxidative metabolism. The main phenotypic characteristic of this genus is the production of fluorescent pigments (Duman et al., 2021), even if in our case the species *P. korensees* did not show a yellow but a white pigmentation. Concerning the growing conditions, these bacteria are known to show different level of tolerance to salinity, temperature and pH, and they persist in the aquatic environment also in absence of nutrients (Liu et al., 2015; Palleroni NJ, 2015). *Pseudomonas* spp. are common bacteria in the water of aquaculture farms (Duman et al., 2021), and so finding these bacteria in the mucus of the skin of farming rainbow trout is a normal evidence. Bacteria of the genus Pseudomonas are potentially pathogens for fish, and at the infectious dose they cause eye lesions and an increase of the mortality rate (Shahi N and Mallik SK, 2014). In particular, *Pseudomonas fluorescent* is a pathogen bacterium for the rainbow trout species, and the typical symptoms of the infection are haemorrhages at the bases of fin and anal region, and petechiae at the intestine walls inside the abdomen (Sakai et al., 1989). Moreover, haemorrhagic skin lesions, deep ulcers on the caudal fin base and tail rot were described when the infection is associated with *Yersinia ruckeri* (Dinçtürk E and Tanrikul TT, 2021). In our case, even if this potentially

pathogen bacterium was isolated, it was not observed any sign of disease or lesion, that probably means that the trout involved in the trial were capable to contrast pathogens spontaneously. Furthermore, it is interesting that *Pseudomonas fluorescens* was recognised as capable of inhibiting the growth of Saprolegnia (Bly et al., 1997), one of the most common parasitic disease in fresh water fish. This observation validates the thesis that preserving the best balance between commensal bacteria and pathogens in fish skin mucus is a fundamental factor to preserve fish health. Moreover, *Pseudomonas* spp. have been counted as possible probiotic organisms with beneficial and protective effects, because they seem to play a role in countering mycological bacterial agents and avoiding the aging of important pathogens on the animal's skin, such as Saprolegnia (González et al., 2011; Liu et al., 2015).

Glutamicibacter protophormiae

The genus *Glutamicibacter* spp. contains bacteria characterized by rod shaped when growing exponentially and coccoid shape when reaching the stationary growth phase. In cell culture the typical shape of these bacteria is white pigmented colonies that become yellow when getting older (Whitman et al., 2015). According to the current literature bacteria of this genus are not connected to fish pathologies. They were isolated in soil, wastewater, sewage, Antarctic lake sediment, and cheeses (Whitman et al., 2015), without any connection to fish disease. The fact that a so harmless and innocuous bacterium represented one of the largest bacterial populations in the H group should be correlated to the absence of potential pathogens, contaminants or parasites in the farming water in which fish lived, and a sign of a very high-water quality in the farming tanks in which fish lived during the trial.

Mannheimia haemolytica

Mannheimia haemolytica is a Gram-negative and rod-shaped coccobacillus bacterium, not motile and non-spore-forming (Tabatabaei M and Abdollahi AF, 2018). In Veterinary Medicine this bacterium is predominantly connected to another animal species. In fact it is the pathogen agent of the bovine respiratory disease (Rice et al., 2007; Confer AW and Ayalew S, 2018), without any reference or connection to fish pathologies according to the current literature. Similarly to above, if a so harmless for fish bacterium is so well represented in the skin of the trout of H group, it should be correlated to the absence of fish pathogens and the state of wellness of the trout of H group.

Aeromonas salmonicida

Aeromonas salmonicida is a gram-negative, non-spore-forming and non-motile bacterium, single or paired rods of different lengths (Kozinga et al., 2002). As the name suggests, it is pathogen for fish and the main susceptible hosts are salmonid. The disease is recognised as a Foruncolosis because the pathognomonic sign of this infection is represented by skin boils, associated with haemorrhagic areas at the bases of fins, mouth, lateral abdominal, and the affected internal organs, often liver, cecum, and heart. Other described alterations that can occur are enlarged spleen, soft kidneys, irregular swimming, lack of nutrition (Cipriano RC and Bullock GL, 2001; Crawford SS, 2001). *Aeromonas salmonicida* is indeed well known in fish clinic to cause several septicemia and acute mortality. The degree of disease severity is strictly correlated to the quality of aquatic environment and the innate resistance of the host (Cipriano RC and Bullock GL, 2001). In our case, this bacterium was isolated in the mucus of trout of S group but, even if this so pathogen for salmonid bacterium was presented on fish skin, it was not found any sign of disease. This fact is probably explained by the innate ability

of these animals to naturally contrast a potential pathogen attack without getting sick thanks to a well state of the immune system and physical well-being.

Shewanella baltica

Considering the greater diffusion of *Shewanella putrefaciens* and the only recent identification of bacterial strains different from it (Beaz-Hydalgo et al., 2015; Paździor E, 2016; Paździor et al., 2019; Saticioglu et al., 2021), the result of the bacterial identification was repeated several times for a correct and certain bacterial identification. According to literature, only the bacteria species Shewanella putrefaciens is a pathogenic bacterium for aquatic organisms (Panagiotis et al., 2013; Pekala et al., 2015; Sood et al., 2019).

The evidence of the change of the composition of the bacterial population in the skin mucus of farming rainbow trout confirms the hypothesis that handling, capture, transfer, and suction could modify the composition of the skin defence barrier, and so predispose to pathogens attack and disease. As declared, the trout of H group were captured from the last tank using the same net and the same method of capture daily adopted in the involved farm.

On this way the manipulation to which the trout of H group were subjected represented an event already well known by the animals, and which therefore did not represent a stressful event for them, because already known. Therefore, looking to the obtained results, if trout live in a very good lifestyle conditions based on high water quality, respect of animal wellness and good zootechnical practises, which completely respect fish welfare and fish welfare (as happened in this case), despite the not evitable manipulations that occur in the practises of a fish farm, animals didn't show any sign of disease or lesions. In fact, every rainbow trout involved in the present trial was subjected to external (and also internal for S

group) inspection analysis, without showing any kind of alteration or sign of disease. Similarly to our results, in farming Atlantic salmon changes of the bacterial community of the fish skin were observed comparing fish before and after a stress condition represented by netting and transfer (Minniti et al., 2017). That study was conducted in the *Salmo salar* species, and the three phyla *Proteobacteria*, *Firmicutes* and *Acidobacteria* were identified on skin mucus, with intra-species variations in the proportion of bacteria compared handled and not handled fish. The most evident shift of the skin mucus community was associated with the genus *Burkholderia* (Minniti et al., 2017). Another study on rainbow trout was conducted in 2009 in the southern region of the Black Sea, in which the evaluation of the bacteria population mostly presented in the water was performed (Kayis et al., 2009). *Yersinia ruckeri*, *Areomonas hydrophila* and *Areomonas salmonicida* were isolated from abdomen organs (kidney, liver, spleen) and skin lesions, and recorded as the major bacteria population that caused several cold-water disease, such as Yersiniosis, furunculosis, vibriosis, motile *Aeromonas septicemia*, and *Pseudomonas* spp. infection.

In European seabass, recognised as another important species for the aquaculture production, skin bacteria were studied from a conventional fish farm where antibiotics were used and compared to those from an antibiotic-free farm (Ramljak et al., 2022). In both farms, the bacteria identified in the skin belonged to the genera *Pseudomonas* and *Vibrio*, and some pathogen bacteria were identified in either type of farms.

3.1.5. Conclusion

Rearing fish in good farm conditions means helping fish to grow strongly and letting them developing a natural resistance to pathogens. This means that the use of drugs, especially antibiotics, is not necessary if animals live in wellness condition thanks to the high quality of

lifestyle in which they live. Animal welfare and optimal living conditions are basic elements to obtain healthy fish and high quality of fish productions, and they are also preliminary concepts for a sustainable aquaculture production based on the blue economy principles. To reach this goal, additional studies are necessary to evaluate the actual possibility of cold-water fish to develop disease caused only by the not respect of protocols of farm biosecurity, such as quality of lifestyle conditions, water quality, stocking density, etc. Furthermore, because fish skin mucus looks to have antimicrobial skills (Kumari et al., 2019), more investigations are required to better understand the possible application of this tissue as an alternative to antibiotics in animals in the next future.

3.2. EFFECTS OF STRESS CONDITIONS ON PLASMA PARAMETERS AND GENE EXPRESSION IN THE SKIN MUCUS OF FARMED RAINBOW TROUT (*Oncorhynchus mykiss*). A CASE STUDY.

3.2.1. Introduction

Stress can be defined as an organism's response to the external forces and environmental conditions that exert influence over the organism and alter its osmotic balance. As reported in previous studies (Krasnov et al., 2005; Momoda et al., 2007; Tacchi et al., 2015; Yarahmadi et al., 2016), physiological changes in fish in response to stress occur primarily with the release of catecholamines and corticosteroids (Bartone BA, 2002). As a result, there is an increase in the blood cortisol level, which is recognized as the stress indicator in all animal species, including fish (Pickering AD, 1993; Momoda et al., 2007; Carbajal et al., 2019). Cortisol has immunomodulatory effects, inhibiting the activity of the immune system in fish. High cortisol level in blood is related to stress, causing negative effects on the immune function (Segner et al., 2012). In fish farming, operations such as handling, food deprivation and high stocking density could have an impact on fish welfare and so, as an hypothesis, can be considered as stress inducers (Yarahmadi et al., 2016; Reverter et al., 2018). Consequently, the alteration of the health and welfare status of farmed fish can be evaluated by analysing plasma parameters such as glucose, total proteins, albumins, total globulins, total lipids, and cholesterol (Řehulka et al., 2005; Yildiz et al., 2009; Lone at al., 2012; Dezzutto et al., 2016). The chemical analysis of blood plasma can indicate the health status of different fish species by assessing physiological changes caused by stressful conditions related to pollutants, pathogens, heavy metals, etc. (Maita M, 2007). Changes in serum and other biochemical parameters are often indicators of modifications of the physiological state (Řehulka et al., 2005; Dezzutto et al., 2016) and generally represent a valid method to evaluate the health status of wild and domestic animals (Lone et al., 2012). Furthermore,

stress conditions could modify the production of skin mucus and its composition, negatively affecting fish health (Reverter et al., 2018; Carbajal-González et al., 2011). Skin mucus is a key natural barrier that protects fish from prospective microbiological attacks caused by pathogens originating in the aquatic environment in which they live (Takeuchi et al., 2021). Furthermore, some genes pertaining to the response of skin mucus and other mucosal barriers to environmental stress factors are differently expressed in fish (Krasnov et al., 2005). The skin mucus plays an essential role in preventing parasitism by bacteria and fungi, thereby acting as a chemical defence barrier in fish (Ingram GA, 1980; Jakowska S, 1963; Benhamed et al., 2014). Moreover, secreted mucus contains certain elements of the immune system, such as specific immunoglobulins (Yarahmadi et al., 2016; Yu et al., 2020; Zhang et al., 2021). However, skin mucus can change in response to external stimuli and stress (Krasnov et al, 2005; Slominski et al., 2013; Tacchi et al., 2015; De Marcado et al., 2017), facilitating pathogen entry (Kulczykowska E, 2019).

In fish, a stress condition is characterized by the change in the quantity of skin mucus produced (Tacchi et al., 2015), as well as in its composition of immunoglobulins, complements, and antimicrobial peptides (Reverter et al., 2018). Considering that the effects of stress on salmonids have been studied more than on other fish species (Schreck CB, 1982; Pickering AD,1993; Momoda et al., 2007; Webster et al., 2018; Hoseinifar et al., 2020), the present study was focused on farmed rainbow trout (*Oncorhynchus mykiss*), which is one of the main freshwater fish species reared on a commercial scale (Alizadeh et al., 2016; Singh et al., 2017).

This investigation was focused on the last phase of rainbow trout fattening process, with the aim of analysing if the standard farming procedures which occur at the end of a standard rainbow trout fattening cycle all together could represent a situation of stress to which fish were exposed, without any experimental infection. In the rainbow trout farming cycle,

animals are most exposed to possible stressful condition in last phase of the farming process, in which fish are captured and moved to smaller tanks (Tacchi et al., 2015; Minniti et al., 2017) with higher density than the breeding tanks. After the required fasting time (Bermejo-Poza et al., 2019), fish are caught using a pumping system and undergo stunning before being finally slaughtered (Yarahmadi et al., 2016; Takeuchi et al., 2021). Collecting, handling, sorting, holding, and transporting are routine practices that could have significant effects on fish physiology and welfare state. It is not completely clear how these potential stress factors influence all together the welfare and the health state of farmed rainbow trout all together. Hence, the analysis of plasma parameters as indicators of wellness was taken into consideration to assess the state of welfare and health of farming rainbow trout in the last phase of a standard fattening cycle. Furthermore, the levels of expression of several immunity genes involved in the stress response and related to T and B cells were assessed and compared, i.e., and the transcriptional modulations of CD8, IL-6, IL-10a, IL-8, CD4, IgT, IgD, IgM, and IFN-γ, evaluated using the qRT-PCR technique.

Because good farming practices can make the difference in fish health and fish welfare, the aim of the present study was to check the welfare of farmed rainbow trout under the standard slaughtering protocol that fish farm adopt in Italy, based on the consideration that skin mucus represents a milieu found in direct contact with the aquatic environment, leading to the immune responses being detectable earlier than in other anatomical structures of fish (Benhamed et al., 2014; Takeuchi et al., 2021). Furthermore, the study of gene expression is a common investigation method used for understanding the specific response of animal species to stress factors (Espinosa-Ruíz et al., 2021). The present study wants to refer a systematization of knowledge on farming rainbow trout, evaluating risk factors and welfare indicators during the last phase of a standard fattening cycle in order to check the real applied health and welfare principles in a fattening rainbow trout farm in Italy.

3.2.2. Material and Methods

3.2.2.1. Farming conditions and sampling

Rainbow trout (*O. mykiss*) were sampled from the fattening farm of the Erede Rossi Silvio Trout Company located in the Apennine area of Central Italy, based on 1,000-L in parallel raceways (120 m^3 each) containing circulating groundwater. The inlet water came to the farm system with a constant velocity of 0.25 m/s and flowed through four parallel raceways. The maximum density at which the fish were grown was 25 kg/m³. Water monitoring took place daily to check the levels of oxygen, temperature, pH, ammonia, nitrogen, and nitrates. Fish were fed with extruded feed distributed with a semi-moving wagon up to levels close to satiety at around the same time every day, twice a day. For the trial, two groups of rainbow trout were considered. One group (group 1) was made up of ten fish randomly captured with a net from the last tank, using the same net and the same capture procedure on a daily basis. The fish were subsequently placed in a basin containing anaesthetic in an aqueous solution and water from the same breeding tank. The anaesthetic was used at the concentration of 0.04 mg/L and was composed of essential oils extracted from cloves and supplied by the Rainbow Trout Company employed in the trial and commonly used in there. The second group (group 2) was made up of ten fish collected at the end of the production line, soon after slaughtering. The potential stress-causing agents represented usual procedures encountered in a rainbow trout farm, including fish capture and transfer to the pre-slaughtering tank. Trout were captured with the same method described above and transferred from farming tanks (20 kg/m^3) to the pre-stunning tank, where trout remained in a fasted state for 4-6 h in high density (35 kg/m^3) tanks. Then, fish were sucked by a fish and water system, and finally transferred to the stunning tank, where fish lose consciousness before being slaughtered. Fish from both groups had about the same commercial size, i.e., weights of ca. 350 g each.

3.2.2.2. Skin mucus sampling

For each fish, the body surface was swabbed from head to tail using a sterile plastic spatula from the antero-dorsal to the posterior surface, without including the ventral portion (to avoid intestinal and urogenital contaminations). Prior to the skin mucus sampling, an examination of a general health status of the fish was done for every sampled fish from both groups through the visual inspection of the entire body surface and the observation of the shape and condition of fins, gills, oral cavity, eyes, and urogenital opening. The visual inspection was conducted by avoiding any type of contamination of fish skin, using disposable gloves and a clean plastic table, and reducing the handling and execution time as much as possible. All the fish under investigation exhibited healthy-looking skin and clear mucus. After the visual inspection of all the fish, the pure mucus was sampled in clean Eppendorf® tubes, immediately frozen in dry ice, and sent to the Department of Fish Clinic of the University of Vienna (VETMEDUNI) in dry ice bags. Each mucus sample underwent microscopic examinations in the prepared slides for the detection of the prospective presence of parasites. The slides were next sent to the Laboratory of the University of Camerino (UNICAM) for microscopic observations.

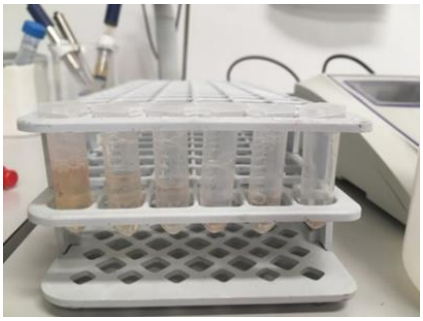
3.2.2.3. Blood sampling

After the skin mucus sampling, blood sampling was performed by endocardial puncture, using a new syringe for each fish (Picture 8). After having removed the needle from the syringe, the blood samples (about 1-2 cc) were placed in tubes containing a drop of anticoagulant (heparin) and sent to UNICAM laboratories. The blood samples underwent centrifugation for 20 min at 3000 RPM (SI-TRON TINCA 3003M) (Picture 9 and 10). The obtained supernatant represented the plasma, which was collected with a pipette and placed in individual tubes for subsequent analyses using spectrophotometric equipment (BT3500VET,

Microtech 648 Electrophoresis) to determine the parameters of interest, which were: cortisol, glucose, total proteins, cholesterol, total triglycerides, albumins, and total globulins. For the present research project, plasma parameters were quantified using the GOD-PAP method for glucose, the Biuret method for total proteins, the CHOD-POD method for cholesterol, and the colorimetric method GPO for total triglycerides. Plasma globulin and albumins values were determined using the BGC protein assay kit after the addition of saturated ammonium sulfate solution and the dissolution in carbonate-bicarbonate buffer. The plasma cortisol level was determined using commercial enzyme immunoassay (ELISA) kit (cortisol ELISA RE52061 IBL International GmbH, Hamburg, Germany).

Since the experiment was conducted on a rainbow trout fattening farm, it was possible to conduct a visual inspection of the internal fish abdomen during the slaughter phase. The visual inspection took place soon after the skin mucus had been sampled from the fish from group 2 and after one day for the fish from group 1, which concluded the last phase of the process the following day. The internal abdomen inspection enabled the detection of possible alterations in the abdominal cavity and organs of the fish.

Picture 8 - Blood sampling from rainbow trout.



Picture 9 and 10 - Samples processing of rainbow trout skin mucus.

3.2.2.4. RNA extraction and qRT-PCR Analysis

In the Department of Fish Clinic, VETMEDUNI, RNA extraction from all skin mucus samples was performed following the RNeasy tissue kit manual of instructions (Qiagen, Hilden, Germany). In short, samples were lysed in RLT buffer containing β-mercaptoethanol. Steel beads were then added to the sample and homogenized using TissueLyser II (Qiagen) for 3 min at 25 Hz. Finally, RNA concentration was quantified using a NanoDrop 1000 spectrophotometer (LabTech International). Reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) on 1 μg of total RNA isolated

from H group and S group samples. Finally, RNA were eluted from the columns in RNase-free water and stored at –80 °C before use. PCR primers specific to the target genes were selected based on the scientific literature, and their nucleotide sequences are displayed in Table 5.

Table 5 - List of qRT-PCR primers used in the present study.

Nr.	Oligoname	Forward Sequence (5´->3´)	Reverse Sequence	Reference
1	CD8	AGTCGTGCAAAGTGGGAAAG	GGTTGCAATGGCATACAGTG	Leal et al., 2016
2	IL-6	TTTCAGAAGCCCGTGGAAGAGA	TCTTTGACCAGCCCTATCAGCA	Iliev et al., 2007
3	IL-10a	GGATTCTACACCACTTGAAGAGCC	GTCGTTGTTGTTCTGTGTTCTGTTGT	Harun et al., 2011
4	IL-8	AGAGACACTGAGATCATTGCCAC	CCCTCTTCATTTGTTGTTGGC	Iliev et al., 2007
5	CD4	CCTGCTCATCCACAGCCTAT	CTTCTCCTGGCTGTCTGACC	Leal et al., 2016
6	IgT	AACATCACCTGGCACATCAA	TTCAGGTTGCCCTTTGATTC	Leal et al., 2016
7	IgD	AGCTACATGGGAGTCAGTCAACT	CTTCGATCCTACCTCCAGTTCCT	Leal et al., 2016
8	IgM	CCTTAACCAGCCGAAAGGG	CCAACGCCATACAGCAGAG	Leal et al., 2016
9	IFNϒ	GAAGGCTCTGTCCGAGTTCA	TGTGTGATTTGAGCCTCTGG	Leal et al., 2016

The quantitative Real-Time PCR (qRT-PCR) was conducted using CFX96 Touch Real-Time PCR detection system (Bio-Rad, München, Germany). Trout beta-actin was used as a reference gene for normalization (Rucker U and El-Matbouli M, 2007; Kumar et al., 2015).

The qRT-PCR assay was performed in a total volume of 10 µL containing 5 µL of 2× SsoAdvanced™ Universal SYBR Green SuperMix (Bio-Rad), 0.5 µL of forward and reverse primer, 3 µL of nuclease free water, and 1 µL of 1:5 dilution of cDNA samples for every gene for the H and S fish groups. The PCR temperature cycling conditions for all investigated genes were as follows: initial pre-denaturation at 95 °C for 15 min, followed by a denaturation at 94 °C for 15 s; 50 cycles of denaturation at 55 °C for 15 s, annealing at 72 °C for 15 s, and elongation at 55 °C for 31 s. The final cycle was followed by extension at 55 °C for 5 s. Each qRT-PCR assay was performed in duplicate.

3.2.2.5. Statistical analysis

For the plasma parameters, data collected for the cortisol, glucose, total proteins, cholesterol, total triglycerides, albumins, and total globulins levels underwent a one-way ANOVA using SPSS 25 (IBM Corporation, 2017) to check for statistical differences between the two considered groups. Means and standard deviations were subsequently calculated. The means were compared using the Student-Newman-Keuls (SNK) test.

Concerning the qRT-PCR analysis, the expression of the genes of both groups was normalized to the geometric mean of the reference genes (ß-actin). The relative gene expression between the H and S groups was calculated using the 2-DealtaDealta Ct method as the fold increase or decrease of the exposed group relative to the unexposed control group (mean expression level adjusted to 1). The statistical difference between groups was determined using the two-tailed unpaired Student's t-test with Welch's correction. For all statistical tests, a P-value < 0.05 was regarded as significant. The data were analysed using R statistical software (version 3.5.1) [R Core Team. R: A language and environment for statistical computing. Vienna, Austria; 2018. www.r-project.org].

3.2.3. Results

3.5.3.1. Experimental conditions, body examination and inspection

The monitoring of physicochemical parameters of the fish farm water rendered values considered suitable for the rainbow trout species (Fiordelmondo et al., 2020).

During the sampling stages, all fish exhibited good body condition and skin integrity. No superficial skin lesions, abrasions, lacerations, or any other alterations of the skin mucus layer were found. The skin mucus appeared homogeneous, uniform and well-distributed in both groups. The microscopic examinations did not reveal signs of the presence of parasites or of any kind of infection. The visual inspection of the internal abdomen did not show any sign of inflammation, infection, or other alteration for any fish of the two groups.

3.2.3.2. Gene expression in skin mucus

The mRNA expression levels were used to examine whether the handling that had occurred in the last phase of rainbow trout standard fattening cycle caused differences in the transcriptomic responses of stress- and immunity-related genes in skin mucus. The levels of expression of several immune genes related to T and B cells were tested and compared including those coding for CD8, IL-6, IL-10a, IL-8, CD4, IgT, IgD, IgM, and IFN-γ. In Figure 10, the expressions of the genes in rainbow trout are shown for the group 1 (labelled H1-H10) and group 2 (labelled S1-S10). Particularly, the expression of the gens coding for CD8, IL-10a, IL-8, CD4, IgT, IgM, and IFN-γ was evaluated in every trout of both groups (group 1 and group 2), and then compared to investigate upregulations or downregulations in group 2 compared to group 1. Data elaboration revealed a statistically significant difference between the two considered groups with respect to IL-6 and IgD. The expression of the IL-6 gene was upregulated (> 2.5 fold) in group 2 when compared with group 1; for the IgD gene,

the level of expression was increased (> 1.2 fold) in group 2 compared to group 1. Concerning the other genes under investigation, no statistically significant differences were recorded between the two fish groups.

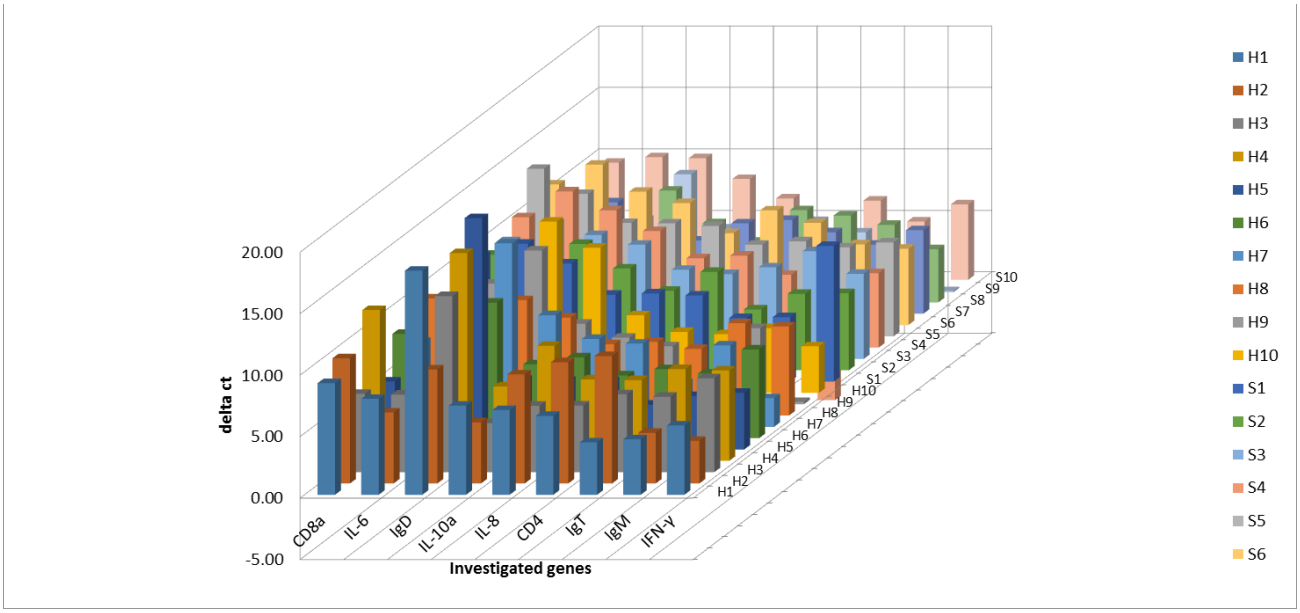


Figure 10 - Gene expression level in skin mucus before (group 1, labelled H1-H10) and after (group 2, labelled S1-S10) fish handling in the last phase of the rainbow trout standard fattening cycle.

3.2.3.3. Plasma parameters

Plasma analysis revealed that glucose and albumins values were modulated with significant statistic differences. For glucose, group 2 showed a mean value of 49.38 ± 7.52 mg/dl, while group 1 showed a mean value of 81.56 ± 10.08 mg/dl. For albumins, group 2 showed a mean value of 1.59 ± 0.05 mg/dl, whereas group 1 showed a mean value of 1.79 ± 0.17 mg/dl. Results for the plasma cortisol level comparing between the two considered group showed

that it ranged between 10.40 ng/ml in group 1 and 15.10 ng/mL , in group 2 between 10.50 ng/ml and 14.80 ng/ml. The value of total proteins showed a mean of 4.41 ± 0.61 g/dl in group 1 and a mean of 4.18 ± 0.16 g/dl in group 2. Referred to cholesterol, a mean of 266.82 ± 23.04 mg/dl was recorded in group 1 and a mean of 248.55 ± 9.39 mg/dl in group 2. Total triglycerides showed a mean of 401.28 ± 102.54 mg/dl in group 1 and a mean of 511.52 ± 107.51 mg/dl in group 2; total globulins showed a mean of 2.76 ± 0.43 g/dl in group 1 and a mean of 2.54 ± 0.11 a g/dl in group 2. No significant statistic differences were founded compared the levels of cortisol, total proteins, cholesterol, total triglycerides, and total globulins between the two groups. Table 6 shows the means of the plasma parameter values measured for group 1 and group 2.

Table 6 - Means ± standard deviation of plasma parameters of the group 1, represented by rainbow trout captured before the potentially stressful farming operations, and of group 2, represented by rainbow trout captured after all the potentially stressful farming operations and before slaughtering.

Plasma parameter	**Group 1**	**Group 2**
Cortisol (ng/ml)	12.67±1.50 a	13.35±1.11 a
Glucose (mg/dl)	81.56±10.08 a	49.38±7.52 b
Cholesterol (mg/dl)	266.82±23.04 a	248.55±9.39 a
Total triglycerides (mg/dl)	401.28±102.54 a	511.52±107.51 a
Albumin (g/dl)	1.79±0.17 a	1.59±0.05 b
Total proteins (g/dl)	4.41±0.61 a	4.18±0.16 a

Total globulins (g/dl)	2.76±0.43 a	2.54±0.11 a

Different letters (a, b) on the same line show statistically significant differences ($p < 0.05$).

3.2.4. Discussion

Rainbow trout (*O. mykiss*) was selected for this study because of its commercial relevance as one of the main farmed fish species in aquaculture. Over the past 10 years, various studies have focused on analyses of the skin of this fish species (Slominski et al., 2013; Benhamed et al., 2014; De Marcado et al., 2017; Reverter et al., 2018). Many studies emphasized the functions of skin mucus as a mechanical buffer and barrier against infections, while simultaneously fulfilling many other physiological functions (Carbajal-González et al., 2011; Reverter et al., 2018; Fajardo et al., 2022). Other studies (Momoda et al., 2007; Tacchi et al., 2015; Yarahmadi et al., 2016; Hoseinifar et al., 2020) dealt with the effect of stress on gene expression and immune parameters in rainbow trout but, to the best of our knowledge, no studies have been conducted to investigate the effects of potentially stressful farming operations stress over the skin mucus of reared rainbow trout during the last phase of a standard farming cycle. Only a methodology for assessing welfare in sea bass and sea bream was validated (Grant Agreement: 727610), and other authors wanted to confirm the truthfulness of the good breeding practices adopted until now (Saraiva et al., 2022).

The impact of bacterial infections on fish skin mucus had already been described in other studies using RT-PCR (Krasnov et al., 2005; Momoda et al., 2007) for evaluating gene expression under stress conditions. Another study revealed that several genes were transcriptionally altered during the stress response in the rainbow trout liver (Momoda et al.,

2007). Studies on the impact of stress have also been conducted on other farmed fish species. For instance, the expression of stress proteins in gilthead sea bream (*Sparus aurata*) in response to experimental wounds was studied by evaluating the expression of stress-related genes in the fish skin matrix (Espinosa-Ruíz et al., 2021).

Apart from skin and skin mucus (Carbajal et al., 2019), blood is another tissue that is readily collectible and potentially insightful with respect to health assessment (Řehulka et al., 2005; Başusta A. and Dağ M., 2016). In the present study, the blood concentrations of cortisol, glucose, total proteins, cholesterol, total triglycerides, albumins, and total globulins were measured in rainbow trout before (group 1) and after (group 2) potentially stressful handling procedures which normally occur at the end of a standard fattening cycle. The obtained results largely overlapped with the ranges of the main chemical parameters measured in rainbow trout blood that had been communicated in previous research papers (Manera M and Britti D, 2006; Başusta A and Dağ M, 2016; Dezzutto et al., 2016).

3.2.4.1. Gene expression

The gene expression of relevant stress- and immune-related transcripts (CD8, IL-6, IL-10a, IL-8, CD4, IgT, IgD, IgM, IFN-γ) was investigated in fish before (group 1) and after (group 2) the handling phase (before slaughtering) to assess the impact of handling on fish welfare. The obtained results indicate a statistically significant difference between the two considered groups pertaining only to IL-6 and IgD expression in fish skin mucus. Moreover, different expressions of genes related to skin immunology were observed when comparing fish living in standard raceways (group 1) with fish assessed before the commencement of slaughtering (group 2), with an increment of the IL-6 and IgD gene expression in group 2. IL-6 represents pro-inflammatory cytokines involved in the regulation of the immune system by controlling immunoglobulin production and the differentiation of lymphocytes and monocytes (Iliev et

al., 2007). IL-6 promotes the production of B lymphocytes (leukocytes responsible for the production of antibodies), and its participation in response to pathogens is well-known (van der Poll et al., 1997). Its effects were also tested in fish (Reyes-Cerpa et al., 2012; Wei et al., 2018), including rainbow trout (Bureau et al., 1999; Costa et al., 2011). Three classes of antibodies have been identified in fish: IgM, IgD, and IgT. The IgD class is an indicator of previous exposure to pathogens involved in the adaptive response by activating B cells, basophils, and mast cells for the production of active antimicrobial agents (Ramirez-Gomez et al., 2012). Its prevalence in group 1, along with the higher expression of IL-6 genes, suggests a reaction of the fish immune system to handling. Considering that the difference between the blood cortisol levels in the two considered groups was not statistically significant, it can be inferred that the fish reacted to the handling without having previously experienced a stressful state or an infection, as indicated by the absence of any signs of inflammation during the inspection of the internal abdomen of fish from group 2.

Considering that significant statistic differences were observed limited to IL-6 and IgD, whereas the values referred to CD8, IL-10a, IL-8, CD4, IgT, IgM, and IFN-γ were statistically the same in the two considered groups of rainbow trout, it is possible to conclude that the farming process that occurred at the end of the rainbow trout fattening phase did not negatively influence the fish welfare conditions.

3.2.4.2. Plasma parameters

Blood glucose is the primary source of energy involved in metabolic pathways. Under stressful conditions, a surge in swimming motions and metabolic activity leads to the reduction of the plasma level of glucose. Consequently, the blood glucose level was lower in group 2 than in group 1, as expected. All the handling that took place in the experimental trials during the last phase of the fattening cycle, before slaughtering, was linked with the

reduction in the glucose plasma levels. Furthermore, the fact that fish had gone through a fasting period lasting between 6 and 12 h must also be taken into account. As previously demonstrated, the fasting time alone is one of the main causes of the reduction of plasma glucose levels (Machado et al., 1989; Pottinger et al., 2003; Dai et al., 2022; Fernández-Muela et al., 2023).

Similarly, with glucose, which represents the main short-term source of energy, total triglycerides represent the main long-term energy reserves (Babin et al., 1989; Gallagher et al., 2017; Olsen et al., 2021). A decrease in triglyceride count could also indicate a state of malnutrition or of long-term stress, but that is not the case under the present circumstances.

The maintenance of a balanced level of total plasma proteins is also considered an indicator of fish welfare. Nevertheless, a stressful situation could lead to a decrease in the total plasma proteins because of increased energy demand (Coeurdacier et al, 2011). The albumins values, in particular, were assessed under the assumption that in fish, as in mammals, the plasma albumins values decrease during pathological states such as inflammations and stress conditions (Řehulka et al., 2005). The plasma level of albumins is usually constant in fish, with low values likely indicating the induction of a pathological state (Mutlu et al., 2015; Chernyavskikh et al., 2019; Pastorino et al., 2022). The plasma level of albumins in this study was lower for the S group than for the H group, which is in accordance with the expected outcome. Globulins are a group of proteins responsible for immunoglobulin production, and their abundance values provide insight into the potential immune response of an organism (Sahu et al., 2006).

Cortisol is considered to be the main stress hormone and the principal indicator of the deterioration of the welfare state of an organism (Sadoul et al., 2019). The plasma level of cortisol could also be increased under stress conditions, triggering a higher release of

cholesterol into the blood. Cortisol and cholesterol act as stimuli for the increased release of energy-carrying molecules. Comparing the cortisol and cholesterol values in the two considered groups, there is no statistically significant variation. This observation suggests that the living conditions of rainbow trout in both groups were similar from the point of view of their impact on the fish welfare state. That means that the good farming procedures adopted in the rainbow trout farm system are good practices that must be adopted to respect the welfare of farming rainbow trout in Italy.

The measured plasma cortisol values in the present study varied between 10 and 15 ng/ml among all rainbow trout under investigation, without statistically significant differences between the two considered groups. The minimum value of plasma cortisol measured was of 10.40 ng/ml, while the maximum value was of 15.10 ng/ml. On the contrary, other studies pertaining to rainbow trout reported a plasma cortisol level of 17.2 ± 4.2 ng/ml that increased up to 108.0 ± 30.7 ng/ml after an acute stress event (Gesto et al., 2015). Another instance of higher values of plasma cortisol level was reported in a case of poor water quality (Satoshi et al., 2011). The plasma cortisol level from the present study was consistent with the results reported in other studies for this parameter (Pottinger et al., 2003; Satoshi et al., 2011; Gesto et al., 2015; Bermejo-Poza et al., 2019; Carbajal et al., 2019; Sadoul et al., 2019) and within the standard range for the rainbow trout values for both the two considered groups, suggesting similar welfare states for both groups, and so the standard farming procedures adopted in Italy look as good practises to respect the rainbow trout welfare in fish farms.

3.2.5. Conclusion

In conclusion, the results of the present study improve the knowledge regarding the fish welfare state in the last phase of a standard fattening cycle in the case of rainbow trout (*O. mykiss*), in addition to providing an insight into the skin mucus and its functionalities. Moreover, the plasma parameters were successfully used to assess the health status of farmed fish. According to the present results, it can be stated that short-term stressors could modify the plasma levels of glucose and albumins, in addition to altering the skin mucus activity, by regulating the transcriptomic response of some immune mediators (i.e., IL-6 and IgD) found within the skin mucus of farmed rainbow trout. In this study the state of welfare and health of farming rainbow trout in the last phase of a standard fattening cycle was assessed by analysing the mRNA expression levels of several immunity genes and plasma parameters between fish before and after potentially stressful farming operations group. The obtained results suggest that the fish farming system in use in Italy for rainbow trout fattening could potentially discard any handling operations that may otherwise cause harm to the fish or lead to body surface alterations. These results must be taken into consideration concerning the evaluation of the fish farm quality, as just conducted for other fish species for same aspects (Pacoring et al., 2022).

TRIAL SECTION ON RAINBOW TROUT (*Oncorhynchus mykiss*) GROWING USING DIETS WITH ALTERANTIVE PROTEIN SOURCES

The constant increase of the world population requires an upper demand of food products and the world aquaculture sector is constantly growing. To support this trend, the demand for specific and balanced fish feeds increases, and finding alternative raw materials to be used in fish feeding is necessary to reduce the amount of fish meal required to make fish feeds for the main fish species reared in aquaculture. The replacement of fishmeal and fish oil, as the main protein and lipid source for aquaculture feed, has the priority in the view of sustainability prospective and because of the high costs and the limited availability of these raw materials. Therefore, many substitute ingredients have been tested in different fish species, as discussed below. It is known that the feed ratio of almost all fish species is characterized by a high protein level (Kim et al., 2016; Sankian et al., 2017; Hua et al., 2019). Thus, the main scope of scientific research is to identify valid alternative protein sources to be used in fish feeding, and which at the same time give good zootechnical performances and provide high quality of fish fillet. The feeding of the main fish species of interest in aquaculture plays a central role because it influences not only the fish body growth but also the composition of the meat of fish fillet, its nutritional value and its dietary qualities, its aptitude for conservation and transformation, as well as the organoleptic characteristics such as flavour, smell, and colour (Johnsen PB and Dupree HK, 1991; Bureau DP and Hua K., 2008; Duma et al., 2010). Nowadays the problem of fish nutrition in aquaculture becomes a priority also in relation to the fact that 50-60% of the management costs of a fish farm could be attributed to the nutrition item.

From the point of view of the sustainability of the raw materials to be used in fish feeding, the employ of vegetable proteins in fish feed poses the discussion on the nutritional quality of that protein and the content of essential amino acids, the presence of anti-nutritional factors,

fibre, and the low diet palatability. Compared different vegetable proteins, the biological value of soy looks the highest for the amino acid composition (Kaushik SJ and Hemre GI, 2008; Chen et al., 2019; Vélez-Calabria et al., 2021; Wang et al., 2022). In fact, soybean was employed for years as the main protein source (32-33%) to substitute fish meal followed by gluten and wheat meals (Hertrampf JW and Piedad-Pascual F, 2000). A part soybean meal, because the soy is largely employed also in human and animal nutrition (Messina M and Messina V, 2010; Rakita et al., 2021; Janocha et al., 2022; Pingxu et al., 2022), many scientists have been looking for other vegetable protein sources in order to promote sustainable aquaculture development (Dorothy et al., 2018; Parisi et al., 2020; Fiordelmondo et al., 2022). In fact, the aquaculture sector, especially referred to carnivorous species, is currently dependent on the capture of fish to produce fish meal and fish oil. Mainly due to the rising prices of both of them, the levels of their inclusion in the fish diet are nowadays lower. In this condition, insect meal is another alternative as a source of protein rich in essential amino acids, minerals and vitamins (Müller et al., 2017; Chemello et al., 2020; Giannetto et al., 2020; Stejskal et al., 2020). Furthermore, the oils contained in insects are rich in polyunsaturated fatty acids (Oonincx et al., 2020; Stejskal et al., 2020; Zhou et al., 2022).

In the context of circular economy and sustainable development, another way to produce fishmeal comes from the processing of by-products deriving from the transformation industry (Gasco et al., 2020; Nawaza et al.,2020; Pateiro et al., 2020).

In conclusion, as discussed, several ingredients are currently considered as alternatives to fishmeal in fish diets, including plant-based proteins and animal-based protein sources (insect meals, by-products etc.). Another interesting way is opening up, that is the dry yeast products, called single-cell proteins (SCPs) (Glencross et al., 2020; Agboola et al., 2022).

4. DUCKWEED

Trial on the Effects of Partial Substitution of Conventional Protein Sources with Duckweed (*Lemna minor*) Meal in the Feeding of Rainbow Trout (*Oncorhynchus mykiss*) on Growth Performances and the Quality Product

4.1. Introduction

According to Food and Agriculture Organization (FAO), average fish consumption in the world has reached a new record of 20.5 kg per capita per year and it will increase further in the next decade (FAO 2020). In this context, sustainable development of aquaculture and effective management of fish resources are the key to support this trend. The increase of fish production needs the use of new raw materials to be included in fish feeding, and the adoption of new technology and new strategies to produce greater quantities of fish in a sustainable way, thus avoiding natural resources exploitation. Nowadays, it is increasingly important to find alternative and innovative raw materials to be used in fish feeding, and to understand the level of the possible substitution without a negative influence in fish growth and fish meat quality, without forgetting the importance of the environmental sustainability. In particular, some non-conventional feeding sources are becoming strategically important from economic and environmental sustainability point of view; indeed, the use of expensive fish flesh for fish feeding is leaving space for cheaper and less impactful alternative diets, based on protein sources able to replace this ingredient with others (insects, vegetables, algae, by-products from aquatic organisms) to combine fish growth and environmental sustainability (Gasco et al., 2020; Parisi et al., 2020). The research of proteins of vegetable origin in the formulation of aquafeed has underlined that the crop-based agriculture can help the aquaculture to become more sustainable through expanding the variety of different plant sources (Hua et al., 2019; Napier et al., 2020). The soybean meal is the conventional protein

source mostly used as a complement to fish meal and consequently the demand for this feedstuff has significantly increased to skyrocket worldwide its price in these last five years. It is clear that this has made its use less and less sustainable both in economic terms, as many countries also have to import it, and in environmental terms. Therefore, the properties of other plants have been investigated to evaluate their potential use in fish feed as protein source (Dorothy et al., 2018; Sudiarto et al., 2019; Ceschin et al., 2020). Recently, the attention has been focused on small aquatic plants, known as duckweeds, appreciated for their ability to reduce nutrient concentrations in water absorbing nitrogen compounds (Sońta et al., 2019; Ceschin et al., 2020) and for their nutritional properties (Bog et al., 2019).

Duckweeds (*Lemnaceae*, species *Lemna minor* as the mayor common duckeed) (Landolt E, 1986; Tippery et al., 2021) are free-floating aquatic plants, occurring spontaneously in standing or slow-flowing waters. They grow very rapidly and widely in nature, showing to be one of the fastest growing higher plants (Lemon et al., 2001; Ziegler et al., 2015), and their supply is easy to recover. These plants are characterized morphologically by a tiny (a few mm) leaf shaped vegetative body (frond) in which the stem is not distinguishable from the leaves and with a root system consisting of a single root (Picture 11 and 12).

Picture 11, 12 – *Lemnaceae, Lemna minor,* common duckweed.

Concerning the cultivation, duckweeds can be produced quite easily and cheaply even without the necessity to use growth media and/or fertilizers since they are characterized by a high Relative Growth Rate (RGR) (Lemon et al., 2001; Ceschin et al., 2016). This means they are able to produce large quantities of biomass in a short time and in relatively small ponds filled with a few tens of centimetres of natural water (30-50 cm deep) (Picture 13). Obviously, their productivity can increase the more the optimal ecological conditions for their growth are present. In optimal growth conditions, duckweeds show high concentrations of nutrients but pathogens, heavy metals and organic pollutants can be accumulated in the plants' tissues (Coughlan et al., 2021). Controlling and monitoring the aquatic environment in which the plants grow is particularly important. Duckweeds productivity increases more if the optimal ecological conditions for growth are respected, which however are generally wide. These, while varying slightly from species to species, generally consist of moderately warm, sunny and nutrient-rich waters, as documented in ecological studies on some duckweed species of the *Lemna* genus (Landolt et al., 1986; Ceschin et al., 2018; Sharma et al., 2019). However, a good productive performance of duckweeds can occur in a wide range of conditions with respect to some factors, such as temperature and pH (Sudiarto et al., 2019; Ceschin et al., 2020) for example, which clearly points out how these plants can be easily cultivable in different habitats. It is interesting to consider the hypothesis of growing duckweeds in wastewater from aquaculture systems (Paolacci et al., 2022) that, generally rich in nutrients, could allow a production of them at low cost and eco-sustainable in line with the principles of the circular economy. This is important when considering the huge quantity of plant needed in large scale fish feeding. Other aspects such as the rapid growth and the composition of protein and poly- unsaturated fatty acids (Yan et al., 2013; Appenroth et al., 2017) make duckweeds a good ingredient for feed applications.

Picture 13 - Cultivation of duckweeds in outdoor tanks.

Considering the composition of duckweeds, they provide a good source of protein (up to 45.5 g of crude protein (Stadtlander et al., 2019)), lipid, and minerals (Mbagwu IG and Adeniji HA, 1988; Leng et al., 1995; Men et al., 2001). For their good protein intake, duckweeds were also largely used for feeding ruminants (Huque et al., 1996; Tanuwiria UH and Mushawwir A, 2020) pigs (Leng et al., 1995; Goopy JP and Murray PJ, 2003) and poultry (Mwale M and Gwaze FR, 2013; Aderemi et al., 2018) and for making pet foods, as an alternative source of amino acids (Yan et al., 2013). Duckweeds are also commonly consumed as food by people in some areas in different continents (McCusker et al., 2014). Amino acid profile and fatty acid profile have confirmed the suitability of these plants in the production of aquafeed (Beukelaar et al., 2019). Moreover, macronutrients and other compounds, such as β-carotene and xanthophyll, increase the importance of the duckweeds as a potential ingredient to be essayed in aquafeed (Chakrabarti et al., 2018) for warm water fish species as rohu (Bairagi et al., 2002), carp (Yilmaz et al., 2004) and tilapia (Fasakin et al., 1999). However, in salmonids the dietary duckweeds meal content has been only evaluated during fry stage for the rainbow trout species (Stadtlander et al., 2019).

Based on these considerations, a trial was performed in order to evaluate the effects of duckweed meal as partial replacement of the main conventional protein sources (fish and

soybean meal) in three different low fish meal diets on productive performances of rainbow trout reared during the on-growing phase and compared with coetaneous fish receiving a conventional feed.

4.2. Materials and Methods

4.2.1. Experimental design and fish material

For the experiment, 12 concrete outdoor tanks were used, divided in 3 tanks for each of the four trout groups fed with different diets (LC, L1, L2, L3). Every tank had a length of 5 m, a width of 0.8 m, a depth of 0.5 m, and a volume of 2 m^3 and was filled with well water. During the experiment, the main water physico-chemical parameters (temperature, dissolved oxygen and pH) were daily recorded in every tank using portable electronic devices (YSI mod. 55 and 60, Yellow Springs, OH, USA). TAN, NO_2-N and NO_3-N were weekly analysed following APHA standard methods (APHA, 1995). All the basins were covered with an antifouling net in order to avoid algal development and to keep away ichthyophagous birds.

This experiment was performed during a standard zootechnical cycle, avoiding any animal suffering, and no sample was collected from live animals, according to the Italian Legislative Decree 26/2014. The farm applied an "Antibiotic-free Code of Prescription" to guarantee the quality of the product and respect the "antibiotic-free" approach. During spring season in 2021, a total of 540 rainbow trout (245 days old; mean body weight 124.5 ± 0.7 g) was randomly allocated in the 12 tanks, with 45 fish in each tank, at the initial stocking density of 6.2 kg/m^3. At the end of the experiment (90 days), fish were weighed, and their final length was recorded. Fish were fed by hand twice a day (8 a.m. and 3 p.m.) until the apparent satiation level; then the unconsumed feed was collected.

Palatability of the feeds was calculated according to the formula: ((ingested feed/administered feed) × 100) based on the index reported in previous studies (Kasumyan AO, 1997; Kasumyan AO and Døving KB, 2003). The following zootechnical performances were evaluated in the four different groups: WG (%) = (final weight − initial weight) × 100⁄initial weight; SGR (%⁄day) = {Ln (final weight) – Ln (initial weight) } × 100⁄days; FCR = live weight gain (g)/feed administered (g); SR (%) = final number of fish/initial number of fish × 100 (Steffens W., 1989). In addition, the condition factor KI = ([fish weight/fish length] × 100) (Bagenal TB and Tesch FW, 1978) and the following biometric indices were calculated after having applied standard procedures for fish sampling (U.S. EPA, 2000): VSI = ([weight viscera/whole body weight] × 100), PFI = ([perivisceral fat/body weight] × 100), and HSI = ([liver weight/body weight] × 100). In order to calculate the PFI and the VSI, the fat adherent to the digestive tract was accurately separated and individually weighed.

4.2.2. Plant material and experimental diets

Fronds of a duckweed species of the genus *Lemna*, *L. minor* (common duckweed) species, were collected from ponds of a fish farm. We avoid the use of duckweeds coming from polluted areas or wastewater due to high sensitivity of this plant to a wide range of toxicants (Abdel-Gawad et al., 2020) which could threaten the fish health status (Vinogradskaya MI and Kasumyan AO, 2019). Samples of duckweed were analysed from the botanical point of view by means of stereomicroscope (mod. Stemi 305, Zeiss), that confirmed the above mentioned species. The duckweed was submitted to washing, air exposure and oven-dried at 60 °C for 6 h, and finally milled modified according to the recent literature (Aderemi et al., 2018). In order to employ the duckweed meal for the on-growing phase of rainbow trout, the proximate composition and the amino acid profile were determined on a duckweed frond sample (Table 7). The proximate composition (moisture, protein, lipid and ash) and the amino acid profile of duckweed meal were performed according to international methods. In

particular, moisture and ash content were determined using the procedures described by the Official Analytical Chemists (AOAC, 1990). The protein content was determined using the standard Kjeldahl copper catalyst method. The amino-acid profile was determined by acid hydrolysis (6 N HCl for 24 hrs, at 110 °C) followed by ion ex-change chromatography with an amino-analyser (L-8800 Auto-analyser, HITACHI, Japan).

Table 7 - Proximate composition (%) and amino acid profile (g/100 g) of duckweed meal used in the experiment.

Composition	**%**
Moisture	92.81
Crude protein	28.13
Crude lipid	5.10
Crude fibre	15.20
Ash	16.40
Amino acid	*g/100 g*
Arginine	4.56
Histidine	3.28
Isoleucine	3.62
Leucine	6.41
Lysine	4.49
Methionine	1.74
Phenylalanine	4.25
Threonine	2.16
Tryptophan	3.89
Valine	3.53

Lemna minor meal was included in the formulation of three feeds (L1, L2, L3) at different rates (10%, 20%, 28%, respectively) of the protein source. At the increasing of the duckweed inclusion, soybean meal, fish meal, wheat flour and gluten wheat meal were reduced or adjusted in order to get similarly an isonitrogenous (41%) and isolipidic (20%) diets. A control diet (LC) was formulated with the same feedstuffs except duckweed meal (Table 8).

This formulation aimed at saving the use of the various conventional protein sources, essaying a local feedstuff derived from duckweed plant; after the pandemic, also soybean meal and not only fish meal are becoming very expensive to get on the market.

Table 8 - Formulation and proximate composition of the control diet without including duckweed meal (LC), and L1, L2 and L3 diets, with different inclusion of duckweed meal (10%, 20%, 28%, respectively).

	LC	**L1**	**L2**	**L3**
Ingredients (%)				
Duckweed meal	**0**	**10**	**20**	**28**
Soybean meal	22.3	21.8	11.0	7.0
Fish meal	21	20	18	17
Wheat flour	20	13	11	8
Haemoglobin meal	10	10	10	10
Gluten wheat meal	7.6	5.6	10.4	11.3
Fish oil	12	12	12	12
Soybean oil	5	5	5	5
L-Lysine	0.4	0.4	0.4	0.4
DL-Methionine	0.18	0.2	0.25	0.28
Vitamin-mineral mix	2	2	2	2
Proximate composition (%)				
Moisture	8.89	8.92	8.97	9.05
Protein	41.50	41.59	41.53	41.27
Lipid	20.00	20.12	20.30	20.00
Ash	6.81	7.28	7.10	7.26

Amino acid profile (% of crude protein)				
Arginine	5.24	4.51	4.00	3.92
Histidine	1.85	1.68	1.67	1.50
Isoleucine	1.39	1.37	1.31	1.30
Leucine	3.61	3.60	3.54	3.51
Lysine	4.99	3.90	3.56	3.01
Methionine	3.33	2.91	2.91	2.91
Phenylalanine	2.28	2.10	1.94	1.80
Threonine	2.06	1.53	1.45	1.42
Tryptophan	0.60	0.50	0.49	0.47
Valine	3.36	2.36	2.31	2.28

The feeds were manufactured in 3.5 mm size using a twin-screw extruder (100 rpm, 110 °C, 50 atm). After the coating, the feeds were stocked in buckets and kept in an aerated room. The proximate composition (moisture, protein, lipid and ash) and the amino acid profile of three samples of each feed type were performed according to the international methods reported for duckweed meal analysis (Burka JF and Johnson G, 2002). For all the four diets, after determining total lipid content, using the procedure described by Folch et al. (Folch et al., 1957), fatty acids were converted to methyl esters following the method described by Christopherson and Glass (Christopherson et al., 1969). The separation of fatty acids was carried out using a GC 3800 gas chromatography (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 × 0.25 mm i.d.; 0.25 μm film thickness; Supelco, Bellefonte, Pennsylvania, USA) and a flame ionization

detector. The essential amino acid profile of the three feeds containing duckweed was obtained as previously indicated for duckweed meal.

4.2.3. Quality traits of fish fillet

After 90 days of experiment, fish have reached the commercial size and are slaughtered by electrical stunning in an authorized slaughterhouse.

Fish were dissected as follow: after a check of the skin status on the whole body to be sure of the absence of skin lesions, the abdomen was opened with a cut starting from the anus to gills, and then a lateral line up the side of the fish allowed to check the status of gills and meat. From the anus the digestive system was tracked and removed; similarly, all the abdomen organs were checked and removed.

A portion of about 50 g of skinless dorsal left muscle from six fish casually selected for each diet group was collected, then homogenized and submitted to proximate composition analyses (moisture, protein, lipid, and ash content). The procedures adopted for these last analyses follow substantially the same methods indicated in the previous paragraph concerning the measurement of proximate composition of the different feedstuffs. The percentage of moisture was determined in duplicate according to the Association of Official Analytical Chemists procedure (AOAC, 1990). The protein content was determined using the standard Kjeldahl copper catalyst method. The ash content was determined using the procedure described by the AOAC (AOAC, 1990). Total lipids were measured using a modification of the chloroform:methanol procedure described by Folch et al. (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 (Christopherson and Glass, 1969). The separation of fatty acids was performed using a Carlo Erba HRGC 5160 gas chromatography (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 mmi.d.; 0.20 μm film thickness; Supelco, Bellefonte, PA, USA) and a flame ionization detector. The operating conditions of the gas chromatography 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 percentage of the total of fatty acids.

4.2.4. Statistical analysis

Data collected (biometric parameters, final productive traits, fish fillet proximate composition, fatty acids categories) were subjected to one-way analysis of variance (ANOVA) using SPSS 25 (IBM Corp. IBM SPSS, 2017) to check differences in productive performances and composition of fillet of rainbow trout fed with different experimental diets. Means and standard deviations were calculated. Means were considered significant with a value of $p < 0.05$ and compared using the Student-Newman-Keuls (SNK) test.

4.3. Results

4.3.1. Water physicochemical characterization

Concerning the physicochemical characteristics of the water in which the fish were reared, in all the groups the temperature ranged from 12 °C, at the beginning of the experiment, to 13.8 °C (L2) at the end of the experiment (mean values: LC 11.05 ± 0.8 °C; L1 11.06 ± 0.9 °C; L2 11.04 ± 0.9 °C; L3 11.05 ± 0.9 °C). The pH ranged between 7.8 and 8.0 without notable

variations among the groups (mean values: 7.9 ± 0.1). The dissolved oxygen was averagely always over 10 mg/L in all the groups (mean values: LC 10.9 ± 1.5 mg/L; L1 10.8 ± 1.6 mg/L; L2 10.9 ± 1.4 mg/L; L3 10.2 ± 1.2 mg/L). Water total nitrogen ammonia (TAN) was included between a minimum in LC and L3 tanks (0.11 - 0.12 mg/L) and a maximum in L2 and L3 (0.19 - 0.20 mg/L) (mean values: LC 0.16 ± 0.1 mg/L; L1 0.15 ± 0.05 mg/L; L2 0.15 ± 0.06 mg/L; L3 0.16 ± 0.06 mg/L). Nitrites (NO_2-N) ranged from 0.02 mg/L in LC to 0.03 mg/L in L3 tanks (mean values: LC 0.025 ± 0.004 mg/L; L1 0.029± 0.002 mg/L; L2 0.026 ± 0.003 mg/L; L3 0.028 ± 0.002 mg/L) while nitrates (NO_3-N) from 0.9 mg/L (L2) to 0.14 mg/L (L3) (mean values: LC 0.12 ± 0.2 mg/L; L1 0.12 ± 0.1 mg/L; L2 0.10 ± 0.1 mg/L; L3 0.12 ± 0.2 mg/L).

4.3.2. Rainbow trout productive performances fed with different experimental diets

The productive parameters are reported in Table 9. The final mean body weight and length did not show significance differences among trout fed with L1 (340.53 ± 4.3; 31.2 ± 1.3 cm), L2 (339.4 ± 4.7 g; 31.6 ± 1.5 cm) and LC group (348.80 ± 4.4 g; 31.0 ± 1.2 cm) but were different from L3 group (302.16 ± 2.2 g; 28.2 ± 1.6 cm) that showed the lowest significantly final weight and size. Growth parameters, weight gain (WG) and specific growth rate (SGR), recorded similar performances among L1 (216.03 ± 2.8; 1.26 ± 0.04), L2 (214.92 ± 2.9; 1.2 ± 0.03) and LC (224.3 ± 2.6; 1.29 ± 0.03) and were significantly higher than L3 (177.66 ± 2.7; 1.11 ± 0.01). Food convertion rate (FCR) gave a favourable result without statistically differences among L1 (1.18 ± 0.02), L2 (1.18 ± 0.03) and LC (1.13 ± 0.02) but significantly better than the one recorded in L3 group (1.37 ± 0.02).

Table 9 - Productive performances of rainbow trout fed with different experimental diets (mean ± standard deviation).

Parameters	LC	L1	L2	L3	*P*
Initial mean weight (g)	124.5 ± 0.7	124.5 ± 0.7	124.5 ± 0.7	124.5 ± 0.7	-
Initial mean length (cm)	20.0 ± 0.6	20.0 ± 0.6	20.0 ± 0.6	20.0 ± 0.6	-
Final mean weight (g)	348.80 ± 4.4 a	340.53 ± 4.3 a	339.42 ± 4.7 a	302.16 ± 2.2 b	<0.05
Final mean length (cm)	31.0 ± 1.2 a	31.2 ± 1.3 a	31.6 ± 1.5 a	28.2 ± 1.6 b	<0.05
WG (%)	224.3 ± 2.6 a	216.03 ± 2.8 a	214.92 ± 2.9 a	177.66 ± 2.7 b	<0.05
SGR (%/day)	1.29 ± 0.03 a	1.26 ± 0.04 a	1.25 ± 0.03 a	1.11 ± 0.01 b	<0.05
FCR (g/g)	1.13 ± 0.02 b	1.18 ± 0.02 b	1.18 ± 0.03 b	1.37 ± 0.02 a	<0.05
SR (%)	99 ± 0 a	98 ± 1 a	98 ± 1 a	98 ± 1 a	<0.05
Palatability	100 ± 0.0 a	99.6 ± 0.4 a b	98.8 ± 1 b	98.2 ± 1.1 b	<0.05
KI	1.17 ± 0.12 a	1.12 ± 0.13 a	1.08 ± 0.14 a	1.35 ± 0.22 b	<0.05
VSI	10.06 ± 0.41 c	10.28 ± 0.59 b c	11.57 ± 0.68 b	14.57 ± 0.54 a	<0.05
PFI	3.00 ± 0.36 b	2.91 ± 0.04 b	3.05 ± 0.12 b	3.68 ± 0.03 a	<0.05
HSI	1.05 ± 0.06 b	1.31 ± 0.08 a	1.35 ± 0.03 a	1.24 ± 0.06 a	<0.05

Different letters (a, b, c) on the same line show statistically significant differences ($P < 0.05$). WG: weight gain, SGR: specific growth rate, FCR: feed convertion rate, SR: survival rate, KI: condition index, VSI: viscerosomatic index, PFI: perivisceral fat index, HSI: hepato-somatic index.

The somatic indices, condition index (KI), viscerosomatic index (VSI), perivisceral fat index (PFI), hepatosomatic index (HSI), were significantly affected by diets with different levels of duckweed meal. KI exhibited differences when the inclusion was at the highest substitution (28%) in L3 group (1.35 ± 0.22) respect to all the other groups (L1: 1.12 ± 0.13; L2: 1.08 ± 0.14; LC 1.17 ± 0.12). VSI significantly increased from L1 (10.28 ± 0.59), at an intermediate level between LC (10.06 ± 0.40) and L2 (11.57 ± 0.68), to L3 (14.57 ± 0.54). PFI had not notable variations among L1 (2.91 ± 0.04), L2 (3.05 ± 0.12) and LC (3.00 ± 0.36), that were all different from L3 (3.68 ± 0.03). HSI appeared similar among the three experimental groups (L1: 1.31 ± 0.08; L2: 1.35 ± 0.03; L3: 1.24 ± 0.06) but significantly higher than LC (1.05 ± 0.06). No significant differences were observed in the survival rate (SR), with values ranging between 98 and 99% in all groups. Palatability of L1 (99.6 ± 0.4) was at an

intermediate level respect to LC (100 ± 0.0) and L2 (98.8 ± 1) and L3 (98.2 ± 1.1); these last two experimental diets showed a similar acceptance.

The proximate composition of the fillets of rainbow trout fed with diets without (LC) or with different percentages of duckweed meal (L1, L2, L3) is reported in Table 10. For all the macronutrients considered (protein, fat, moisture and ash content) no significant differences were shown among the duckweed meal diets (L1, L2, L3) and the conventional control diet (LC).

Table 10 - Proximate composition (% ww) (mean ± st. dev.) of the fillet of rainbow trout fed with the control diet (LC) and the three experimental diets (L1, L2, L3) at the end of the trial.

Parameters	**LC**	**L1**	**L2**	**L3**	***P***
Moisture	77.73 ± 1.4	77.76 ± 1.2	77.35 ± 1.1	77.41 ± 1.2	>0.05
Protein	19.44 ± 0.9	19.78 ± 1.0	18.46 ± 1.1	18.82 ± 0.8	>0.05
Fat	2.33 ± 1.1	2.54 ± 0.8	3.17 ± 0.9	3.31 ± 0.9	>0.05
Ash	1.37 ± 0.2	1.29 ± 0.1	1.25 ± 0.2	1.18 ± 0.2	>0.05

Due to no significant differences from the statistical point of view, no letter was reported among the parameters on the same line.

With regard to the main categories of fatty acids in the final composition of the rainbow trout fillet, the saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) categories

were similar among all the four groups without notable differences (Figure 11). Furthermore, the polyunsaturated fatty acids (PUFAs) (n-3 and n-6 series) did not show significant differences ($P < 0.05$) between the experimental (L1, L2, L3) and the control (LC) group (Figure 11).

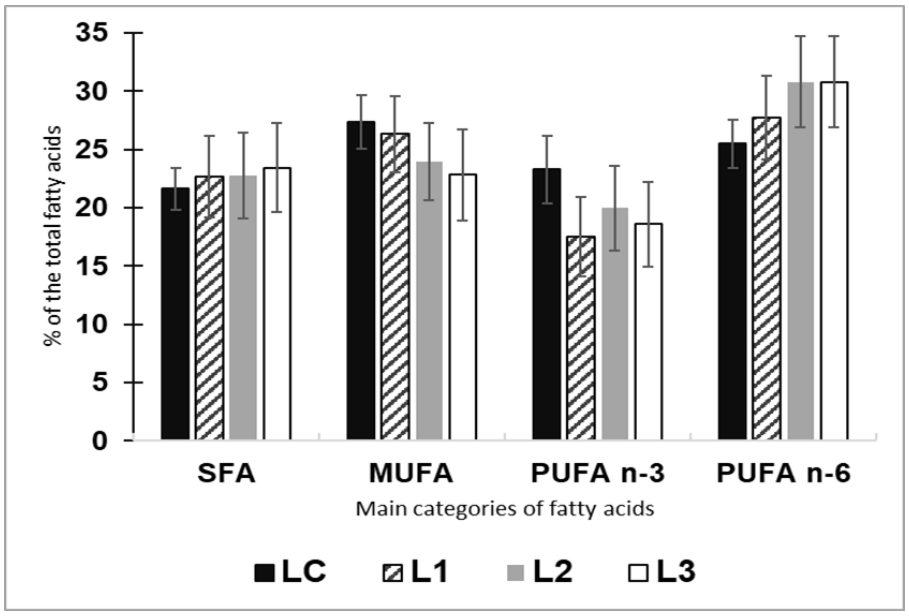


Figure 11. Categories of fatty acids (SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: n-3, n-6 polyunsaturated fatty acids) determined in the fillet of trout fed with LC and L1, L2, L3 diets (% of the total fatty acids). Due to no significant difference from the statistical point of view, no letter was reported in the groups of column charts.

4.4. Discussion

For decades, duckweed has captured the interest of scientists (Van Dyke JM and Sutton DL, 1977) due to its organoleptic characteristics. It is still a current topic due to the urgent problem of finding new protein sources as alternatives to the standard ones to be used in aquafeed. In particular, the properties of duckweed meal have been studied for their protein content in freshwater and marine fish.

In this trial, duckweed meal was included in three experimental diets for rainbow trout as partial replacement of the two main protein feedstuffs, fish meal and soybean meal, in order to evaluate an alternative protein source less expensive and more sustainable than conventional ones. The substitution has mostly concerned soybean meal and secondly fish meal, because in the last years the strong increase of the production costs of aquafeeds has also concerned the price of feedstuffs of vegetable origin such as soybean meal. Besides, the maintenance of a fraction of fish meal was considered prudent by authors to assure good growth results for rainbow trout. To perform the study, the duckweed employed was collected in freshwater areas, strictly monitored in terms of quality as demonstrated by the analyses of physicochemical parameters which were within the range considered optimal for the rainbow trout species in all the tested fish groups (Fiordelmondo et al., 2020). The survival rate was very high in all the experimental groups.

The meal obtained by the common duckweed employed in the experimental feeds had a protein content within the range specifically reported for that plant species (Fasakin et al., 1999). The amount of essential amino acids was detected in a good quantity, except methionine. Because of that, DL-methionine was added in the formulation of all the three experimental diets used in the present trial, with the aim to administer balanced diets according to the specific requirements of the rainbow trout species (NRC, 2011). The

percentages of duckweed substitution were also evaluated and decided making a gradual increase of duckweed in the experimental diets. In duckweed meals used in the present study, the fat content (5%) was in agreement with the literature reporting ranges between 4% and 7% (Leng et al., 1995).

The productive results showed that no adverse effects were observed in mean body weight, weight gain, and final length of rainbow trout when fish receives diets including up to 20% of duckweed meal, whereas highest levels affected the growth performances and the FCR. These differences could be related to the diet palatability that slightly decrease at increasing the duckweed meal. As Table 9 shows, the decrease of the palatability of the duckweed meals corresponds to an up inclusion of *Lemna* meal. The rainbow trout receiving the feed with the highest duckweed meal content exhibited reduced productive performance and unfavourable FCR. In another study on the taste of various aquatic plants for tilapia (Vinogradskaya et al., 2019), duckweed showed a low attractive effect due to the presence of flavonoids and triterpene compounds considered as not feed stimulating for fish. However, in common carp, diets with 20% of duckweed replacement gave results similar to conventional feeds in terms of growth performances (Bairagi et al., 2012). In the Indian carp species (rohu *Labeo rohita*), the replacement of 30% of fish meal dietary with common duckweed did not affect the growth of fingerlings in comparison with coetaneous fed with other macrophytes (Goswani et al., 2020). Based on literature, studies on common duckweed in fish diets were mostly performed on cyprinids and tilapia, so fish with feeding (omnivorous) and living (warm water) habits different respect to salmonids. In rainbow trout, a 4 weeks trial using another species of duckweed (*Spirodela polyrhiza*) at low and high (respectively 6.25% and 12.5% of feed) substitution for 4 weeks, had the same acceptance of the control diet although both the duckweed meal treatments resulted in 5 and 10% poorer growth traits (Stadtlander et al., 2019). As concerns the somatic indices, KI and PFI were affected by the highest percentage

of duckweed inclusion followed the trend of growth increased, whereas VSI and HSI discriminated differences also among the three experimental diets as well as with the control feed. These variations in VSI and HSI could be associated to the carbohydrate fraction not used as energy source, and therefore accumulated in the liver and transformed in lipids and glycogen, resulting in an increase of this index as documented in rainbow trout fed with diets including alternative plant ingredients rich in indigestible carbohydrates, in the form of oligo- as well as polysaccharides (Kaushik et al., 2022). To overcome this drawback, other works proposed to employ duckweed after a fermentation process that could considerably reduce the anti-nutritional factors and the crude fibre content (Goopy et al., 2023). Regarding the effects of dietary duckweed on the fish fillet quality, the proximate composition did not show notable differences among the macronutrients of the groups. In fact, as reported in Figure 1, the proximate composition of the fillet of rainbow trout fed with LC, L1, L2 and L3 doesn't show significant differences from the statistical point of view. In terms of fatty acid categories in the meat, trout fed diets including *Lemna* appeared very similar among them and the control. It is well known that the final nutritional flesh quality is strongly affected by the diet composition administered to fish (Sargent et al., 2001). In the current study, the experimental feeds maintained the blend of fish and vegetable oil unchanged respect to the control diet with the aim to show the only effects of the duckweed meal ingredient. According to a study on nutritional value of different duckweed genera, *Lemna minor* is reported as the species with an intermediate proportion (27.99%) of SFA, a very low MUFA level (4.6%) and a very high PUFA n-3 rate (46%), surprisingly higher than content of PUFA n-6 (20%) (Appenroth et al., 2017). In the current experiment, the fillet of rainbow trout had good content of fatty acids especially in terms of PUFA n-3, considered essentials of a balanced diet in humans providing beneficial effects on neural development (Campoy et al., 2022) and in mitigating several pathological conditions (Calder PC, 2014).

4.5. Conclusion

The current study aimed at evaluating the use of a local duckweed, collected in not contaminated waters, as protein source in partial substitution of the conventional feedstuffs (fish meal and soybean meal) to find out how could be used in animal feeding respecting the nature of the animal species. In particular, it provided useful information on the effects of duckweed meal diet on rainbow trout performances under on-growing phase and the quality fillet. This has great implication on responsible and sustainable aquaculture because it is essential to preserve fish biometric indices, rearing parameters and quality product, mainly when fish are reared under antibiotic-free approach. Through this study it has been possible to essay a protein source substitution with an alternative plant feedstuff, showing that the replacement should be done using *Lemna minor* at 20% of the protein sources, without negative consequences in the growth performance of the fish and quality fillet. At this rate of protein content, the feed with duckweed meal has shown satisfactory results. In this view, the present study could represent a challenge for further investigations aimed to analyse possible variations of duckweed composition in different seasons, in different areas or considering different *Lemna* species occurring locally.

4.6. Notes

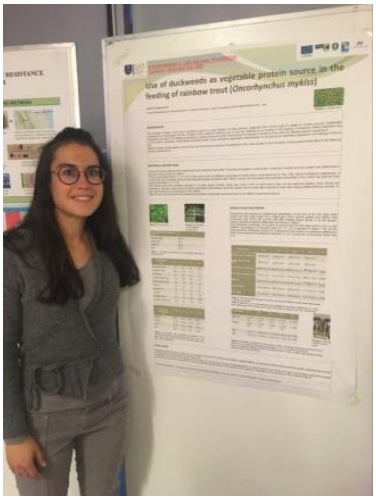
Publication

The study on the employ of *Lemna* spp. in fish feeding was the subject of a research article published in the Plant journal on April 2022 in the Special Issue "Duckweed: Research Meets Applications" (Fiordelmondo, E.; Ceschin, S.; Magi, G.E.; Mariotti, F.; Iaffaldano, N.; Galosi, L.; Roncarati, A. Effects of Partial Substitution of Conventional Protein Sources with

Duckweed (*Lemna minor*) Meal in the Feeding of Rainbow Trout (*Oncorhynchus mykiss*) on Growth Performances and the Quality Product. Plants 2022, 11, 1220. doi.org/10.3390.).

Poster presentation

Part of the results on the use of duckweeds as alternative protein source in the feeding of rainbow trout was the object of a Poster presentation at the PhD WORKSHOP in LIFE and HEALTH SCIENCES 2022 (Camerino - November 25th 2022) (Picture 14) entitled "Use of duckweeds as vegetable protein source in the feeding of rainbow trout (*Oncorhynchus mykiss*)".



Picture 14 – The author at the Workshop with her poster.

4.7. Supplementary insight

During the PhD triennium the Advanced Course on "Aspetti Molecolari della Nutrizione: dalla Nutrigenomica alla Nutrizione Funzionale" was concluded at the University of Camerino a.y.2021/2022. The duckweed inclusion in human diets, the properties related to the consumption of this group of plants and its application as ingredient in human foods was the topic of the final defence entitled "Use of Duckweeds (*Lemna* spp.) as an alternative vegetable proteins". The aim of the thesis summarized information on duckweeds, considered that it is helpful to understand the possible utilisation of these plants in modern applications in the food and feed industries and highlight the characteristics and the properties of duckweeds, their uses and employs as novel food and super food, as well as showing results obtained on their uses in animal feeding techniques until now. Also, in human nutrition a transition towards diets containing less animal-derived protein and more plant-derived protein would benefit the conservation of biodiversity, land, water, energy, climate, human health and animal welfare (Aiking H, 2011).

The summary of the thesis in Nutrigenomics "Corso di Perfezionamento in Aspetti Molecolari della Nutrizione: dalla Nutrogenomica alla Nutrizione Funzione" is reported at the end of the manuscript as an attached chapter.

Acknowledgments: A special thanks to Dr. Stefania Ruggeri of CREA Roma for her collaboration, support and tutoring, and in particular for her help during the elaboration of the Nutrigenomics thesis. She was the supervisor of the Nutrigenomics thesis, and after that a research group was create in order to continue the study on this specific topic. Our collaboration is still continued to study the composition and the chemical-phisical differences between the American duckweed species *Lemna minuta* and the Italian ones, *Lemna minor*, in particular conserning the aminoacids contents..

5. GUAR POWDER

Trial on the use of guar meal as an alternative protein source in the feeding of rainbow trout (*Oncorhynchus mykiss*)

5.1. Introduction

The galactomannan polysaccharide Guar gum (*Cyamopsis tetragonalobus*) derives from the endosperm of an Indian cluster bean. It was initially cultivated in India and Pakistan for feeding cattle and nowadays is being re-evaluated as additive in animal feeding due to its properties and organoleptic characteristic. In literature, guar was studied both for its technological properties and its use as feedstuff. In 1963, Vohra and Keatzer (Vohra P and Keatzer FH, 1963) showed that guar gum could replace soybean meal satisfactorily in chicken feeding. The capacity of guar to increase faecal stability in water was one of the main properties observed by Janphirom and colleagues in 2010 (Janphirom et al., 2010). That study showed the ability of this indigestible binder to reduce the breakdown of faeces that caused the dispersion of nitrogen and phosphorus sources into the wastewater. It was observed that the addition of guar reduced faecal leaching and increased faecal stability in water (Brinker A, 2009). Therefore, guar gum had a significant potential effect on improving the treatability of fish faecal waste. Furthermore, in rainbow trout it was observed that the addition of guar gum in fish feed stabilised faecal solids making them easier to remove (Brinker A, 2007). It is known that the quality of the aquatic environment in which farmed fish live impacts directly on the quality of the fish fillet and on the health of the animals themselves (Mu G, 2014; Fiordelmondo et al., 2020; Loan et al., 2021). In rearing conditions, water faeces are the main sources of organic nitrogen and phosphorus, which are transformed by microorganisms into toxic compounds such as ammonia, nitrites and nitrates (Wan Ahmad PC, 2001). In a study performed by Brinker et al. (2004), guar gum showed high viscosity and its addition to fish feed appeared not digestible and unabsorbable by fish. In the last years, guar meal has been

submitted to processing systems and purification technology able to reduce saponin, tannin, phytates and protease inhibitor concentration that are considered to negatively affect salmonids (Pach F and Nagel F, 2017). It could slow the decomposition of faeces and reduce the dispersion of the mentioned toxic compounds into farming water, improving the quality of the water where trout live. Moreover, larger and more stable faecal particles are more resistant to hydro-mechanical manipulations of the flow-through systems and remain more stably suspended in water (Reid et al., 2009; Brinker et al., 2009). Recently, an increase in faecal particle size through diet manipulation improved solids removal efficiency (Welker et al., 2018), reduced the impact of effluents on the environment and ensured compliance with environmental regulations. As previously mentioned, guar meal could also be considered for its chemical properties, in particular for its protein content. For this reason, it was used for trials in other animal species as a potential protein source. In one experiment on growing pigs, guar powder was added to the feed and did not negatively affect the feed efficiency, but resulted in lower average daily feed intake and daily weight gain (Hasan et al., 2020). In another study an aquaculture trial conducted on Nile tilapia fingerlings showed that guar gum provided a good protein source in substitution of dietary soybean meal (Abdel-Fattah et al., 2016).

Based on these considerations, a trial was performed to investigate the possible use of guar meal, submitted to the hydrolization process, as alternative protein source to conventional ones in the fattening phase of rainbow trout and its effects on zootechnical performances.

5.2. Materials and Methods

5.2.1. Experimental design

For the experiment, 12 concrete tanks were used; every tank had a length of 6 m, a width of 1 m, a depth of 0.5 m, and a volume of 3 m^3 and were located in a hatchery where the water supply came from the adjacent river (Picture 15). During the experiment, the main water physical-chemical parameters (temperature, dissolved oxygen and pH) were daily recorded in every tank using portable electronic devices (YSI mod. 55 and 60, Yellow Springs, OH, USA). TAN, NO_2-N and NO_3-N were weekly analysed following APHA standard methods (APHA, 1995) by means of a spectrophotometer (HACH mod. DR 6000 UV-VIS, HACH Lange Gmbh Düsseldorf, Germany). The trial was performed during a standard zootechnical cycle. Every morning (8 a.m.) all the tanks were submitted to cleaning water by siphoning and fish dead were removed.

Picture 15 – Tanks involved in the trial.

Fish

A total of 2700 rainbow trout (mean body weight 50 ± 1.4 g) were reared at the initial stocking density of 15 kg/m3. Fish were fed by hand twice a day (8 a.m. and 3 p.m.). At the end of the experiment (90 days) (Picture 16), fish were weighed, and their final length was recorded. Palatability of the feeds was calculated according to the formula: ((ingested feed/administered feed) × 100) based on the index reported in previous studies (Kasumyan AO, 1997; Kasumyan AO and Døving KB, 2003). Weight gain (WG), specific growth rate (SGR), food conversion rate (FCR) and survival rate (SR) were calculated according to the following formulas:

WG (%) = (final weight − initial weight) × 100⁄initial weight;

SGR (%⁄day) = {Ln (final weight) – Ln (initial weight)⁄days} × 100;

FCR= live weight gain (g)/feed administered (g);

SR (%) = final number of fish/initial number of fish × 100 (Steffens W., 1989).

Palatability of the feeds was calculated according to the formula: ((ingested feed/administered feed) × 100) based on the index reported in previous studies (Kasumyan AO, 1997; Kasumyan AO and Døving KB, 2003).

Picture 16 – Capture of rainbow trout at the end of the trial.

5.2.2. Experimental diets

Before starting the trial, the hydrolized guar meal was obtained by Panghea (Milan) and analysed in terms of proximate composition, amino acid profile and fatty acids content (Table 11). Then, a growing feed, available in the trout farm and characterized by a protein level of 43% and a lipid content of 25.3% as it was, was considered as Control diet (CD). It was compared with two experimental feeds (D5; D15) that were manufactured according to two different formulations: D5 included guar meal at 5% of replacement of fish meal (1%), chicken meal (0.81%) and soybean meal (3.19%); D15 included guar meal at 15% instead of fish meal (4%), chicken meal (5.71%) and soybean meal (5.39%).

In a productive feed mill plant, one line of extruders dedicated to the preparation of the experimental diets was employed. All the ingredients were mixed according to the target

formulation. The feeds were 4.5 mm in size. After the coating, the three diets were stocked in buckets and maintained in an aerated room. Samples of each diet were taken for proximate composition analysis. All the analyses of feeds were performed (Table 12) according to procedures recognized at international level and used in other studies (Fiordelmondo et al., 2022). The feeds were transported to the trout farm and subsequently the growth trial was performed.

Table 11 - Proximate composition (%) and amino acid profile (g/100 g) of guar meal used in the experiment.

Proximate Composition (% as it was)	
Moisture	6.0
Crude protein	66.0
Crude lipid	10.0
Fibre	6.0
Ash	4.0
Fatty acid content	
Linolenic Acid	39.34%
Oleic Acid	29.98%
Palmitic Acid	15.34%
Stearic Acid	6.93%
Linolenic Acid	2.95%
Aminoacids profile (% as it was):	
Aspartic acid	6.51%
Glutamic acid	13.1%
Alanine	2.30%
Arginine	9.23%
Phenylalanine	2.61%
Glycine	3.34%
Isoleucine	1.92%
Histidine	1.71%
Leucine	3.56%
Lysine	3.30%
Proline	2.35%

Tyrosine	2.00%
Threonine	1.94%
Cysteine	0.75%
Valine	2.22%
Tryptophan	1.00%
Methionine	0.70%

Table 12 - Formulation and proximate composition of CD, D5 and D15 diets.

	CD	**D5**	**D15**
Fish meal	17.5	16.5	13.5
Chicken meal	16.81	16	11.1
Soybean meal	13.99	10.8	8.6
Guar meal hydrolized	0	5	15
Haemoglobin	12.6	12.6	12.6
Wheat meal	12.53	12.53	12.53
Wheat distillers	3.2	3.2	3.2
Wheat gluten	2.5	2.5	2.5
Soybean lecithin	0.5	0.5	0.5
Fish oil	10	10	10
Soybean oil	8.5	8.5	8.6
L-Lysine	0.3	0.3	0.3
DL-Methionine	0.22	0.22	0.22
Mineral-vitamin premix	1	1	1
Choline liquid 75%	0.35	0.35	0.35
Proximate composition:			
Moisture	7.75	7.5	7.12
Crude Protein	43.04	43.5	43.01
Crude fat	25.31	25.01	25.01
Fibre	1.4	1.5	2.01
Ash	8.66	8.7	7.74

5.2.3. Fish histological analysis

To investigate hypothetical differences in the intestinal structure among the considered groups at the end of the experiment, the tract of the proximal intestine of 10-15 rainbow trout per diet was sampled (Picture 17), fixed in 10% buffered formalin, and then processed for histological examination. Tissues were serially sectioned at 4 μm using a rotary microtome (Leica RM2235, Leica Microsystems, Wetzlar, Germany). Histological sections were stained with haematoxylin and eosin (HE) and evaluated under a light microscope in a blinded fashion. An optical microscope (Leica DM 2500, Leica Microsystems Srl, Buccinasco, Italy) equipped with a camera (Leica DFC 7000T, Leica Microsystems Srl, Buccinasco, Italy) was used to acquire images. Every section was entirely analysed at low (4-10×) and medium (20×) magnification considering the following pathologic traits, namely: mucinous hyperplasia; inflammatory change, shown by the presence of infiltration characterized by macrophages and lymphocytes; presence of IEL (intraepithelial lymphocytes); steatosis, in order to show lipid degeneration of enterocytes. For each of these parameters has been assigned a score related to the level of severity of modification: 0 = not observed; 1 = mild and/or focal; 2 = moderate and/or multifocal; 3 = severe and/or diffuse.

Picture 17 – Sampling of a tract of the proximal intestine.

5.2.4. Statistical analysis

Data collected (biometric parameters, final productive traits, fish fillet proximate composition, fatty acids categories) were subjected to one-way analysis of variance (ANOVA) using SPSS 25 (IBM Corp. IBM SPSS, 2017) to check differences in productive performances and composition of rainbow trout fillet fed with different experimental diets. Means and standard deviations were calculated. Means were considered significant with a value of $P < 0.05$ and compared using the Student-Newman-Keuls (SNK) test.

Histological data were analysed using GraphPad Prism 9 software (GraphPad Software Inc., La Jolla, CA, USA). Data are expressed as median values by using box and whisker plots. For the overall histological score and for each single histological parameter, a Kruskal-Wallis test followed by a Dunn's multiple comparisons test was used to analyse differences among the three groups.

5.3. Results

The water physicochemical parameters had a very similar trend in terms of temperature, dissolved oxygen and pH during all the time of the trial. The nitrogen compounds showed significantly differences in TAN having the highest ammonia concentration in D15 tanks (0.44 ± 0.01 mg/L) respect to CD (0.24 ± 0.09 mg/L) and D5 (0.22 ± 0.06 mg/L). Nitrites ranged between 0.01 ± 0.001 mg/L (D15) and 0.02 ± 0.002 mg/L (CD). Nitrates varied between 0.9 ± 0.1 mg/L (D15) and 1.1 ± 0.4 mg/L (CD) without significant differences from the statistical point of view (Table 13).

The productive parameters are reported in Table 14. The final mean weight of trout receiving the three diets showed results similar between D5 (201.00 ± 3.7 g) and CD (198.8 ± 3.8 g),

both significantly different in comparison with D15 (171.2 ± 10.1 g) that had the lowest mean weight. The same trend of difference was observed considering WG and SGR. FCR had the most convenient performance in D5 (1.18 ± 0.01) and in CD (1.15 ± 0.02) respect to D15 (1.56 ± 0.17). SR ranged between 98.07% (D5) and 97.3% (CD) without notable differences. Feed palatability resulted very high in CD and D5, differently from D15 that was slightly lower than the other two diets.

Histology of the proximal intestine of the three groups is presented in Figure 12, 13 and 14. Figure 2 shows the histology of the proximate intestine whit the guar ingestion, instead Figure 3 shows the normal histology of the proximate intestine obtained from rainbow trout fed with the control diet (CD). The results of the histological scoring performed on proximal intestine are reported in the Table 15. The overall histological score showed statistically significant differences between the three groups (Figures 15) with higher values in the CD group and lower values in D15 group. Regarding the single histological parameters considered, statistically significant differences were observed among groups for goblet cells hyperplasia (Figures 12,13,14) with higher values in the CD group and lower values in D15 group.

Table 13 – Main physicochemical parameters of water sampled in the three groups of tanks used for the trial.

	CD	D5	D15
Temperature °C	13.5 ± 0.4	13.5 ± 0.6	13.5 ± 0.7
Dissolved oxygen	8.7 ± 0.5	8.7 ± 0.6	8.7 ± 0.4
pH	7.86 ± 0.4	7.82 ± 0.6	7.84 ± 0.5
TAN	0.24 ± 0.09 a	0.22 ± 0.06 a	0.44 ± 0.01 b
NO_2-N	0.02 ± 0.002	0.02 ± 0.01	0.01 ± 0.001
NO_3-N	1.1 ± 0.4	1 ± 0.2	0.9 ± 0.1

Different letters (a, b, c) on the same line show statistically significant differences ($P < 0.05$).

Table 14 - Productive performances of rainbow trout fed with different experimental diets (mean ± standard deviation).

Parameters	CD	D5	D15
Initial mean weight (g)	50.0 ± 1.4 a	50.0 ± 1.4 a	50.0 ± 1.4 a
Final mean weight (g)	198.8 ± 3.8 a	201.00 ± 3.7 a	171.2 ± 5.1 b
Final mean length (cm)	23.2 ± 3.5 a	25.1 ± 0.9 a	23.75 ± 2.1 a
WG (g)	148.8 ± 26 a	151 ± 24 a	121.2 ± 39 a
SGR (%/day)	1.65 ± 0.18 a	1.68 ± 0.11 a	1.35 ± 0.3 a
FCR	1.15 ± 0.02 b	1.18 ± 0.01 b	1.56 ± 0.17 a
SR (%)	97.33 ± 2.89 a	98.07 ± 1.29 a	98 ± 1.7 a
Palatability	100 a	100 a	98 a

Different letters (a, b) on the same line show statistically significant differences ($P<0.05$).

Table 15 – Results of histological samples of the proximal intestine tract and differences (0 = none, 1 = mild and/or focal, 2 = moderate and/or multifocal, 3 = severe and/or diffuse).

	Mucous cells hyperplasia	Inflammation	IEL	Steatosis	**Final score**
CD					
1	2	1	0	0	**3**
2	2	1	0	0	**3**
3	3	1	0	0	**4**
4	3	1	0	0	**4**
5	1	1	0	0	**2**
6	3	1	0	0	**4**
7	2	1	0	0	**3**
8	2	1	0	0	**3**
9	3	1	0	0	**4**
10	3	1	0	0	**4**
D5					
1	0	1	0	0	**1**
2	1	1	0	0	**2**
3	0	0	0	0	**0**
4	1	0	0	0	**1**
5	1	1	0	0	**2**
6	0	1	0	0	**1**
7	2	1	0	0	**3**
8	2	1	0	0	**3**
9	2	1	0	0	**3**
10	2	1	0	0	**3**
11	1	1	0	0	**2**
12	1	1	0	0	**2**
13	1	1	0	0	**2**
14	3	1	0	0	**4**
15	1	1	0	0	**2**
D15					
1	0	0	0	0	**0**
2	1	1	0	0	**2**
3	1	1	0	0	**2**
4	0	2	0	0	**2**
5	1	0	0	0	**1**
6	1	1	0	0	**2**
7	1	1	0	0	**2**
8	0	0	0	0	**0**
9	0	0	0	0	**0**
10	0	0	0	0	**0**
11	0	0	0	0	**0**
12	0	0	0	0	**0**
13	0	0	0	0	**0**
14	0	1	0	0	**1**
15	0	1	0	0	**1**

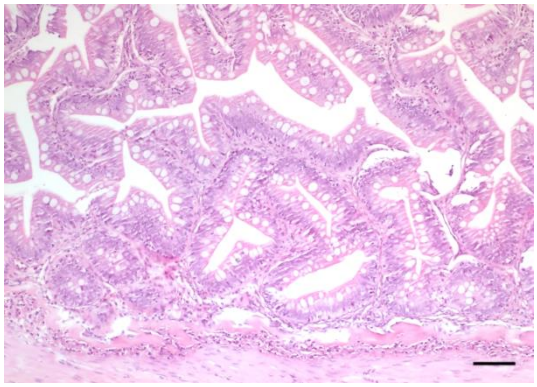


Figure 12 - Proximal intestine (CD group) whit the guar ingestion. Diffusely within crypts and villi enterocytes are hypertrophic and numerous goblet cells are present. (HE, bar: 100 micron).

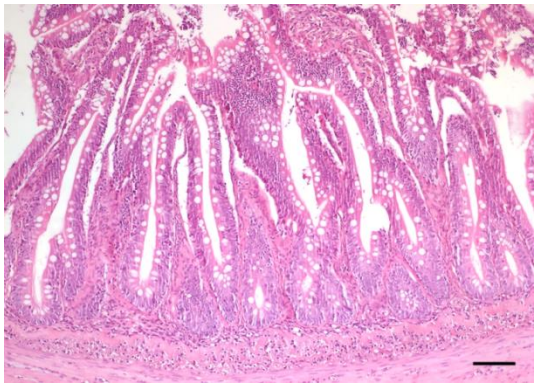


Figure 13 - Proximal intestine (D5 group). Intestinal mucosa with focal mild globet cells hyperplasia. (HE, bar: 100 micron).

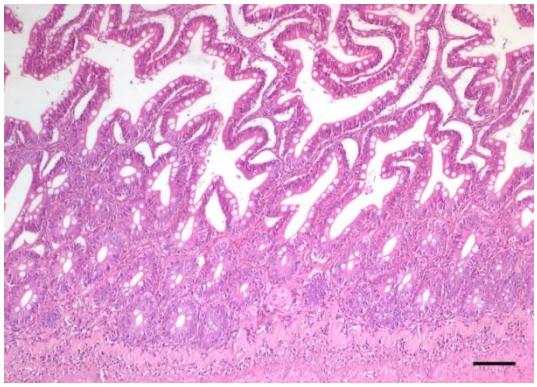


Figure 14 - Proximal intestine (D15 group). Normal mucosa. (HE, bar: 100 micron).

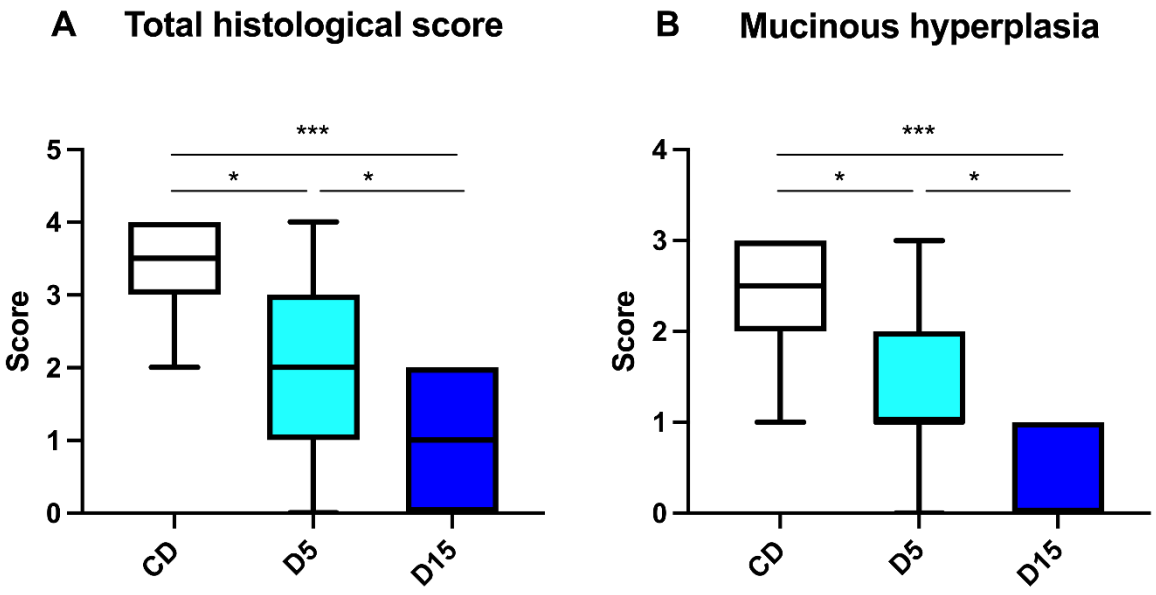


Figure 15 - Histological analysis. Schematic representation of (A) the overall histological score in the CD group, D5 group and D15 group; (B) mucous cells hyperplasia in the CD group, D5 group and D15 group. *P < 0.05; **P < 0.01; ***P < 0.001.

5.4. Discussion

In this trial hydrolized guar meal, a very common feedstuff in aquafeeds in the feeding of rainbow trout during the grow-out phase at international level, was essayed with the aim to get dietary ingredient with a very high protein content able to replace high proportions of protein source, not only represented by fish meal, but also others of vegetal and animal origin, such as soybean meal and chicken meal. If the first two feedstuffs (fish meal and soybean meal) represent the most important and traditionally feedstuff used as protein supplement (Kaushik et al., 2022), the chicken meal has been recently considered for its good source of essential aminoacids and wide availability (Seong et al., 2015). In the current study, the guar meal employed had a very high protein content (66%) indeed higher than those reported in literature in the past years (Vohra P and Keatzer FH, 1963.), when the guar meal ranged around 45% of protein. Moreover, a good aminoacids profile is now available in guar meal enough to cover the requirements of fish as reported in a study showed the successful effect of dietary guar meal at different rates and total replacement of soybean meal in feed for rainbow trout (Yadollahi et al., 2018). This property is one of the targets of sustainability in feed formulation.

In the current trial, the highest substitution of these three ingredients (fish meal, soybean meal and chicken meal) with guar meal reduced the final zootechnical performances as shown by the final mean body weight lower than trout receiving the other experimental feed and the fish receiving the conventional one. The D15 thesis showed the most unfavourable FCR and the relative tanks showed TAN levels higher than the other two groups that compromised the farming water quality. The presence of faeces on the bottom was observed in the tanks of this group and the daily cleaning activities were essential because of the grids clogged by faeces (Picture 18) respect to D5 and CD groups that appeared free. The cause of more dense and more solid composition of fish faeces is a consequence to use guar meal has been also found

by other authors (Brinker A and Friedrich C, 2012; Schumann et al., 2018), who had observed a considerable increase of suspended solids in fish farm waters at the increase of guar meal in feed. In the present study, the guar meal employed had been submitted to a fermentation process that should have avoided the inconvenience of inadequate water quality and the FCR less favourable in the diet with the highest guar meal inclusion. Other researchers (Brinker A and Fredrich C, 2012) focused the attention on the effects of the use of dietary guar meal on the mechanical quality of resulting faecal wastes in recirculating aquaculture systems, observed negative effects of fine solid particles among which clogged biofilters, increase oxygen demand and ammonia production. The authors underlined that this situation could favour the onset of pathogens and directly cause fish diseases. The same result was observed by Yadollahi and colleagues (2018) in replacing soybean meal with guar meal in rainbow trout. In this trial authors observed the lowest level of the fish condition factor in fish fed with 100 of protein replacement respect to the fish that received other diets. The minimum survival rate was observed in fish fed with the 75% of substitution, and the eviscerated fat of fish in the groups that received the 75% and the 100% of substitution were significantly higher than animals submitted to other diets.

In rohu fish, carcass composition analysis revealed the highest moisture and the lowest crude protein, fat and gross energy levels in fish from 90% of replacement; on the other hand the lowest moisture level with the guar inclusion and the highest crude protein, fat and energy levels were observed in fish from 30% of replacement (Barlaya et al., 2021). In juvenile largemouth bass, a recent study (Liu et al., 2022) ascertained that guar gum diet adversely affected intestinal morphology, decreased intestinal digestive and absorptive enzyme activities, besides caused poor nutrient digestibility and low growth performance.

Regarding the present study no pathological changes in the gut histology were observed in fish receiving the guar meal on the contrary fish fed with conventional diet had goblet cells

hyperplasia, a condition that may be induced in response to pathogens (particularly helminths), oxidants and toxins. (Dezfuli et al., 2016). Fish receiving the experimental diet showed a better intestinal health status compared to control.

Picture 18 - Grids clogged by faeces in the tanks in which rainbow trout were fed with D15.

5.5. Conclusion

The present study confirmed that hydrolized guar meal can be used in the feed formulation for rainbow trout in the growing phase. The inclusion of this feedstuff was satisfactory at a moderate rate, mainly 5% of the main protein sources. Considering the most important zootechnical performances, no difference was observed in comparison with the conventional diet. However, if the cost of feedstuffs is evaluated, the 5% replacement was less convenient (92.36 euro/100 kg) respect to the other experimental diet (92 euro/100 kg) although more affordable than the conventional one (93.98 euro/100 kg). In this situation, fish farmers have

to pay attention and evaluate every decision to take in relation to the productive performances and the cost of production of rainbow trout size-portion.

Considering the zootechnical results obtained with the 5% of guar gum inclusion, this trial represents a challenge for other studies to perform trials with intermediate guar inclusion, hypothetically between 5% and 15%.

6. USE OF HEMP IN THE FEEDING OF THE MAIN FISH SPECIES

Characterization and use of hemp in the feeding of the main animal species of zootechnical interest. State of the art.

6.1. Aim of the study

In order to evaluate the possible use of hemp (*Cannabis sativa L.*) in the feeding of the rainbow trout species (*Oncorhynchus mykiss*), a survey in the current literature was conducted to know the state of the art concerning the nutritional characteristics of hemp and the use of this plant in the feeding of the main animal species of zootechnical interest**.**

6.2. Introduction

In a world scale the food production industries are seeing an increase in the demand of food from different origin (animals, vegetable, protein, vegan etc.) (Clark et al., 2019; Daszkiewicz T, 2022). The aquaculture sector is growing very fast and constantly to respond to the increasing market demand (Føre et al., 2018; Sumaila et al., 2022). To support such a constantly growing trend, and to guarantee the sustainability of the entire supply chain, it is essential to identify new and sustainable raw materials to be used in the formulation of fish feeding. For this purpose, in recent years scientific researchers focused the attention on innovative feedstuff to guarantee at the same time the welfare of farmed animals, the high quality of the final product obtained, and a suitable value of feed conversion index according to the considered animal species. Among the most recent raw materials tested in the feed formulation for livestock production, hemp (*Cannabis sativa L.*) was taken into consideration as a partial substitute of the oily fraction and also as an alternative protein source.

In fact, considering the profile of essential amino acids and polyunsaturated fatty acids (PUFA) that the plant show and their importance for fish growth and fish health, in recent years it was hypothesized the potential use of hemp by-products in the feed formulation for aquatic organisms. The inclusion of whole hemp seeds or by-products obtained from their processing in the diets of farming animals could be correlated to the transfer of bioactive substances and nutrients to humans through foods from animal origins reared using hemp as feedstuff in animal feeding. This hypothetical correlation represents a challenge for other studies.

6.3. The plant *Cannabis sativa L.*

As marijuana, hemp derives from the *Cannabis sativa L*. plant, with the difference that hemp contains less than 0.3% of the psychoactive component of the plant (tetrahydrocannabinol or THC). Hemp is an annual herbaceous plant used in the past as a raw material for food, fibre and medicines. Due to its use for both medical and food purposes, there is a growing demand for hemp seed oil, whose production gives large volumes of by-products, such as hemp seed husks and hemp flour. The hemp oil obtained from the seeds of *C. sativa L.* is nowadays largely used in cosmetics, pharmaceuticals, and also in various human food products. When the oil is extracted from hemp seeds with chemical or mechanical methods, a dough with a high protein content remains; from this by-products an extracted flour can be obtained. For this reason, in recent years hemp saw an increasing interest as a potential source of functional food ingredients and nutraceuticals in the feeding of animals of zootechnical interest. The use of hemp by-products in livestock and aquaculture feeding could be possible from the circular economy perspective. However, other aspects should also be taken into consideration. First of all, the fact that hemp seeds contain anti-nutritional compounds, in particular the phytic acid

which could reduce the absorption of proteins, minerals and vitamins and other micronutrients. Therefore, it is fundamental making feeds well balanced in all the nutrients and microelements needed according to the animal species taken into consideration. This is fundamental for maintaining the efficiency of the metabolic processes that support growth, development, and health of animal organisms.

6.4. Features and nutritional values of hemp

Nutritional characteristics, amino acid profile and lipid profile of products derived from hemp are strictly related to the following conditions: the characteristic of the habitat in which plant grow, the variety of seeds considered, its geographical origin, conditions of the treatment process, and finally to the method of conservation of the final products. Anyway, as showed by Banskota and colleagues (Banskota et al., 2022), the most determining factor in the variability of the composition of the hemp by-products is related to the part of the plant considered (Occhiuto et al., 2022). According to Banskota and colleagues, the crude protein content is about 30.4% in hemp flour, 8.6% in hemp seed walls, 31.6% in the germ portion of the seeds. Considering the common alternative protein sources in animal feeding, and compared them with hemp, hemp seeds show an intermediate value of crude protein, exactly between the soy meal (39.2 ± 5.4%) and sunflower seeds (19.2 ± 4.2%) (Bailoni et al., 2022). Table 14 shows the composition of different hemp products. Table 15 reports the differences in the amino acid profile in different part of hemp plant (Bailoni et al., 2022).

Table 14 – Proximate composition expressed in g/100g (mean ± St.Dev.) (Banskota et al., 2022).

Parameter	Heart of hemp seeds	Whole hemp seeds	Flour of hemp seeds	Flour of hemp seed walls
Moisture	5.1 ± 0.1	3.1 ± 0.0	8.2 ± 0.1	6.7 ± 0.0
Ash	5.3 ± 0.5	4.5 ± 0.2	6.1 ± 0.2	2.4 ± 0.0
Crude Protein	31.6 ± 0.2	27.1 ± 0.2	30.4 ± 0.5	8.6 ± 0.1
Total Lipid	154.7 ± 2.3	48.0 ± 2.8	13.1 ± 0.3	17.5 ± 0.1

Table 15 - Amino acid profile in different part of hemp expressed in g/100g (mean ± St.Dev.) (Bailoni et al., 2022).

Aminoacid	Heart of hemp seeds	Whole hemp seeds	Flour of hemp seeds	Flour of hemp seed walls
Histidine (His)	23.7 ± 1.5	25.0 ± 2.8	23.7 ± 2.1	18.0 ± 0.0
Serine (Ser)	12.3 ± 3.5	10.0 ± 0.0	13.7 ± 3.8	8.0 ± 0.0
Arginine (Arg)	112.0 ± 6.6	115.0 ± 8.5	109.0 ± 7.9	81.3 ± 1.2
Glycine (Gly)	37.7 ± 2.5	39.5 ± 3.5	38.0 ± 2.6	29.7 ± 0.6
Aspartate (Asp)	98.0 ± 5.6	95.5 ± 4.9	88.7 ± 1.2	2 72.3 ± 0.6
Glutamate (Glu)	156.0 ± 9.2	159.0 ± 4.2	146.7 ± 5.5	117.0 ± 0.0
Threonine (Thr)	16.3 ± 2.1	15.0 ± 0.0	17.7 ± 2.9	12.0 ± 0.0
Alanine (Ala)	37.7 ± 2.5	38.0 ± 1.4	34.7 ± 1.2	28.0 ± 0.0
Proline (Pro)	32.3 ± 2.1	33.5 ± 0.7	32.0 ± 1.7	25.0 ± 0.0
Cysteine (Cys)	-	-	1.0 ± 1.0	-
Lysine (Lys)	26.3 ± 2.1	24.0 ± 2.8	22.7 ± 1.2	19.3 ± 0.6

Tyrosine (Tyr)	22.0 ±3.5	24.0 ± 2.8	18.0 ± 3.5	17.0 ± 1.0
Methionine (Met)	24.0 ± 3.6	27.0 ± 0.0	19.0 ± 6.1	17.7 ± 2.9
Valine (Val)	51.3 ± 3.1	53.5 ± 0.7	48.7 ± 2.1	39.0 ± 0.0
Isoleucine (Ile)	42.3 ± 3.1	43.5 ± 0.7	40.0 ± 1.7	32.0 ± 0.0
Leucine (Leu)	61.3 ± 4.0	63.5 ± 0.7	58.0 ± 2.6	46.0 ± 0.0
Phenylalanine (Phe)	43.3 ± 3.1	44.5 ± 6.4	42.0 ± 4.4	32.3 ± 0.6
Total Aminoacid (mg/g)	796.7 ± 48.0	810.5 ± 13.4	753.3 ± 30.6	594.7 ± 1.5
Crude Protein	85.1 ± 0.2	86.4 ± 0.1	87.8 ± 0.3	77.0 ± 0.4

6.5. Characteristics of hemp oil

Speaking about the lipid profile of hemp oil, the palmitic acid and the stearic acid are the main fatty acids, with respectively an average of 65% and 24% of the total lipid content. Polyunsaturated fatty acids (PUFA) represent about 75% of the total lipids, with a prevalence of linoleic and alpha-linoleic acids. The amount of saturated fatty acids compared to total lipids are different, depending on the different products, from 8.2% to 14.5% in whole hemp seeds, from 7.7% to 13.% in hemp flour, and from 7% to 11.6% in hemp oil. Hemp oil is also rich in antioxidants, essential fatty acids and vitamins (Leizer C, 2000).

6.6. Use of hemp seeds flour in fish feeding

In literature studies on the characterization of lipids and proteins extracted from hemp by-products showed a possible application of these products in fish feeding due for its digestibility. In particular in salmonids the digestibility of proteins extracted from hemp was recorded between 83%, as the minimum value, and 95%, as the maximum value. With in-

vitro experiments it was shown that the digestibility of proteins extracted from hemp was strongly influenced by the extraction process (Bailoni et al., 2022). On the other hand, with an in-vivo trial on the Nile Tilapia species it was demonstrated that the replacement of 10% of soybean oil with hemp oil and the total replacement of soybean oil with industrial hemp oil induced an increase of the metabolism velocity in the animals fed with the experimental diets compared to the control (Saoud et al., 2017). The results showed in this trial (Saoud et al., 2017) were related to the effects of cannabinoids on the fish body. The increase of the metabolism velocity caused a significant reduction in the final weight, which decreased from 49.96 g in the control group, fed with soybean oil and any hemp oil, to 43.19 g in the group fed with 10% of hemp inclusion, and to 40.26 g in the group of fish that received only hemp oil. Also, the food conversion index was significantly increased, from 1.8 in the control group to 2.1 in the fish fed with 10% of hemp inclusion, up to 2.3 in the group of fish fed with 100% of hemp inclusion. Survival index, final length, and blood parameters did not show significant differences between the experimental and the control groups.

6.7. Use of hemp seeds flour in ruminants

The inclusion of hempseed meal as a source of crude protein was studied in ruminants by Banskota and colleagues (Banskota et al., 2022). Their study showed that the fat and the protein inclusion in dairy milk decreased with the parallel increase of the hemp inclusion in the diet. Furthermore, a significant linear increase of urea in milk was correlated to the increase of the quantity of raw protein feed intake. Other trials were conducted on the effects of the inclusion of whole hemp seeds in dairy cow forage (Jacobson et al., 2021). The results showed an increase of omega-3 fatty acids and linoleic acid, well known as beneficial substances for human health, into milk and milk-derived products. Finally, an in-vivo

evaluation of the digestibility of crude proteins added to the forage for dairy cows gave the result of 87.8% as a mean value, with a minimum value of 88% and a maximum value of 98%.

6.8. Final consideration

Nowadays the small number of publications in the literature concerning the use of hemp in animal feeding does not allow to indicate or suggest an ideal dosage of hemp or its by-products to be used in the inclusion of animal feed. However, no adverse effects related to any anti-nutritional factors were observed. Furthermore, any information is actually published in literature relating to differences in the use of the whole plant or just its fractions in the feeding of the main animal species of zootechnical interest. In particular, with specific reference to fish species, the limited numbers of trials published in literature on aquatic species should be considered only preliminary, and so it represents a challenge for other studies.

7. FISH BY-PRODUCTS

7.1. Use of fish by-products as raw material to be used in animal feeding

In the last few years, scientific researchers gave attention on the study of innovative raw materials to be used in animal feeding, especially for protein substitutes. The attention to this issue came from the observation that food supply chains have significant environmental impacts in terms of emissions and waste produced. According to the European Directive 98 of 2008, the term “waste” means all substances that producers do not use anymore in the production chain. Researchers started to indicate that organic material whit the term of "co-products" (Newton et al., 2014). In particular speaking about rainbow trout farming, it must be considered that the main product obtained from trout is the fillet, through the filleting process, which produces a significant amount of waste. However, these “wastes” are still composed by quality organic material and therefore should be re-evaluated as noble ingredients (Gehring et al., 2011; de la Caba et al., 2019). Therefore, the term “wastes” was substituted with the term of “residues”, or better “by-products”, to indicate all those fish parts which are not used for the primary production and which represent from 20 to 80% of the starting raw material (Islam et al., 2021). FAO declares in 2018 that over 20 million tons of by-products are generated from fish processing (FAO, 2022).

Fish by-products are represented by all the fishing waste (Stevens et al., 2018) and the parts derived from the fillet processing, therefore fish head, viscera, skin, fins and blood. Each fish portion has different nutritional characteristics (Gehring et al., 2011). In rainbow trout, fish head is particularly rich in saturated fatty acids, which are also abundant in fish skin and fins, while polyunsaturated fatty acid are more present in viscera and in trimming. Even in gilthead seabream viscera, and in particular the liver, is the fish portion richest of fat (Pateiro et al., 2020).

Considering their composition and the quality of fish by-products components, fish by-products could be processed to make animal feed (Gasco et al., 2020), but it is important to take in mind that they are composed by fresh material, that it is highly perishable. Therefore to avoid the multiplication of microorganisms fish by-products must be processed immediately. They are subjected to grinding and cooking, and then a centrifugation allows to separate the solid part, called fish paste, to the liquid component, represented by raw fish oil.

Observing proteins values, in both species, rainbow trout and sea bream, a higher protein content is present in skin and head compared to fillet; this finding can be explained considering that during the fish threading portions of muscle remain adhered to the fish skin. The lipid fraction can be used directly in fish feed as raw fish oil (Goosen et al., 2014) because it is rich in polyunsaturated fatty acids of the omega 3 series, of which the waste from the processing of sardines, mackerel and cod are particularly rich. In this way it is therefore possible to produce sustainably fish oil avoiding the use of fresh fish (Zeller et al., 2017). For its quality that lipid fraction could also be used to produce pet food and food supplements. Concerning the fish paste, similarly to raw fish oil it can be used in the formulation of pet food or subjected to various bioprocessing methods to isolate substances such as: minerals (calcium, potassium, magnesium, sodium, phosphorus, zinc, iron), vitamins (especially vitamin A), chitin and collagen, molecules of interest of the pharmaceutical industry (Gasco et al., 2020).

Since the amino acid profile is still well balanced and the protein content is high, fish by-products could be subjected to a protein hydrolysis process to obtain protein hydrolysates. This means that from by-products it is possible to produce fish meal intended for feeding of aquatic organisms in a sustainable way, avoiding the conventional method which instead involves the use of fresh raw materials, taking advantages of the high quality protein content of fish by-products.

In this context, a study was performed to investigate the possible use of fish proteins hydrolysed (FPH) derived by rainbow trout processing as a protein source in the diet of gilthead sea bream juveniles in the pre-growing phase. FPH seems to have beneficial effects on the non-specific fish immune response and on the stimulation of intestinal enzyme activity (Bui et al., 2014; Kotzamanis et al., 2007).

7.2. Effects of high substitution of conventional protein sources with Fish Proteins Hydrolysates by-products from rainbow trout processing, in feeding for gilthead sea bream in pre-growing phase

7.2.1. Introduction

Food supply chains have significant environmental impacts due to the use of resources and the production of emissions, effluents and waste. The importance of food waste stretches from environmental pressures to economic and social impacts, including negative effects on food and nutrition security (Otles et al., 2015). According to the circular economy approach, to meet the international aim to increase the sustainability of production chains, waste prevention and minimization is the main priority, followed by reuse, recycling and energy recovery. In this context, waste from food industries should be revalorized leading to the production of proteins (Gasco et al., 2020) and other valuable compounds such as collagen and minerals (Nawaza et al., 2020). According to the European Union (EU) Commission Council Directive 2008/98/EC, “waste” is defined as “any substance or object which the holder discards or intends or is required to discard”. Fishing refuses should not be considered as waste but revalued and enhanced in order to minimize the final waste of food production. In fact, it represents a quality raw material that can be used as raw materials for other productions by a circular economy point of view. Nowadays the term "fish waste" is replaced

with more appropriate terms such as fish "by-products" come from fisheries and aquaculture. That term intends to mean fish or bycatch products of no commercial value or of little value (Felici et al., 2020; Gasco et al., 2020), portions of fish (head, fins, scales, skin, bones, viscera) or crustaceans (carapace, exoskeleton, shells etc.) not used in the process of the main production. Moreover, a by-product must have a market value and the final use should be integral without a negative impact on human health or environment. For example, seafood processing generates an amount of fish by-products between 20 and 60% of starting raw materials (Ferraro et al., 2010), that is a huge amount of organic material that should be reused. The management of fish by-products represents an attracting topic to discuss, due to the value compounds contained in that unused mass of organic material. Fish by-products should be a potential source of feed and bioactive compounds such as proteins firstly, oils rich in polyunsaturated fatty acids (PUFAs) secondary, and moreover, astaxanthin and chitin (Gasco et al., 2020). The fatty acid profile, amino acids profile, digestibility and palatability can vary significantly depending on the raw material employed (Ferraro et al., 2010; Gasco et al., 2020). In fact, each portion of the animal has different nutritional characteristics, as clearly Pateiro and colleagues showed in sea bream (Pateiro et al., 2020). In literature different studies focused the attention on the investigation of using fish by-products as an alternative protein source to traditional feedstuffs (Gasco et al., 2020). Furthermore, a part of fish by-products, vegetable proteins are another example of possible protein substitution (Fiordelmondo et al., 2020), even if their addiction in fish feeding could be restricted by the reduction of digestibility, scarce palatability, not balanced amino acid profile, and the presence of anti-nutritional factors (Parisi et al., 2020). Anyway, all these negative aspects can be reduced by feeding fish with an excellent balanced diet at the right percentage of substitution of alternative proteins, and using high quality of raw materials. In particular, for the feed formulation of carnivorous species the challenge is to identify alternatives protein

animal sources, and at the same time maintaining optimal levels of digestibility, palatability, amino acid profile and lipid profile (Parisi et al., 2020). In this context, the aim of the present study is to investigate the possible use of fish proteins hydrolysates (FPH) obtained from rainbow trout by-products as a protein source in gilthead sea bream (*Sparus aurata*) diet in the pre-growing phase.

7.2.2. Materials and Methods

7.2.2.1. Experimental design and fish employed

Three groups of 170 gilthead sea bream each, with initial mean body weight of 37.8 ± 0.5 g, were employed in triplicate indoor tanks of 2 m^3 of volume. During the experiment, in all the tanks water temperature was maintained at 20°C, salinity was 37 ppt, dissolved oxygen was at 6.8 mg/l and pH was 7.8. Water parameters were daily recorded in every tank using portable electronic devices (YSI mod. 55 and 60, Yellow Springs, OH, USA). During all the time of the trial, carried forward 85 days, fish were fed twice a day (8 a.m. and 3 p.m.) until the satiation level; then the unconsumed feed was collected. This experiment was performed during the pre-growing phase of a gilthead sea bream in a standard zootechnical cycle.

Specific Growth Rate, Feed Conversion Rate, Survival Rate, Palatability, Condition Index, and Perivisceral Fat Index (PFI) were calculated as follow:

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 (SR, %) = final number of fish/initial number of fish*100 (Steffens W, 1989);

Palatability (P) = (ingested feed/administered feed) x 100 (Kasumyan AO, 1997; Kasumyan AO and Døving KB, 2003);

Condition index (KI) = (fish weight fish/length3) x 100 (Bagenal TB and Tesch FW, 1978);

Perivisceral Fat Index (PFI) = (perivisceral fat/body weight) x 100; and

Hepatosomatic index (HSI) = (liver weight/body weight) x 100 (U.S. EPA, 2000).

7.2.2.2. Formulation and experimental diets

Three experimental diets were formulated to be isoproteic (47.1%) and isolipidic (16%). One group was fed with a diet including 354 g/kg of FPH (L1), one group with a diet including 177 g/kg of FPH (L2) and one group received the control FPH-free diet (LC). In this latter, the protein source was mainly provided by fish meal and soybean meal. FPH was obtained by rainbow trout by-products come from a rainbow trout slaughterhouse. LC represented the control diet, free from FPH and in which the protein fraction was mainly given by fish meal and soybean meal (Table 16). Proximate composition and amino acid content of the experimental diets are reported in Table 17. Feeds were manufactured at the feed mill of the same fish Company (Erede Rossi Silvio Trout Company) involved in the trial, that also acquired all the feedstuffs to be included in the feeding plan.

In the feed mill a dedicated line of extruder was used for the preparation of the experimental diets. All the ingredients were mixed according to the target formulation. The feeds were manufactured in 3.0 mm size using a twin-screw extruder (100 rpm, 110 °C, 50 atm). After coating, the feeds were stocked in buckets and kept in an aerated room. Samples of each diet were taken for proximate composition analysis. The feeds were transported to labs in Porto Conte Ricerche to perform the growth trial.

Table 16 - Feedstuffs used in the experimental diets (g/kg).

	LC	L1	L2
Fish meal	220	0	110
FPH	0	354	177
Soybean meal	185	185	185
Wheat meal	122	122	122
Gluten corn	100	100	100
Gluten wheat	80	80	80
Tuna meal by-products	80	0	40
	LC	**L1**	**L2**
Haemoglobin	54	0	27
Fish oil	80	80	80
Soybean oil	48	48	48
L-Lysine	5	5	5
Choline powder	4	4	4
DL-Methionine	2	2	2
Phosphate mono-calcium	10	10	10
Vitamin premix	10	10	10

Table 17 - Proximate composition and amino acid profile of the three experimental diets.

	LC	L1	L2
Moisture (%)	7.0 ± 0.3	6.9 ± 0.3	6.8 ± 0.3
Protein (%)	46.7 ± 1.3	47.1 ± 1.6	47.1 ± 1.4
Lipids (%)	16.5 ± 0.6	16.5 ± 0.6	16.2 ± 0.6
Fibre (%)	2.0 ± 0.6	2.0 ± 0.6	2.3 ± 0.4
Ash (%)	8.41 ± 0.27	9.41 ± 0.27	8.84 ± 0.46
Aminoacid profile (g/100 g):			
Arginine	2.37 ± 0.51	2.50 ± 0.4	2.44 ± 0.39
Aspartic acid	3.99 ± 0.58	3.81 ± 0.61	3.69 ± 0.59
Cysteine	0.69 ± 0.11	0.66 ± 0.1	0.69 ± 0.11
Glutamic acid	7.18 ± 1.13	7.24 ± 1.16	7.15 ± 1.14
Histidine	1.04 ± 0.16	1.14 ± 0.18	1.07 ± 0.17

Isoleucine	1.55 ± 0.26	1.54 ± 0.25	1.53 ± 0.24
Leucine	3.86 ± 0.49	3.90 ± 0.62	3.82 ± 0.61
Lysine	2.96 ± 0.4	2.72 ± 0.44	2.70 ± 0.43
Methyionine	0.82 ± 0.14	0.84 ± 0.13	0.85 ± 0.14
Phenylalanine	1.93 ± 0.33	2.12 ± 0.34	2.01 ± 0.32
Threonine	1.55 ± 0.24	1.60 ± 0.26	1.55 ± 0.25
Tryptophan	0.49 ± 0.07	0.49 ± 0.08	0.48 ± 0.05
Tyrosine	1.20 ± 0.22	1.29 ± 0.21	1.25 ± 0.2

7.2.2.3. Traits of FPH employed in the experimental diets

The mixture of rainbow trout by-products used in this experiment was obtained from a rainbow trout slaughterhouse located close to the fattening farm situ. All the rendered portions and any possible residue obtained by the filleting process were collected and transported to the feed mill. In this place, the mass of rainbow trout by-products was ground into a powder using a grinder and after a centrifugation phase the oily part and the solid part (fish paste) were separated. The solid part, rich in essential amino acids of high quality and short chain peptides, was used to made the diet included FPH. Then, FPH powder was added to the other ingredients at the inclusion of 354 g/kg in L1, and at 177 g/kg for L2. The obtained mixtures were extruded with an extruder machine using a module of 4.5 mm diameter and air dry for 3 days. Dried feed was sieved and packaged in bags and stored at – 20 °C until the feeding trial started.

7.2.2.4 Water quality analysis

During all the time of the experiment the main water physicochemical parameters (temperature, dissolved oxygen and pH) were daily recorded in every tank using portable

electronic devices (YSI mod. 55 and 60, yellow Springs, OH, USA). TAN, NO2-N and NO3-N were weekly analysed following APHA standard methods (APHA, 1995).

7.2.2.5. Histological analysis of fish

To investigate hypothetical differences in the hepatic structure among the considered groups, at the end of the experiment the liver of five gilthead sea bream per diet was sampled and fixed in 10% buffered formalin, then processed for histological examination. Tissues were serially sectioned at 4 μm using a rotary microtome (Leica RM2235, Leica Microsystems, Wetzlar, Germany). Histological sections were stained with hematoxylin and eosin (HE) and evaluated under a light microscope in a blinded fashion. An optical microscope (Leica DM 2500, Leica Microsystems Srl, Buccinasco, Italy) equipped with a camera (Leica DFC 7000T, Leica Microsystems Srl, Buccinasco, Italy) was used to acquire images. Every section was entirely analysed at low (4-10×) and medium (20×) magnification considering the following pathologic traits, namely circulatory changes, regressive change (presence of degeneration and/or necrosis of hepatocytes and/or bile ducts), progressive change (presence of hypertrophy and/or hyperplasia of hepatocytes and/or bile ducts, and infiltration of adipose cells in the peripancreatic fat), and inflammatory changes (i.e. presence of inflammatory cells). For each histopathologic change detected a score was assigned as follow: 0 (not observed), 1 (presence of 1 to 3 foci), 2 (presence of 4 to 10 foci); 3 (more than 10 foci) (Silva et al., 2015; Bonvini et al., 2018; Pacorig et al., 2022). The fish involved for the liver samples were obtained from the number of fish objected to the regular veterinary check of fish health and welfare in the facility of Porto Conte Ricerche.

7.2.2.6. Statistical data analysis

Data collected referred to zootechnical parameters (Specific Growth Rate, Feed Convertion Rate, Survival Rate, Palatability, k-index, Perivisceral Fat Index) were submitted to one-way

analysis of variance (ANOVA) using SPSS 25 (IBM 2017) to check differences in productive performances and composition of fillet of rainbow trout fed with different experimental diets. Means were considered significant with a value of $P < 0.05$ and compared using the Student-Newman-Keuls (SNK) test. Also, the histological analysis was considered and differences among diets for histopathological changes were analysed using a Kruskal–Wallis's test followed by Dunn's multiple comparison test. The level of significance was set at $P < 0.05$.

7.2.3. Results

Throughout the trial (85 days), in all the tanks water temperature was maintained at 20°C, salinity was 37‰, dissolved oxygen was at 6.8 mg/L and pH was 7.8.

At the end of the pre-growing trial, productive and zootechnical parameters were collected from the three groups. Similar performances were observed between L1, L2 and LC: the final mean weight ranged from 76.6 g to 78.0 g without significant differences; specific growth rate (SGR %, day-1) was from 1.26 to 1.31%; FCR (food conversion rate) was between 1.24 ± 0.04 and 1.26 ± 0.01; SR (survival rate) was high in all the groups with a percentage between 98.83% and 100%. All the three diets exhibited high palatability (P). Somatic indices did not show differences in terms of condition index (KI, 1.50-1.52); PFI (perivisceral fat somatic index, between 7.5 and 8.1) and HSI (hepato-somatic index, between 1.62 and 1.66) were slightly high in all the groups. Table 18 show the zootechnical parameters at the end of the trial.

Table 18 - Zootechnical parameters at the end of the trial as mean ± standard deviation.

	LC	L1	L2	P
Initial body weight (g)	37.8 ± 0.5a	37.8 ± 0.5a	37.8 ± 0.5a	-
Final body weight (g)	78.8 ± 12.2a	76.6 ± 11.2a	78.0 ± 12a	P > 0.05
Average weight gain (g)	41.10 ± 2.46a	39.13 ± 0.32a	39.50 ± 1.56a	P > 0.05
Weight gain (%)	108.91 ± 5.77a	104.64 ± 0.45a	102.93 ± 5.80a	P > 0.05
SGR (%)	1.31 ± 0.05a	1.28 ± 0.01a	1.26 ± 0.05a	P > 0.05
FCR	1.25 ± 0.03a	1.26 ± 0.01a	1.24 ± 0.04a	P > 0.05
Survival rate (%)	100 ± 0.0a	99.40 ± 1.01a	98.83 ± 2.02a	P > 0.05
Palatability	100 ± 0.0a	100 ± 0.0a	100 ± 0.0a	P > 0.05
KI	1.51 ± 0.01a	1.52 ± 0.01a	1.50 ± 0.02a	P > 0.05
PFI	7.5 ± 1.12a	8.0 ± 1.23a	8.1 ± 1.36a	P > 0.05
HSI	1.62 ± 0.24	1.63 ± 0.31	1.66 ± 0.41	P > 0.05

The same letter (a) shows no significant differences (P > 0.05) on the same line.

7.2.3.1. Histological investigations

The results of the histological scoring concerning the fat infiltration in the tissue performed on the livers of fish fed with L1, L2 and LC diet are reported in Table 19. In all the fish fed with different diets (L1, L2, LC) a diffuse cytoplasmic vacuolization of the hepatocytes was observed with nuclei frequently located at the periphery (Figure 16, 17, 18). In all the groups fish liver also showed the presence of adipocyte infiltrates associated with intrahepatic

pancreatic tissue. No statistical difference was observed among the three groups for both hepatocyte vacuolization and peripancreatic fat infiltration.

Table 19 - Histological scores of the livers of fish fed with L1, L2 and LC diet. The score of 0 represents the minimum level of fat infiltration in the hepatocytes, the score of 3 the maximum.

	Diets		
	L1	L2	LC
Hepatocyte vacuolization	3-3-3-3-3	3-3-3-3-3-	3-3-3-3-3
Peripancreatic fat infiltration	2-2-1-3-0	3-3-3-0-1	2-3-3-2-2

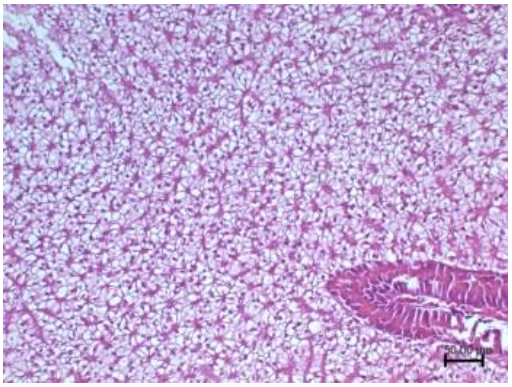


Figure 16 - Liver from fish fed with LC. Diffuse hepatocellular degeneration with intracytoplasmic lipid accumulation (HE).

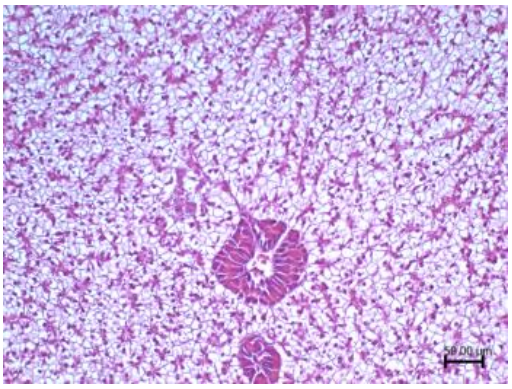


Figure 17 - Liver from fish fed with L1. Diffuse hepatocellular degeneration with intracytoplasmic lipid accumulation (HE).

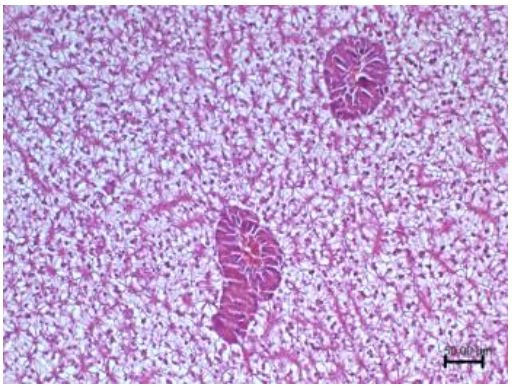


Figure 18 - Liver from fish fed with L2. Diffuse hepatocellular degeneration with intracytoplasmic lipid accumulation (HE).

7.2.4. Discussion

As reported in previous studies (Aletor VA and Onibi OE, 1990), fish by-products can be used for other productions in the feeding for aquatic organisms and poultry sector. An example is the reuse of not common sea snail species accidentally fished by fishermen, which should be re-evaluated as protein source in aquafeed (Felici et al., 2020; Fiordelmondo et al., 2020). Recent investigations have shown that sea snail shells can be employed to produce a powder particularly rich in calcium and carotenoids after fragmentation and drying, and that powder can be used as a food supplement in the feeding of laying hen (Proudfoot FG and Hulan HW, 1987; Houndonougbo et al., 2012; Alm et al., 2017). Similarly, fishery by-products can be used in the formulation of feeds for the main fish species of interest to aquaculture, as an organic material of excellent quality rich in proteins.

Fish meal can be replaced greater than 29.7% by enzymatically hydrolysed tuna backbone by-products in the diets of juvenile rainbow trout (Bae et al., 2019), or by a mixture of animal by-products (approximately 23%) in the growth of juvenile snapper *L. argentimaculatus* (Khalid et al., 2007).

In this context, the aim of the present study was to formulate a new diet that contained fish by-products from rainbow trout as FPH powder intended for gilthead sea bream in the pre-fattening phase. This study investigated the effects of the experimental diets characterised by complete or halved substitution of the fish meal with sustainable feed ingredient as hydrolysed proteins obtained by farmed rainbow trout processed. At the end of the trial, good productive results were obtained with similar performances between fish fed with and without the FPH substitution. The zootechnical and histological results did not show significant differences among the three groups; although the final mean weight was not very high in all the sea bream. The reason of these acceptable but not fully satisfactory

performances are presumably due to the moderate lipid content (16%) of the three diets that could also limit the negative effects of an extra deposition of lipids in gilthead sea bream liver.

According to liver histology, our results showed that FPH can be included in the feeding for gilthead sea bream in pre-growing phase without significant negative modification on the liver structure compared to fish fed with LC.

In the present trial the histological aspect of the liver of fish fed with the FPH inclusion (L1 and L2) did not significantly differ from one of fish fed with the control diet (LC). Our results are similar to the trial conducted by Bae and colleagues (Bae et al., 2019), who indicated the level of substitution of hydrolysed tuna by-product to replace fishmeal in juvenile rainbow trout diet.

Fish by-products are very heterogeneous and presents a highly variable composition, which depends on the species involved and the percentage of inclusion. Moreover, recent investigations have shown that organs and different part of the same fish species have a different proximate composition (Gasco et al., 2020; Munekata et al., 2020; Pateiro et al., 2020). In fact, as reported in literature, into fish skin proteins are present as 32% in yellow tuna (Gasco et al., 2020), 24.78% in gilthead seabream (Pateiro et al., 2020), 25.95% in seabass (Munekata et al., 2020). In salmon, the total lipids percentage is 22% in the skin, and 44% in the viscera (Gasco et al., 2020). Animal by-products show also other interesting applications. Concerning fish by-products, they are not only used as feedstuff in animal feed but they are also used to produce foods for human consumption (Palmeira et al., 2015; Amir RS, 2015). An example are snacks with an extra-protein intake conferred precisely by an integration with fish bone power (Nawaza et al., 2020).

7.2.5. Conclusion

The exploration of alternative sources and the utilization of by-products coming from the food industry are gaining more importance. Fish by-products (Picture 19) can be used in the formulation of feeds for the main fish species of interest in aquaculture as a good raw organic material. In this context, this study showed that FPH derived from rainbow trout as sources of alternative proteins is feasible in the feeding of gilthead sea bream (*Sparus aurata*) juveniles in the pre-growing phase.

Poster presentation. Preliminary data were presented as a poster presentation at the International Fish Nutrition Symposium in Sorrento in 2022.

Picture 19 – Fish and shrimps by-products

8. CIRCULAR ECONOMY

8.1 General introduction

For several years scientific researchers turned their attention on the study of innovative raw materials to be used in animal feeding. The attention on this issue came out from the observation that food supply chains have significant environmental impacts in terms of emissions and waste production. According to the European Directive n.98 of 2008 year, the term “waste” means substances not used anymore by producers in the production chain. In aquaculture, some researchers started to refer to these organic materials as "co-products" due to the fact that fishing waste should not be considered as waste material to discard but should be revaluated in order to minimize the final waste of the supply chain. In fact it represents a quality material that can be used as raw materials for another production. The circular economy is a model of production and consumption that reproduces a cyclical processes that normally occurs in nature yet, where waste from one species is reused by another one. The concept of circular economy is based on three aspects: reusing, recycling and energy saving. This process is applied avoiding a negative impact on human and animal health, and on the global environment. Moreover, the reuse in these terms of bio-waste and by-products is crucial for converting a low-value waste into new materials and products. Concerning aquaculture, this concept means that fish by-products and fishing waste should be considered as quality raw materials to be used in another process thank to the high biological value of their components. It is necessary to consider that seafood consumption is globally increasing, resulting in large amounts of waste, waste accumulation, and environmental pollution. Therefore management solutions are needed to administer this seafood waste as better as possible.

Considering the increase of the amount of fish waste produced in the world, recycling wastes has become a key issue, and from the circular economy point of view marine material can represent the starting point of productions of materials with high biological, commercial and economic value (Coppola et al., 2021). Fish processing by-products and wastes, including skins, heads, guts, bones, scales, and fins, are sources of several potentially valuable molecules, such as proteins and peptides, oils and lipids, vitamins, minerals, pigments, and enzymes (Shaidi et al., 2019). The definition of "fish waste" includes many fish species or by-catch products with no or low commercial value, viscera from fish processing occurred directly at sea, undersized fish species and non-target fish species (Mozumder et al., 2022). Following the principles of circular economy, the utilization of seafood waste to produce bioactive compounds and functional ingredients destined to food or feed industry is a sustainable way for their new utilization (Zao et al., 2022).

8.2. Sea snail as food: Evaluation of Long Sea Snail *Hinia reticulata* (Gastropod) from the Middle Adriatic Sea as a possible alternative for human consumption

8.2.1. Introduction

Along the coasts of the mid-Italian Adriatic Sea of Emilia Romagna, Marche, Abruzzo and Molise, the common sea snail (*Nassarius mutabilis*) is considered the most important target species among marine snails and is particularly appreciated for traditional gastronomic dishes, known as "tiny snail" ("bomboletto" or "chiocciolino" in Italian). Gastropods are considered a valuable source of precious nutrients as protein and essential amino acids. On the Mediterranean coasts, cases of the marine snails showed high rates of n-3 long-chain polyunsaturated fatty acids, essential in preventing disorders and cardiovascular disease (Zarai et al., 2011). Since the 1950s, the sea snail has been harvested by commercial fishery

using trawls and common cuttlefish traps. Currently, it is carried out using a basket trap called “nassino,” from the beginning of autumn to the end of spring. In relation to recent years, catch rates have decreased and an increase in the minimum landing size has been proposed (Balducci et a., 2006; Grati et al., 2010). At the same time, fishermen are recording an increase in the capture of another sea snail species, called the long sea snail or false sea snail (*Hinia reticulata*) that is considered to have no commercial value compared to the common sea snail and is thus thrown back into the sea. Both gastropods species have a similar biology, particularly living on the sandy-muddy bottoms (Fisher et al., 1987). An increase in the fishing efforts for the common sea snail (*Nassarius mutabilis*) and re-entry of the long sea snail (*Hinia reticulata*) have contributed to modifying the population stock of marine snails in different Adriatic districts (Felici et al., 2015). Up to now, the gastronomic use of the long sea snail has not been considered because meat fraction removing the shell is more difficult than with the common sea snail. All the traditional plates are only based on the common sea snail (Fabi et al, 2006). Besides, the long one was less abundant in captures until the last few years.

Since January 2019, a ban on discards (EC Reg. 1380/2013) in professional fishing has comeinto force and, therefore, the entire bulk of the capture operation, including non-target species, such as the specimens of the false snail, *Hinia reticulata*, must be landed (EC Reg. 1380/2013) (Fiori et al., 2008). In this situation, some fishing cooperatives have considered the possibility of employing the long sea snail species as a resource in terms of meat for human consumption (Hilborn et al., 2015) and shell composition in calcium content. Increasing interest in the fishery value chain and valorisation of seafood and fishery discards

has promoted research on related topics as well as sustainability and a circular economy (Jurgilevich et al., 2016; European Commission Regulation (EU) N. 142/2011). In addition to fishermen, other stakeholders or actors operating along the coasts are increasingly focusing

on discussing the sustainable management of seafood by-products and discards. For example, after the entrance in force of the Port State Measures (FAO 2016), besides the adoption of new measures to prevent illegal fishery, administrators of harbours are going to re-think the purpose of harbours in a sustainable manner. In the last few years, new models of fishery ports as infrastructure are going to be reconsidered from the environmental, economic, and social points of view (FAO 2018). At the international level, fishery harbours are moving towards a "green approach" with reduction of emissions and energy saving, and are equipped with areas dedicated to activities with a social role, such as education and training or cultural spaces, as well as processing of fish by-products (Kienker et al., 2018; Chen et al., 2019).

From the perspective of using the two (common and long) sea snail species for purposes different to food for human consumption such as recycling by-products from the shells or meat, characterization of their quality traits in terms of meat fraction and shell was considered in this paper. Moreover, being gastropods classified as bio-indicators in an aquatic environment (Phillips DJH, 1990), some elements were determined in the soft tissue of both species. Therefore, the edible fraction of the most appreciated common sea snail (*Nassarius mutabilis*) was evaluated in terms of proximate composition, fatty acid profile, mineral content, and heavy metals in comparison with the same traits in meat of the long sea snail (*Hinia reticulata*) species according to the catching sample. The recovery of value-added compounds, such as calcium carbonate from the shell, was also investigated in both species. The possibility of performing these processing activities in public re-designed infrastructures, such as in harbours equipped for the recovery of seafood by-products, was also proposed.

8.2.2. Materials and Methods

8.2.2.1. Samples and sampling areas

Sea snail samples belonging to the common (*N. mutabilis*) and long (*H. reticulata*) species were provided by fishermen catching within three miles from the seashore, along the coast of San Benedetto del Tronto at two different catching times in the first decades of November 2018 and March 2019. At each sampling, specimens of the two species of sea snail were transported on ice to the laboratory at the University of Camerino and were submitted to determination of total weight (meat + shell) and the fractions of meat and shell, after having extracted the edible part. All weight measurements were made using an electronic scale (mod. CP224S Sartorius, Gottigen, Germany). The shell yield on the total body weight was calculated (weight of shell in g/total weight of sea snail g x 100) in both groups. The samples were kept frozen and were stored at –18 °C until analysis.

8.2.2.2. Proximate composition

The edible part was removed from 50 specimens/species/sampling time to determine the proximate composition and fatty acid profile. Pools of each group were homogenised and subjected to proximate analysis (moisture, protein, lipid, and ash content). The moisture percentage was determined in duplicate following the procedure of the Association of Official Analytical Chemists (AOAC, 1990). Proteins were determined using the standard Kjeldahl copper catalyst method (AOAC, 1990). Ash was determined using the AOAC procedure (AOAC, 1990). Total lipid content was measured using a modification of the chloroform:methanol procedure described by Folch et al. in 1957.

8.2.2.3. Fatty acid profile determination

After determining the total lipid content of the two species of sea snails, fatty acids were converted to methyl esters following the method described by Christopherson and Glass in 1969. Separation of fatty acids methyl esters was carried out on an Agilent Technologies GC/MS (6890N)/ MSD (5973inert) system (Agilent, Palo Alto, CA, USA) equipped with a db5 column (60 m x 0.25 mm) and calibrated. The operating conditions of the gas chromatograph were as follows: oven temperature was kept at 170°C for 15 min, 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 temperature of the injector was 280°C. Helium was used as the carrier gas at a constant flow of 1.0 mL/min. The identification of individual fatty acids was accomplished by comparing the observed retention times to fatty methyl esters of standard mixtures (37 FAME Mix, Supelco) and NIST MASS SPECTRAL DATABASE (NIST MS SEARCH 2.3) for mass spectrum.

8.2.2.4. Determination of elements in meat and calcium content in the shell of the two Sea snail species

In the two sea snail species, the concentration of essential (Se, Fe, Ca, Zn, Mg, K) and non-essential elements (Cd, Cr, Pb) in the meat fraction of a pool of samples of the two species collected in the two seasons was determined using an Agilent inductively coupled plasma with mass spectrometer (ICP-MS) system Model 7800 (Agilent, Palo Alto, CA, USA). Calibration curves were set up for quantitative determination using standard element solutions obtained by diluting the mother solution in HNO_3 3% + HCl 0.05% with yttrium, scandium, terbium, and bismuth as internal standards.

For shell analysis, 50 g samples of shell fraction/species, as a pool of samples in November and March, were subjected to grinding using a blender (Optimum mod. 9400 Vortex Blender)

(Optimum, Bayswater, VIC, Australia) and by sieving to obtain particles less than 1mm in size. The powdered form was then processed and the degree of $CaCO_3$ was determined by treating the compound with a 1 N solution of hydrochloric acid; the excess acid was titrated with a 1 N solution of sodium hydroxide (Yoon et al., 2004).

8.2.2.5. Statistical analysis

Biometric parameters (total height, total weight, shell weight, meat weight) of the specimensof the two species of collected sea snails were subjected to one-way analysis of variance (ANOVA) using SPSS 25 (Version 25.0, Armonk, NY, USA) (IBM Corp. 2017) and the means were considered significant at $P < 0.01$. The results concerning proximate composition, fatty acid profile, were subjected to two-way ANOVA considering the sea snail species (SP) and the season of sampling (SE) and their interaction (SP x SE) as fixed effects. Due to sample organization, element contents were subjected to one-way ANOVA considering only the snail species. In all categories of compounds, differences were considered significant at $p < 0.01$ and the means were compared using the Student–Newman–Keuls (SNK) test.

8.2.3. Results

In Table 20 the mean values of shell height, total weight, shell weight and mean weight werereported according to month of sampling and species. Considering the month of sampling, total weight showed significant differences with higher values in March (2.53 g) respect to November (1.38 g) whereas the meat weight was similar between the two sampling periods. In relation to the species of sea snails, the total weight showed higher values (3.86 g) in the long sea snail (*H. reticulata*) respect to the common sea snail (2.84 g). The meat weight

was notably higher in the common (1.38 g) respect to the long sea snail (0.97 g). Consequently, the meat yield was double in the common compared to the long one whereas the shell yield was heavier in the long sea snail.

Table 20 - Mean values of shell height, total weight, shell weight and meat weight of the two species of sea snails according to month of sampling (SE) and species (SP).

	Month of Sampling (SE) for both species		**Species (SP)**		**Error**			
	November	**March**	***N. mutabilis***	***H. reticulata***	**MSE**	**SE**	**SP**	**SE × SP**
Shell height (mm)	25.5 ± 0.3	29.8 ± 0.5	28.6 ± 0.4	30.2 ± 0.6	1.350	2.19	0.282	n.s.
Total weight (g)	2.43 ± 0.9 B	3.45 ± 0.6 A	2.84 ± 0.6 B	3.86 ± 0.8 A	0.304	0.001	0.001	n.s.
Shell weight (g)	1.38 ± 0.8 B	2.53 ± 0.9 A	1.46 ± 0.9 B	2.89 ± 0.9 A	0.037	0.001	0.001	n.s.
Meat weight (g)	1.05 ± 0.3	0.92 ± 0.4	1.38 ± 0.3 A	0.97 ± 0.2 B	0.042	0.098	0.019	n.s.
Shell yeild (%)	56.79 ± 2.4 B	73.33 ± 1.5 A	51.41 ± 1.4 B	74.87 ± 1.7 A	0.02	0.001	0.001	n.s.
Meat yeild(%)	43.21 ± 1.1 A	26.67 ± 1.3 B	48.59 ± 1.6 A	25.13 ± 1.5 B	0.04	0.001	0.004	n.s.

MSE = Mean standard error; A, B = $p < 0.01$; n.s. = not significant.

In Table 21 the proximate composition was reported showing contents of macronutrients very similar without notable differences independently of the season of sampling and the species of sea snail considered.

Table 21 - Proximate composition (% as it was) of the two species of sea snails according to season of sampling (SE) and species (SP).

	Month of Sampling (SE) for both species		Species (SP)		Error			
	November	**March**	***N. mutabilis***	***H. reticulata***	**MSE**	**SE**	**SP**	**SE × SP**
Moisture	73.95 ± 0.9	73.72 ± 1.1	73.73 ± 1.2	72.65 ± 1.4	0.205	0.33	0.62	n.s.
Protein	21.13 ± 1.4	22.28 ± 1.3	21.24 ± 1.2	22.02 ± 1.1	0.445	0.05	0.68	n.s.
Lipids	1.95 ± 0.8	1.76 ± 0.7	1.79 ± 0.6	1.53 ± 0.9	0.877	1.4	0.16	n.s.
Ash	1.81 ± 0.4	1.83 ± 0.3	1.71 ± 0.6	1.72 ± 0.8	0.007	0.07	0.06	n.s.

MSE = Mean standard error; n.s. = not significant.

With respect to fatty acids (Table 22), considering the season of sampling, the most important category was represented by the saturated fatty acids (SFA), ranging between 45.22% and 46.24%, with palmitic acid (16:0) as the most representative fatty acid (26.29-29.08%), followed by monounsaturated fatty acids (MUFA) (22.44-23.91%), with the 18:1 (10.72-12.45%) as the most representative fatty acid. In these two categories, no significant difference was observed between the sea snails, irrespective of SE and SP. The polyunsaturated fatty acids (PUFA) n6 showed significant differences in arachidonic acid (ARA) with significantly differences between the sample of November (3.90%) and that of March (6.86%) independent of the species. The PUFA n3 exhibited significant differences in eicosapentaenoic acid (EPA) between the seasons, with the highest proportions in November (14.13%) compared to March (12.04%). Consequently, the n3/n6 ratio was significantly higher in November (4.1) compared to March (2.38).

Considering the fatty acid profile in relation to the sea snail species, independently by the season of sampling, no differences were found between the SFA in the common (47.11%) and the long one (46.43%). The two species show similar proportions of MUFA in the common (22.69%) and the long snails (20.17%). By contrast, the species type had significant effect (0.0001) on the n-6 PUFA with the highest percentage in the long species (8.13%) respect to the common (4.64%); these differences were due to the ARA which reached the prevalence in the long sea snail (6.92%) respect to the common sea snail (2.83%). The n-3 PUFA had the highest level in EPA with significantly higher percentages in the common (13.86%) respect to the long (12.89%). The n3/n6 ratio was significantly higher in the common (4.98) respect to the long sea snail (2.68).

Table 22 - Fatty acid profile (% total fatty acids) of the two species of sea snails according to season of sampling (SE) and species (SP).

	Month of Sampling (SE) for both species		**Species (SP)**		**Error**			
	November	**March**	*N. mutabilis*	*H. reticulata*	**SEM**	**SE**	**SP**	**SE × SP**
SFA								
14:0	6.98	7.52	6.90	6.84	0.7230	0.2140	0.4320	n.s.
15:0	0.41	0.49	0.46	0.52	0.0734	0.0400	0.0120	n.s.
16:0	29.08	26.29	29.14	27.72	0.9611	0.930	0.0400	n.s.
17:0	1.22	1.08	1.33	1.62	0.3223	0.014	0.0140	n.s.
18:0	8.20	9.49	8.98	9.45	0.5514	0.246	0.0820	n.s.
20:0	0.34	0.35	0.30	0.28	0.1504	0.160	0.1000	n.s.
Total SFA	**46.24**	**45.22**	**47.11**	**46.43**	**0.243**	**0.147**	**0.0036**	**n.s.**
MUFA								
14:1	0.20	0.22	0.02	0.28	0.14	0.022	0.0220	n.s.
16:1	7.90	7.81	6.93	7.04	0.87	0.385	0.4950	n.s.
17:1	1.23	1.19	1.48	1.04	0.14	0.013	0.0420	n.s.
18:1	10.72	12.45	11.75	9.24	1.70	0.570	1.1600	n.s.
20:1	2.39	2.24	2.51	2.57	0.20	0.121	0.1020	n.s.
Total MUFA	**22.44**	**23.91**	**22.69**	**20.17**	**1.95**	**0.137**	**0.0245**	**n.s.**
PUFA n6								
18:2 n6	1.51	1.42	1.54	1.05	0.23	0.199	0.1730	n.s.
18:3 n6	0.25	0.25	0.27	0.16	0.18	0.047	0.0310	n.s.

20:4 n6 ARA	3.90 B	6.86 A	2.83 B	6.92 A	1.04	0.001	0.0009	n.s.
Total PUFAn6	**5.66 B**	**8.53 A**	**4.64 B**	**8.13 A**	**0.23**	**0.0001**	**0.0001**	**n.s.**
PUFA n3								
18:3 n3	2.92	2.63	3.01	3.08	0.40	0.005	0.4050	n.s.
20:5 n3 EPA	14.13 A	12.04 B	13.86 A	12.89 B	1.02	0.0003	0.0005	n.s.
22:5 n3 DPA	0.99	0.87	0.81	0.86	0.10	0.0260	0.0260	n.s.
22:6 n3 DHA	5.20	4.73	5.45	5.00	0.54	0.3850	0.1050	n.s.
Total PUFAn3	**23.24 A**	**20.27 B**	**23.13**	**21.83**	**0.78**	**0.0001**	**0.2382**	**n.s.**
Others	2.42	2.07	2.43	3.08	0.25	0.4782	0.4710	n.s.
n3/n6	4.10 A	2.38 B	4.98 A	2.68 B	0.26	0.0001	0.0001	n.s.

MSE = Mean Standard Error; A, B = $p < 0.01$; n.s. = not significant. Fatty acids are reported as numbers of C atoms (14–22) and numbers of double bonds (1–6) presents in the molecular structure, while n3 and n6 indicated the position of double bonds. SFA = Saturated Fatty Acids; MUFA = Monounsaturatedfatty acids; PUFA = Polyunsaturated Fatty Acids; ARA = Arachidonic Acid; EPA = Eicosapentaenoic acid; DPA = Docosapentaenoic acid; DHA = Docosahexaenoic fatty acid.

In element concentration (Table 23), selenium content was at an average content of 24 µg/100 g in *N. mutabilis* compared to 35 µg/100 g in *H. reticulata*. The mean concentrations of other elements were similar in the common and long snails (mg/kg): Iron 3.1-4.4; calcium 32-33; zinc 1.7-1.5; magnesium 51-54; potassium 290-310. The other elements were in the ranges (µg/100g), but significantly different with the lowest concentrations in the common sea snail: lead 26-30; cadmium 31-42; chromium 26-36.

In the gastropod shells, analyses showed that calcium was present at 283 g/kg of the long sea snail, whereas the common snail was at 265 g/kg. The purity of calcium carbonate was 96 ± 0.5% in the long snail and 91 ± 0.5% in the common one.

Table 23 - Element concentration determined in the meat of the two species of sea snails (mean value ± std. dev.).

		N. mutabilis	*H. reticulata*
Selenium	μg/100 g	24 ± 7	35 ± 6
Iron	mg/100 g	3.1 ± 2	4.4 ± 3
Calcium	mg/100 g	32 ± 2	33 ± 2
Zinc	mg/100 g	1.7 ± 0.3	1.5 ± 0.5
Magnesium	mg/100 g	51 ± 4	54 ± 6
Potassium	mg/100 g	290 ±13	310 ± 17
Lead	μg/100 g	26 ± 2 B	30 ± 3 A
Cadmium	μg/100 g	31 ± 5 B	42 ± 3 A
Chromium	μg/100 g	26 ± 2 B	36 ± 5 A

A, B = $P < 0.01$.

8.2.4. Discussion

In this study, the main qualitative traits of meat and the calcium shell content of the common sea snail species (*N. mutabilis*) were compared with those of the long sea snail (*H. reticulata*) whose population is increasing to the disadvantage of the most appreciated common species. To our knowledge, this paper is one of a few that deals with the quality traits of meat and the shell characterization of these gastropod species, captured in the Mediterranean Sea, which are mostly considered from the microbiological viewpoint (Serratore et al., 2019). Therefore, data were also compared with gastropods of other species and latitude distribution was investigated in a similar way to the current paper. The choice of monitoring sea snail samples at two different times (November and March) aimed to assess the quality and biometric attributes through the catching season. In the last few years, besides the revision of the

minimum landing size, shortening of the fishing season (from autumn to end winter) was one of the measures proposed to safeguard the common sea snail population (Polidori et al., 2015). In our study, both in November and March, all specimens of both gastropods showed the mean shell height to be higher than that proposed as the revised minimum size (shell height 23–26 mm) (Grati et al., 2010). Based on the results of the current study, the obligation of landing, as contemplated by the aforementioned EC Regulation, can contribute to balancing the stock population of the common species and, presumably, avoiding throwing of long sea snails back into the sea could be enough to balance the dynamics of both gastropod populations.

Analysis of the two fractions (shell, meat) of the common and long marine snails showed that the Common snail has a higher meat proportion compared to that of the Long species, which had a higher shell part, representing about 2/3 of its total weight.

The results showed that both sea snail species are a good source of protein. Among sea snails, *Rapona venosa*, collected from the Marmara Sea (Celik et al., 2014), showed high protein variation, ranging from around 65% (on dry weight) to 12.53% (on wet weight), indicating that the common and long sea snails are in a good ranking position.

With respect to the lipid content, both species exhibited low fat content in a very similar range. In the turban shell, *Turbo cornutus*, a marine snail captured along the coasts of south east Asia Japan, Korea, China, and the Philippines, lipids were determined separately in the foot and viscera and their sum appeared to be slightly higher (2.2-4.5%) than that determined in the common and long sea snails (Saito et al., 2014).

Regarding fatty acids in the soft tissue, both sea snails showed a prevalence of SFA, with palmitic acid as the most important fatty acid. This result was consistent with those in other species of gastropods from Portuguese waters, such as *Aplysia* spp. (Pereira et al., 2013), but

the level was lower than that reported in the hepatopancreas and meat of a *Murex* species (*Heraplex trunculus*) from the Tunisian Mediterranean coasts, collected from the fish market in a single sampling (Modica MV and Holford M, 2010). In our study, the entire edible fraction was examined in both sea snails; however, considering the mean values from those in the *Murex* species, differences were observed in fatty acid categories MUFA and total PUFA (both n3 and n6 series) as being respectively lower and higher compared to those determined in the present paper. The ARA content represented the highest difference between the common and long snail species showing higher proportions in the long compared to the common one, independent of the season. Although the samples considered in the present study were included only at the beginning and at the end of the sampling seasons and not throughout the year, the variable "season" was not significant for both gastropod species, except for ARA in the common snail where less yield was found in March. These two gastropod species, similar to most marine gastropods, are considered carnivorous with a degree of predatory activity that varies from actively seeking prey to grazing on sessile invertebrates to scavenging (Modica et al., 2010; Sajjadi et al., 2011; Pereira et al., 2013; Celik et al., 2014; Saito et al., 2014; Polidori et al., 2015; Serratore et al., 2019). This feeding behaviour can be affected by the substratum and may have affected the fatty acid profile in terms of n-3 PUFA and the n3/n6 ratio in the common sea snail. *Hinia reticulate* could thus have a more generalist food spectrum including detritus and bacteria that are responsible for the higher level of ARA as determined in another gastropod (*Turbo cornutus*), collected during a year at seasonal intervals (Saito H and Hashimoto J, 2014). In this species of sea snail, the authors suggested that the ARA level is derived from bacteria as part of the diet, and that the low EPA level (0.48-6.12%) is probably not necessary for the survival of this mollusc (Go et al., 2002). Other authors (Saito et al., 2010) found that gastropods from the Mediterranean Sea (*Monodonta turbinata*, *Gibula cineraria*, *Littorina neritoides*) contained

high levels of ARA but also of 18:3n-3. In the common and the long sea snails, PUFA n-3, represented by EPA with a low content of 18:3n3 and DHA, were higher in proportion to that reported in a deep-vent gastropod (*Ifremeria nautilei*), defined as herbivorous, where the lack of docosaenoic acid (DHA) was dispensable (Miniero et al., 2014).

The content of various elements was examined to evaluate the benefits and risks of consuming sea snails and to include them in seafood, which is an important source of high-quality proteins and essential fatty acids without adverse health effects in the full respect of food security (European Aquaculture Society, 2012; Silva et al., 2017; Bilanzcic et al., 2018). Regarding selenium, both species of sea snails were a good source of this micronutrient, with levels comparable to those reported in fish from European aquaculture (0.02 mg/100 g and 0.06 mg/100 g fish flesh) (Yamashita et al., 2013) and in Japanese fish (0.12 mg/kg and 0.77 mg/kg tissue) (Iamiceli et al., 2015).

Considering that both species were from the same sampling area, where they occupy the same trophic level, the higher concentration of non-essential elements such as lead, cadmium, and chromium, although only slightly significant, which was exhibited in the long sea snail species, can be explained by a species-specific accumulation mechanism as recognized in other aquatic organisms (Directive 2008/98/EC). Different levels of essential and non-essential elements have been documented and their bioaccumulation in seafood tissues may have unwanted effects on human health. In the current paper, the sampled sea snails showed mean values below the limits of quantification imposed by the European legislation (Directive 2008/98/EC). Both gastropods showed mean values lower than those reported by *N. mutabilis*, collected from the Adriatic Sea, for lead and cadmium as investigated in a previous study (Bille et al., 2015) although sampled further north (north-western Adriatic Sea) compared to our sampling area. In this previous study, toxic elements including lead and cadmium were investigated in edible echinoderms and molluscs, and showed mean values

around 0.09 mg/kg (Lead) and 0.11 mg/kg (Cadmium) in the common sea snail. In our study, these elements along with zinc, iron, and magnesium were significantly lower than those reported in other gastropods (*Murex* spp.), for which continuous monitoring of heavy metals was recommended (Ragi et al., 2017).

In relation to the calcium content in the shell, if the quality traits exhibited by the long marinesnail appeared similar to those of the common species, the higher quantity of the shell in terms of yield on the total body and calcium content, with a concentration similar to that reported in mussel shells (>280 g/kg), suggest the possibility of using the long sea snail as an alternative in food consumption. The common sea snail is requested for gastronomic uses, by contrast with the long sea snail that is considered too gummy. Another study aimed to show eventual differences in the sensory attributes of the soft tissue in these two species of sea snails, as performed for other molluscs that are appreciated just in one gourmet version, as in case of the cupped oyster (Felici et al, 2019).

As part of discarded materials, shells of this gastropod species can be processed to recover precious compounds such as calcium carbonate, to promote recycling of by-products. Recovery of the high calcium concentration in shells from the long sea snail is in line with the valorisation of shells from molluscs occurring in Galicia, where this practice has been promoted to employ valve mussels and to avoid incineration (Iribarren et al., 2010). In bivalves, shell calcium is used as a soil amendment to increase pH in acidic soils (Barros et al., 2009; Iribarren et al., 2010; Felici et al., 2019); recently, it has also been purposed as a sorbent of fluoride, a mineral associated with industrial and agricultural pollution (Quintans-Fondo et al., 2016). The calcium content of the long sea snail shell could allow its inclusion in feed for aquatic species and poultry. In shrimp, growth performances were found to be enhanced by supplementation with marine snail shells, as the calcium source, especially at an inclusion level of 10% in the diet. In fact, calcium affects the moulting frequency in shrimps

and tends to be absorbed into these organisms during the pre-moult period (Moss et al., 2019). In poultry, the potential use of sea snail shells in the feeding of breeding hens could allow poultry farms to differentiate their products, thus increasing the degree of competitiveness on the market of local supply chains, which could benefit from feed supplemented with sustainable additives. In laying hens, calcium could become a supplement with high added value in feed, especially starting from the 25th week (before the deposition peak) when feed supplemented with sources of this element is necessary (Schiavone et al., 2008).

Management of discards derived from fishing and processing of seafood products is of growing interest at the international level (Gasco et al., 2020). Every year, the fishing industry and seafood processing sectors generate wastes mainly represented by body fractions such as head, skin, fins, viscera, mollusc valves, and crustacean exoskeletons, which are removed without any recovery attempts. Enhancement of recovery for unwanted seafood parts would reduce wastes coming from the fish sector, responding to ethical and environmental needs, but also to economic opportunities, linked with obtaining precious compounds as shown in the circular economy. According to the EC Regulation (1774/2002), by-products of fish processing plants destined for human and animal consumption are considered as Category 3 materials. A recent study (Halim et al., 2018) has quantified the amount of these waste quotas at over 60% of the biomass, focusing on the fact that their inactivity poses serious disposal and pollution problems both in developed and developing countries.

Preliminary information derived from this study suggests that in the Adriatic Sea, a “green-harbour” could be equipped as a pilot plant for treating discards of seafood processing plants or from fisheries, as the by-catch or non-target species that are obliged to be landed. This part of the harbour could have rooms equipped to perform the separation of shells for fishery by-

products, in a mixture submitted to grinding, pressing to obtain a cake, followed by packaging and storage. The fishermen, organized in a sort of consortium could collect the long sea snails caught in the period in which fishing is allowed. This action could transform gastropods such as sea snails into a resource for the fishermen, penalized by the scarcity of fishery for most target species represented by the common sea snail species.

8.2.5. Conclusion

The proximate composition, fatty acid profile, and elemental content in the soft tissue of twomarine snail species were investigated along with the calcium content in their shells. Both species of gastropods were found to be a good source of nutrients and calcium concentration. Shell composition indicated that the long sea snail can be processed to recover calcium content thus reducing waste by “closing the loop” of production in perfect agreement with the circular economy approach (Jurgilevich et al., 2016). Operators specialized in fishing for sea snails could thus find the capture of long sea snail species as an additional economic resource by processing their shells and extracting the calcium content, indicating an interesting opportunity for commercial development.

8.3 - Sterol and mineral profiles of the Common Sea Snail *Hinia reticulata* and the Long Sea Snail *Nassarius mutabilis* (Gastropods) collected from the Middle Adriatic Sea

8.3.1. Introduction

Snails are an essential part of dishes across the world because of their palatability and nutritional content. In European countries such as Italy, Portugal, Spain, and Turkey, snails, along with other shellfish such as mollusc and oyster, are a major constituent of seafood diets. In Italy in particular, they attract more attention for their use in traditional gastronomic dishes, in which sea snail meat is used as the main ingredient, and its organoleptic characteristics are stood out. In 2011, the estimated annual consumption in Italy was 306 million snails (Çağıltay F, 2011) and the number has increased considerably in recent years; according to a 2017 Coldiretti (National Federation of Direct Farmers) survey, the consumption of snails by Italians has increased by 325% in the last 20 years.

For a long time, snails were considered to be a traditional dish in Mediterranean cuisine; however, the growing standard of living and the popularity of European cuisine in Asian countries have made sea snails a famous meat in the Asiatic continent, thus increasing its global demand. Similarly, snail meat is consumed in African countries, especially in West Africa, where they are included in the staple diets.

Snails are univalves, and belong to the class Gastropoda of the phylum Mollusca. The phylum Mollusca is one of the largest phyla of animals, it includes more than 80 000 species, most of them aquatic, and very vary in size, from giant squids and clams to little snails. Inside the phylum Mollusca the class Gastropoda is divided into three groups: univalves, which includes sea snails, that has got only one single shell; bivalvia, which includes mussels and oysters, characterized by two shells connected by a flexible ligament; and cephalopoda, which includes octopus and squid, characterized by a set of arms or tentacles. A considerable

amount of literature has been published on the nutritional composition of some molluscs such as oysters (Orban et al., 2004; Venugopal V and Gopakumar K, 2017) and mussels (Orban et al., 2002; Oliveira et al., 2015); however, studies on the nutritional composition of sea snails are limited.

The general knowledge concerning sea snails as food shows that snail meat has a high protein content, a low fat and carbohydrate content, and is an important source of minerals and vitamins, such as sodium, calcium, potassium, phosphorus, and vitamin E (Çağıltay F, 2011; Ozyurt et al., 2012; Ghosh et al., 2017; Felici et al., 2020). Additionally, similar to shell fishes such as mussels (Orban et al., 2002; Venugopal V. and Gopakumar K, 2017) and oyster (Orban et al., 2004; Venugopal V and Gopakumar K, 2017), snail meat is a good source of polyunsaturated fatty acid (PUFA) (Ozyurt et al., 2012), which is an important nutritional element. Therefore, snail meat makes an excellent high-protein low-fat diet. Among snail species, the long sea snail (*Hinia reticulata*) and the common sea snail (*Nassarius mutabilis*) have been used as food in Europe, particularly in Italy. A recent study showed that the long sea snail and the common sea snail could be considered a valuable source of nutrients such as protein and fatty acids (Felici et al., 2020).

In Italy, sea snails are a common product caught from the Adriatic sea. A search detected at the port of San Benedetto del Tronto estimated the annual catches of sea snails caught by the fishermen in Center Italy, showing that the catch of the common sea snail were about kg 780 ± 210 / year and the catch of the long sea snail were about kg 705 ± 190 / year (Felici et al., 2020).

Snails are also in high demand because of their beneficial sterol profile. Sterols such as cholesterol play an important role in human nutrition, such as in the maintenance of cell structure, in the synthesis of steroidal hormones, and in the production of vitamin D; it is

therefore necessary that sterols are included in the diet of humans at appropriate levels (Venugopal V and Gopakumar K, 2017; Carson et al, 2019). According to the World Health Organization (WHO 2003), the maximum recommended intake of cholesterol is 300 mg/day.6 Shellfish are low in saturated and trans fats, and are a good source of healthy polyunsaturated fats (Pereira et al., 2013; Venugopal V and Gopakumar K, 2017). Some shellfish also contain significant amounts of other non-cholesterol sterols that can decrease the absorption of cholesterol (Pereira et al., 2013; Venugopal V and Gopakumar K, 2017). Apart from zoo-sterols such as cholesterol, sea snails are also rich in phytosterols, of which the main ones are sitosterol, stigmasterol, and campesterol. This is because sea snails feed mainly on algae and vegetables, which are rich in these phytosterols. Phytosterols are precious elements contained in foods of plant origin, and play an important role in reducing the LDL-cholesterol level in human blood (Cabral CE and Klein MRST, 2017), and reducing the risk of coronary heart disease (Ozyurt et al., 2012). According to some research, they may also promote the health of the prostate and urinary system, and in general, prevent the development of cancer (Tapiero et al., 2003; Jiang et al., 2019). The main source of phytosterols are vegetable oils and dried fruit, mushrooms, and algae, which represent the main food for sea snails, thus improving their phytosterol profile. Additionally, as a result of their nutrition and environment, sea snails may also serve as a mineral source; however, their mineral content is dependent on their environment. As aquatic animals, apart from algae and vegetables, snails also feed on dead animals, and their health and the chemical characteristics of their meat directly reflect the quality of the aquatic environment in which they live. The accumulation of nitrogenous catabolites and heavy metals have been recorded in the tissues of sea snails living in polluted seas and poor-quality waters (Ab Lah et al., 2016; Bilanzcic et al., 2018; Serratore et al., 2019).

Previous studies on the long sea snail and the common sea snail have been focused on their PUFA profiles and amino acid content. Therefore, to enrich the knowledge of the two species, the present study aimed to examine the sterol profile (total lipid, cholesterol, and phytosterol) and the mineral profile (iron, calcium, zinc, magnesium, and potassium) of the long sea snail and the common sea snail collected from the Adriatic Sea. Additionally, the level of toxic metals and metalloids in the two studied snails was also investigated in order to ascertain the consumer safety eating their meat. For this purpose, the level of cadmium, chromium and lead were evaluated in the common and in the long sea snail.

8.3.2. Materials and Methods

8.3.2.1. Samples and sampling areas

Samples of the two species of sea snails, *H. reticulata* and *N. mutabilis*, were collected at different catching times from November 2019 to March 2020 from the middle Adriatic Italian Sea, and processed according to the procedure of Felici et al. (2020) (Felici et al., 2020). At each time of catching, 50 specimens for each specie were sampled and transported in an icepack to the Chemistry and biochemistry laboratory of the School of Biosciences and Veterinary Medicine at the University of Camerino (UNICAM) for the determination of total weight (meat + shell) and the separated fractions of meat and shell, after having extracted the edible part. From the edible fractions, pools of each group were homogenised and subjected to analysis. To measure the weight, an electronic scale (mod. CP224S Sartorius, Gottigen, Germany) was used (Felici et al., 2020). The moisture content of the snails was determined following the procedure of the Association of Official Analytical Chemists (AOAC 1990). The edible portion was used for the sterol profile and mineral profile analyses and for the heavy metals and metalloids evaluation.

8.3.2.2. Sterol profile determination

Determination of the sterol profile was performed according to the method of Felici et al. (2020) (Felici et al., 2020). The lipid extract of the samples was used for the determination of the sterol profile after a saponification process. Total lipid content was determined using the modification of the chloroform:methanol procedure described by Folch et al. (1957) (Folch et al., 1957).

In the Chemistry laboratory of the University of Camerino (UNICAM), the total sterol content was determined gravimetrically and the individual sterols were determined after derivatisation in trimethyl-silyl derivatives using Agilent 6890 N gas chromatographer (Agilent Palo Alto, CA, USA) equipped with a db5 (60 m x 0.25 mm) with helium (1 ml/min) as carrier gas at a constant flow of 1.0 mL/min. The temperature of the gas chromatographer was 170°C for 15 min at the beginning, then it increased by 1°C/min until reaching the temperature of 190°C, to increase again by 5°C/min until 220°C; finally, it remained at the same temperature for 17 min (Felici et al., 2020).

8.3.2.3. Determination of mineral profile, heavy metals and metalloids in the meat of the two Sea Snail Species

Following the procedure reported by Felici et al. (2020) (Felici et al., 2020), the quantity of iron, calcium, zinc, magnesium and potassium in the two snail species was determined using Agilent Agilent inductively coupled plasma with mass spectrometer (ICP-MS) 7800 model (Agilent, Palo Alto, CA, USA), an inductively coupled plasma with mass spectrometer (ICP-MS) system. With the same procedure the quantity of heavy metals and metalloids (cadmium, chromium and lead) were investigated.

The calibration curve profiles were obtained using standard element solutions obtained by diluting the mother solution in 3% HNO3 + 0.05% HCl with yttrium, scandium, terbium, and bismuth as internal standards (Felici et al., 2020).

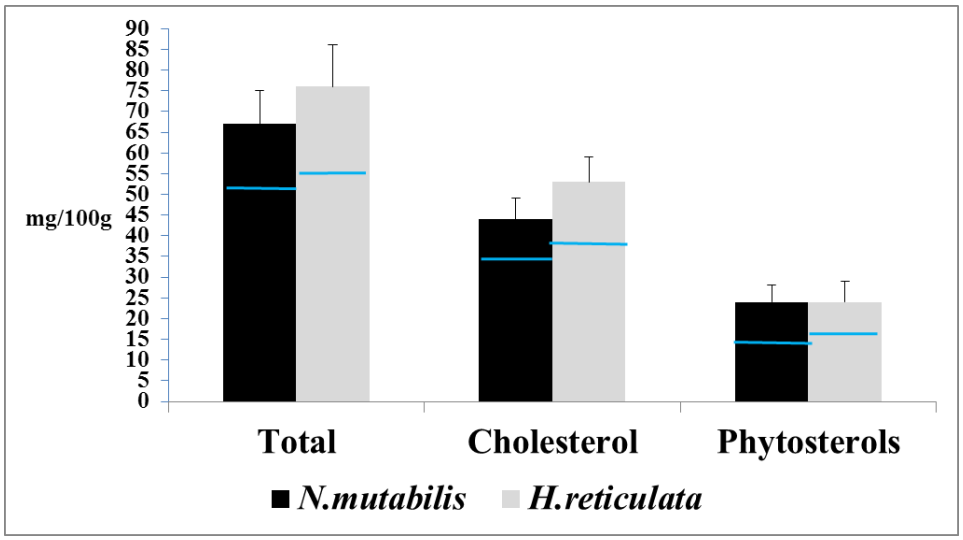
8.3.2.4. Statistical analysis

Data obtained from the study, including data of the mineral content of the samples were subjected to one-way analysis of variance (ANOVA) using SPSS 25 (Version 25.0, Armonk, NY, USA) and significant means were compared using Student–Newman–Keuls (SNK) test of the same software. Means were considered significant at $P < 0.01$.

8.3.3. Results

Results of the total lipid, cholesterol, and phytosterol contents of the sea snails are presented in Figure 19. The total lipid content of the long sea snail ranged from 47 to 67 mg/100 g (mean: 57 ± 10 mg/100g), whereas that of the common sea snail ranged from 58 to 78 mg/100 g (mean: 68 ± 8 mg/100 g). No significant difference was recorded in the values of total lipids between the two species. In addition, the cholesterol and phytosterol contents of the sea snails were evaluated. The result showed that the cholesterol contents of the long sea snail ranged from 32 to 44 mg/100 g (mean: 38 ± 6 mg/100 g) and the phytosterol content from 14 to 24 mg/100 g (mean: 19 ± 5 mg/100 g), whereas the values for the common sea snail ranged, respectively for cholesterol and phytosterol, from 43 to 53 mg/100 g (mean: 48 ± 5 mg/100) and from 16 to 24 mg/100 g (mean: 20 ± 4 mg/100 g). No significant difference was recorded between the two species.

Figure 19 - Total lipid, cholesterol and phytosterols of *Nassarius mutabilis* (*N. mutabilis*) and *Hinia reticulata* (*H. reticulate*) expressed in mg/100 g. The black blocks refer to the long sea snail (*N. mutabilis*), while the grey blocks refer to the common sea snail (*H. reticulata*). The blue lines mark the minimum registered quantity. Standard deviations are also reported on the top of the blocks.



The results of the mineral contents of snail samples are presented as means and standard deviations (Table 24). The elements investigated were iron (Fe), calcium (Ca), zinc (Zn), magnesium (Mg), and potassium (K). In the common sea snail the mean content obtained was 3.15 ± 1.1 mg/100 g for Fe, 31.50 ± 1.5 mg/100 g for Ca, 1.70 ± 0.2 mg/100 g for Zn, 51.08 ± 2.8 mg/100 g for Mg, and 293.50 ± 2.5 mg/100 g for K. Whereas the contents in the long sea snail were 4.40 ± 0.4 mg/100 g for Fe, 32.50 ± 1.5 mg/100 g for Ca, 1.45 ± 0.2 mg/100 g for Zn, 54.00 ± 1.3 mg/100 g for Mg, and 296.44 ± 2.30 mg/100 g for K.

Table 24 - The Fe, Ca, Zn, Mg, and K contents of the two sea snail species *Hinia reticulata* and *Nassarius mutabilis* expressed as mean values and the standard deviations.

		Common sea snail (*Hinia reticulata*)	**Long sea snail** (*Nassarius mutabilis*)
Fe	mg/100 g w/w	3.15 ± 1.1	4.40 ± 0.4
Ca	mg/100 g w/w	31.50 ± 1.5	32.50 ± 1.5
Zn	mg/100 g w/w	1.70 ± 0.2	1.45 ± 0.2
Mg	mg/100 g w/w	51.08 ± 2.8	54.00 ± 1.3
K	mg/100 g w/w	293.50 ± 2.5	296.44 ± 2.30

Concerning the amount of heavy metals and metalloids, the long sea snail showed the maximum registered concentration of 0.031 mg/100g for cadmium (Cd), 0.028 mg/100g for chromium (Cr) and 0.028 mg/100g for lead (Pb). For the common sea snail the maximum registered values were respectively 0.045 mg/100g, 0.041 mg/100g and 0.033 mg/100g.

8.3.4. Discussion

This study confirmed that sea snail can be included into human diet owing to the organoleptic and nutritional characteristics of the meat. As reported in previous studies, snail meat is rich in polyunsaturated fatty acids (PUFA) (Zarai et al., 2011), essential amino acids, and minerals (Çağıltay F, 2011; Pereira et al., 2013; Ghosh et al., 2017; Felici et al., 2020). Our results showed that the both evaluated sea snail are valuable food.

8.3.4.1. Total lipid profile

Previous studies on snails focused mainly on their zoosterol and phytosterol composition (Zhu et al., 1994), fatty acid composition (Çağıltay F, 2011; Ghosh et al., 2017; Felici et al., 2020), and cholesterol content (Pereira et al., 2013). To the best of our knowledge, this study is the first to evaluate the total lipid content of the long sea snail and the common sea snail; the lipid content of both species were similar and were generally low. Comparing our results for sea snails with those obtained for land snails,1 it can be seen that sea snails contain lower lipid content compared to land snails. In comparison with other shellfish such as mussels (Orban et al., 2002; Oliveira et al., 2015) and oysters (Orban et al., 2004), sea snails contain a relatively lower quantity of lipids.

8.3.4.2. Cholesterol

In the current study, cholesterol was the major sterol present in the two sea snail species studied. Studies have shown that cholesterol is the major sterol present in shellfish such as sea snails; however, other sterols such as stigmasterol and desmosterol are also present in limited quantities (Jiang et al., 2019).

8.3.4.3. Phytosterols

To the best of our knowledge, this study is the first to report the phytosterol profile of the common sea snail and the long sea snail. Similar to results in mussels (Orban et al., 2002) and in several mollusc species (Larsen et al., 2011), our results showed that the two studied sea snails had around 50% phytosterols in their sterol-pool. On the contrary, in land snail species, phytosterol content is lower: 6.3 mg/100 g in the northwest hesperian (*Vespericola columbiana*), 6.9 mg/100 g in Helix sp., and 3.8 mg/100 g in Haplotrema sportella (Zhu et al., 1994).

Owing to the importance of fishes in human nutrition and health, the sterol profiles of fishes have been studied extensively. Ozyurt et al. (2012) studied the sterol profiles of some Mediterranean fishes caught in different seasons (Ozyurt et al., 2012). Comparing the result of the present study to results obtained in fishes, the mean content of cholesterol in the two species of evaluated sea snails was similar to the lowest phytosterol content recorded in fishes; the phytosterol content ranged from 15.00 to 36.54 mg/100 g in seabream (*S. aurata*), 27.66 to 48.07 mg/100 g in mackerel (*T. trachurus*), and 14.37 to 28.65 mg/100 g in common sole (*S. solea*) (Ozyurt et al., 2012).

8.3.4.4. Mineral profile

The principal aim of this study was to investigate the sterol profile of the common sea snail and the long sea snail present in the Adriatic Sea. Moreover, the mineral profile analyses of the sea snails were necessary to verify the reports of Felici et al. (2020) (Felici et al., 2020), and the results obtained in the present study were in agreement with their findings.

A study on the mineral content of the Mediterranean marine snail, *H. trunculus*, was carried out by Zarai et al (2011) (Zarai et al., 2011), and the most abundant electrolytes were potassium (224.80 ± 55.10 mg/100 g), sodium (196.10 ± 30.00 mg/100 g) and magnesium (178.70 ± 54.30 mg/100 g). A comparison with the data obtained by Zarai et al. (2011) (Zarai et al., 2011) showed that the Fe, Ca, Zn, and Mg contents of the Mediterranean snail were higher than the values obtained in the long sea snail and the common sea snail in the present study; however, K was higher in the present study. On the contrary, the result of a study on *T. mitilis*, *L. torquata*, and *L. undulata* showed a different mineral concentration from what was recorded in the present study (Ab Lah et al., 2016). In addition, the mineral profile of the edible garden snail (Çağıltay F, 2011) and *Pomacea canaliculate* (Ghosh et al., 2017) were

different from what was obtained in the present study; *Pomacea canaliculate* had a higher P content compared to that of the long and the common sea snails.

The results of the present study showed that the long sea snail and the common sea snail are sources of important minerals such as iron, zinc, magnesium, and calcium, and their inclusion into healthy human diet should be considered. In particular, the sea snails are an excellent source of potassium, which possesses several biological functions. Potassium contributes to the health of the heart and vessels, bone, and kidney (Weaver CM, 2013); and is involved in enzyme activities, protein synthesis, and insulin secretion (Ringer J and Bartlett Y, 2007).

8.3.4.5. Toxic metals and metalloids

Sea water is constantly polluted by contaminants from industrial activities, such as micro-plastics and heavy metals. These pollutants are usually deposited in the meat of aquatic organisms. Residues of heavy metals were found in fish and sea food products in Bosnia and Herzegovina (Djedjibegovic et al., 2020). In the Black, Marmara, Aegean, and Mediterranean Seas, cadmium, arsenic, lead, and mercury were found in selected fish species and marine animals (Kuplulu et al., 2018). Therefore, for public health reasons and for the safety of sea snail food, it is important to investigate the presence and the possible accumulation of these toxic substances in the meat of sea snails (Acquavita et al., 2010; Spada et al., 2013; Felici et al., 2020).

In this study, results of the heavy metal and metalloid content in the two analysed sea snails showed that the content of toxic metals were below the safe recommended standards for human consumption (Spada et al., 2013; Rogan et al., 2018).

8.3.5. Conclusion

The sterol, mineral and toxic metals content in the soft tissue of the two marine snail species, *Hinia reticulata* and *Nassarius mutabilis*, were investigated. Both species of Gastropods were found to be a good source of nutrients and they resulted healthy and safety foods.

As reported in previous studies on shellfish, the organoleptic properties and chemical composition of sea snails in the Adriatic sea differed depending on the location and season; Different seasons influence the water temperature and the metabolism of aquatic animals, which may alter their composition. Differences based on different sea areas or different season were not assessed in the present study.

There are conflicting information on the mineral contents of sea snails, therefore, there is a need for detailed studies, particularly on the quality and characteristics of sea snail meat. Moreover, studies on the metabolic processes and energy requirement of shellfish and their effect on the mineral profile and sterol content should be carried out. As shown in mussels (Ozyurt et al., 2012), seasonal differences in the quality of sea snail meat may be linked to sea water conditions and temperature. However, seasonal differences in phytosterol concentrations in the two sea snails (*H. reticulata* and *N. mutabilis*) studied were not assessed, and is therefore recommended for future studies.

COLLATERAL ASPECTS ANALIZED DURING THE PhD TRIENNIUM

9. ALTERNATIVE MOLECULES TO CONVENTIONAL TREATMENTS

During the PhD triennium the attention was put also on the use of natural and essential oils with a potential effect to boost the immune system of farmed fish avoiding the use of medicated feeds. For example, the rosemary oil showed antibacterial properties (Glenn et al., 2014; Ebrahimi et al., 2019), while basil extract was known to show antimicrobial activities and improve growth performances, probably with a boost effect on the fish organism (El-Dakar et al., 2015; Amor et al., 2020; Chung et al., 2020; Ilić et al., 2021). And more, the tea tree oil had antibacterial, anti-inflammatory and antioxidant properties (Mingyang et al., 2022; Xin et al., 2022), and spirulina was known for its antioxidant and anti-inflammatory effects (Wu et al., 2016; Altmann BA and Rosenau S, 2022; Calella et al., 2022; Faheemet al., 2022).

With the aim to have a completely antibiotic free production, the Erede Rossi Silvio Trout Company started researches on the topic of alternatives to conventional antibiotics for the eggs incubation. The farm of the Erede Rossi Silvio Trout Company involved in the rainbow trout eggs production was located in Colli sul Velino (RI), where experiments were conducted in order to perform the eggs disinfection using only natural substances. The target of the Erede Rossi Silvio Trout Company was to get the attention of consumers toward the beneficial properties of healthy and high fish quality products. The purpose of the conducted experiments was to essay a natural product as the tea tree oil (TTO), used to disinfect rainbow trout eggs during the incubation phase against the Saprolegnia spp. infection, one of the main agents of fungal diseases that causes important economic losses in rainbow trout farming.

9.1. Tea Tree Oil as a natural alternative to antibiotics for the disinfection of rainbow trout eggs (*Oncorhynchus mykiss*) during the incubation phase

9.1.1. Introduction

The most common infection in freshwater fish is a fungal infection caused by the oomycetes Saprolegnia spp. responsible for devastating infections on fish in aquaculture, and nowadays the *Saprolegnia parasitica* is economically a very important fish pathogen. Pathogenic oomycetes such as Saprolegnia spp. have the ability to infect a wide range of plant and animal hosts and are responsible for a number of economically important diseases (Earle G and Hintz W, 2014). Saprolegnia spp. are fungi found in freshwater ecosystems around the world, and most transmission is by zoospores, which are produced by vegetative hyphae (Pavić et al., 2022). The disease is called saprolegniosis and is caused by Saprolegnia spp.; it mainly affects salmonids, from eggs to adult fish, in particular fish eggs and juvenile fish in hatcheries worldwide (Earle G and Hintz W, 2014; Pavić et al., 2022). It is characterised by visible white or grey patches of filamentous mycelium, causing dermal damage and cellular necrosis, respiratory insufficiencies and behavioural alterations such as lethargy and loss of balance, up to being able to cause the death of the infected fish (Hatai K and Hoshiai G, 1992; Hussein MMA and Hatai K, 2002; Van West P, 2006). In fish eggs death caused by a Saprolegnia infection is due to haemodilution, following the failure of the infected eggs to maintain the proper osmoregulation; the hyphae attack the eggs chorionic membrane, and then cause an osmotic shock (Hatai K and Hoshiai G, 1992; Pickering AD and Willoughby LG, 1982). Episodes of saprolegniosis are more evident during the winter season then in the rest of the year, when the water temperature is warmer, which represents a suitable habitat in farms and hatcheries where fish and eggs are found in a state of assembly.

Nowadays the standard treatment protocol to control Saprolegnia infections is represented by daily baths as prophylactic or therapeutic measure against fungal and bacterial infections in fish food eggs based on bronopol (Shepherd et al., 1988; Aller-Gancedo JM and Fregeneda-Grandes JM, 2007; Tedesco et al., 2019) or other chemical substances. The most common molecule used to control Saprolegnia is the bronopol, applied to protect fertilised rainbow trout eggs as a daily bath-flush treatment (Pottinger G and Day JG, 1999). In the past, the infection was under control by using malachite green, but it was not more authorized in the European Union from 2010 (EFSA Journal 2016) in the production of fish destined to human consumption. Besides, in aquaculture the use of chemicals can accumulate in fish meat and spread in the aquatic environment, creating a food and habitat contaminations (Okocha et al., 2018; Okeke et al., 2022).

Looking to a more sustainable aquaculture and following the "One Health" principle, it is necessary to find natural alternatives, safer for fish and human and with a lower environment impact. For Saprolegnia spp, after the banned of malachite green, there is an urgent necessity to find alternative methods to control this pathogen. In this context, TTO was essayed to evaluated its antimicrobial properties and was tested on rainbow trout incubation eggs as an alternative compound to chemical treatment.

9.1.2. Material and Methods

Two experiments were performed with two different concentrations of TTO in the rainbow trout hatchery located in Colli sul Velino (RI) (Picture 20). The first one was performed with the concentration of 50 µg/L of TTO, the second one with the concentration of 2000 µg/L of TTO. For both trials, 5 vertical incubators of 70 L (approximately 200,000 fertilized eggs) (Picture 21) were involved: one incubator represented the positive control (incubator 1), in

which any treatment was applied, one represented the negative control (incubator 2), in which the conventional treatment normally used in the hatchery was maintained, and the other 3 (incubator 3, 4, 5) were employed to essay the solution with TTO. For the negative control, the standard treatment was represented by a first iodine-based bath for 1 hour in absence of water flow, followed by a second disinfection with bronopol once a day.

In both experiments the TTO used was obtained from the Melaleuca alternifolia plant and was performed by the Erbamea Company (Perugia). The TTO emulsion was obtained making a TTO dilution in absolute ethanol ≥99.8% without additives to make it miscible in water.

The farming water came directly in the hatchery from the clear and uncontaminated Santa Susanna River. Before entering into the hatcheries, the income water passed through a series of filtration systems represented by gravels and sands, and then received a UV rays treatment. This process allowed to obtain cleaner water free from microorganisms and natural solid material.

The first experiment was performed with the concentration of 50 µg/L of TTO. The first TTO-based disinfection took place the same evening as the placing of eggs in the hatchery. Once the flow of water on embryos was interrupted, 35 ml of TTO solution were applied, and the eggs remained in contact with it for 30 minutes. After this time, the water flow was opened again. The same procedure was repeated once a day, during all the trial. After 11 days of the first experiment, the invasion of the so called "cotton mold" developed on all the experimental treated eggs (incubator 3, 4, 5), evident sign of the spread of Saprolegnia into the eggs mass. After a valuation of the conditions of the incubator 3, 4 and 5, authors decided to continue the test only in the incubator 3, which looked in more acceptable conditions then the incubator 4 and 5.

As a consequence of the first trial, in the second experiment the test was replicated increasing the dose of the TTO to 200 µg/L of TTO. For the second experiment the procedure was the same followed of the first one.

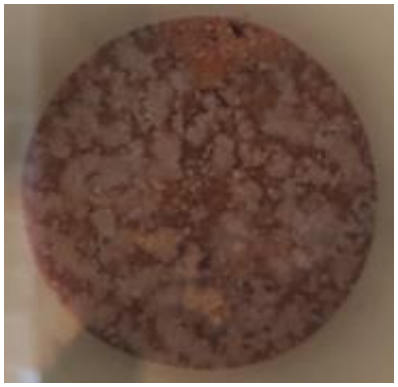
Picture 20 - Breeding and reproduction rainbow trout farm of Erede Rossi Silvio Trout Company, Colli sul Velino (RI).

Picture 21 - Vertical embryos located in the farm in Colli sul Velino (RI).

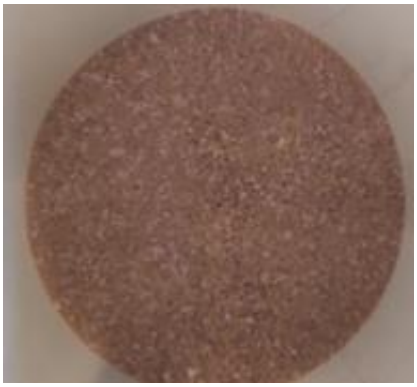
9.1.3. Results

In the first trial after 11 days the “cotton mold” developed on all the treated eggs, and the research group decided to continue the experiment with the TTO application only in one incubator (incubator 3). Pictures from 22 to 26 show the eggs conditions at 11 days of the experiment in each incubator.

Picture 22, 23, 24, 25, 26 – Incubated eggs respectively without any treatment (22-positive control), with the bronopol application (23-negative control), and with the TTO (experimental incubation- 24, 25, 26).

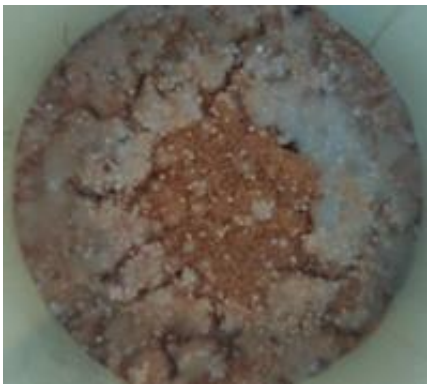


Picture 22 – Positive control, incubator 1. Any treatment was used.



Picture 23 – Negative control, incubator 2. The conventional treatment with bronopol was used.

Picture 24 – Incubator 3 at 11 days



Picture 25 – Incubator 4 at 11 days.

Picture 26 – Incubator 5 at 11 days.

As a consequence of that event, the TTO dosage was increased from 50 to 200 µg/L in the second trial. Despite that, also in this case Saprolegnia spp. grew in the eggs treated with TTO. The molding started to develop after 6 days of treatment, to be well manifested after 10 days of treatment. In consideration of these results, authors decided to interrupt the

experimentation, as the appearance of mold could compromised the percentage of hatching of the eggs.

Analysing the different phases of the experiment, authors supposed that the reason in the different presence of mold between incubator 4 and 5 compared to 3 was caused by an incorrect opening of the water flow, perhaps done too quickly and with a too strong water flow. From the embryonated eggs of hatchery 3, rainbow trout larvae born in good health and without signs of damage or malformations.

9.1.4. Discussion

In both the experiments, the use of only TTO was not sufficient to contrast the development of the typical "cotton mould" caused by Saprolegnia infection on rainbow trout eggs during their incubation. Despite this, authors noticed that the increase of the TTO concentration from 50 to 200 µg/L contrasted the growing velocity of Saprolegnia spp. compared to the first test. Therefore, the higher TTO concentration contrasted the development of the Saprolegnia mycelium, that appeared with a delay of several days. It means that greater quantities of TTO should be used against the antimicrobial agents that could grow on rainbow trout eggs. The right concentrations must be optimized, but it is known that microrganisms such as Staphylococcus spp (fish skin commensals), Micrococcus spp., Enterococcus faecalis, Pseudomonas aeruginosa and several fungal species were found to be sensitive at a TTO concentrations ≤ 1% (Carson et al., 2006). TTO was used on rainbow trout eggs in the past for its antimicrobial properties yet, up to a concentration of 1500 ppm, without toxic effects (Marking et al., 1994).

Furthermore, the possibility of using TTO in association with other essential oils should be considered in order to increase its efficiency through the combination with several essential oils which could work in synergy with each other. In fact it looked to have a greater antimicrobial activity than using only one single component (Davidson PM and Palish ME, 1989; Gill et al., 2002).The use of a combination of essential oils based on *Thymus vulgaris* (thyme), *Salvia officinalis* (common sage), *Eucalyptus* and *Mentha piperita* (peppermint) were tested for the disinfection of rainbow trout eggs during the incubation phase with good results, in fact the mixture of these essences was able to reduce the rate of mold infection and increase the hatching rate. (Mousavi et al., 2009).

9.1.5. Conclusion

In aquaculture the use of completely natural molecules is fundamental to allow the protection of aquatic environment from chemical pollution. Further fish farms could obtain “organic" category products of high quality. TTO should represent a potential antiseptic substance to be used in aquaculture for the disinfection of eggs, thanks to its antimicrobial activities and low level of toxicity. Other natural based-complementary feed was successfully tested on early stages of life of rainbow trout (Aniballi et al., 2020) and these preliminary results open the way to more feeding trials to find the efficiency way to use natural products in substitution of chemical molecules.

10. CONCLUDING REMARKS

The world population continues to grow exponentially, but at the same time the capture fishery production has remained relatively static since the late 1980s. Thus, it is imperative to find a way to provide enough food for such a number. In this context, aquaculture has been responsible for the continuing impressive growth in the supply of fish for human consumption (FAO, 2018). In aquaculture 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 CJ and Jackson AJ, 2013).

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 fish products and to the protection of environmental resources. 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. In the last two decades, new strategies for improving feeding techniques for the main fish species have been developing in order to reach global sustainability. Considering that feed quality also directly affects farming water quality, in the first year of the PhD project a study on the trend of the most important water quality parameters in rainbow trout farming was carried out in a long-term activity (2009–2019). In the optic of sustainable aquaculture, modern researches are oriented towards the partial reduction of fish meal and fish oil in favour of feedstuffs of plant origin. During the PhD triennium the attention was focused on the use of duckweeds and guar gum in rainbow trout feeding as substitutes to conventional protein sources. Furthermore the potential use of

hemp by-products in the feed formulation for aquatic organisms was hypothesized and discussed.

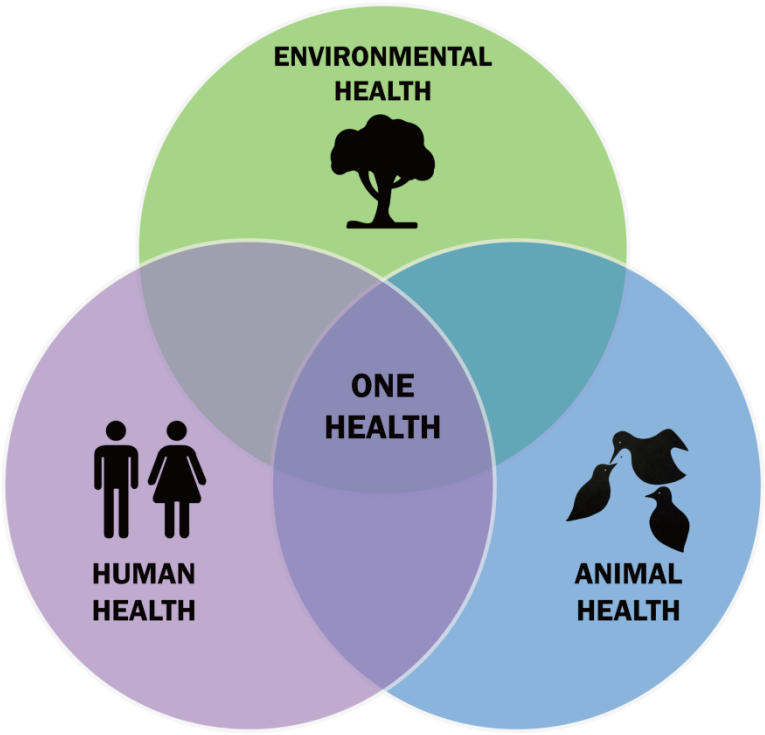
Following the One Health principles, the management of the health status of farmed fish is a fundamental issue in aquaculture. The transmission of pathogens, often associated with overcrowding, which increases the risk of infection, is often the cause of huge economic loss. Antibiotics and various other health care products can no longer be the solution. In aquaculture it is now a day proven that the improvement of fish welfare is an essential factor to prevent diseases and improve non-specific immune response, with consequent improvement of farming and growth fish performances. In fish farming the first step to avoid the use of antibiotic is to improve fish welfare, and therefore to avoid any stress condition that could negatively affect the health status of farming fish. During the PhD triennium, the mechanisms regulating the physiological response to stress conditions before slaughtering in the last phases of farming rainbow trout (*Oncorhynchus mykiss*) were investigated by conducting plasma analyses during the last phase of a standard fattening cycle and by studies on the gene expression in skin mucus. The part of the research on skin mucus analysis was performed in collaboration with the University of Veterinary Medicine of Vienna (VETMEDUNI, Austria), Department for Farm Animals and Veterinary Public Health, Clinical Division of Fish Medicine, under the supervision of the head of the Clinical Division of Fish Medicine, Prof. Monsour El-Matbouli. For a complete antibiotic free fish production, the use of the tea tree oil emulsion was tested for the disinfection of rainbow trout eggs during the incubation phase against the Saprolegnia spp. infection.

Other aspect to consider in the One Health prospective is the respect of the aquatic environment. 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 possible use of fish proteins hydrolysates (FPH) obtained from rainbow trout by-products as a protein source in gilthead sea bream (*Sparus aurata*) diet in the pre-growing phase was analysed. Concerning this topic, the evaluation of organoleptic characteristic and nutritive principles of two sea snails, *Hinia reticulate* and *Nassarius mutabilis*, was performed as food destined to human consumption.

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, the Erede Rossi Silvio Trout Company, in its process of innovation.

THE ONE HEALTH APPROACH



ENVIRONMENT HEALTH

FARMING WATER QUALITY to reduce aquatic pollution

CIRCULAR ECONOMY for waste reduction

ANTIBIOTIC FREE PRODUCTION to reduce environmental contaminants

HUMAN HEALTH

FISH MEAT QUALITY

FISH FEEDIND

ANTIBIOTIC FREE PRODUCTION

ANIMAL HEALTH

FISH WELAFRE

WATER QUALITY

FISH FEEDIND

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ABBREVIATIONS AND ACRONYMS

AA	Arachidonic acid
AMR	Antimicrobial resistance
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
ARA	Arachidonic acid
BOD5	Biochemical Oxygen Demand
Ca	Calcium
Cd	Cadmium
Cr	Chrome
COD	Chemical Oxygen Demand
DHA	Docosahexaenoic acid
Egcs	Embryonic germ cells
EPA	Eicosapentaenoic acid
FAO	Food and Agriculture Organization
FCR	Food conversion rate
Fe	Iron
FHP	Fish hydrolysed products

HE	Intracytoplasmic lipid accumulation
HSI	Hepatosomatic index
IEL	Intraepithelial lymphocytes
K	Potassium
KI	Condition index
MUFA	Monounsaturated fatty acids
Mg	Magnesium
N	Nitrogen
NO2-N	Nitrites
NO3-N	Nitrates
OH	One Health
OIE	World Organization for Animal Health
P	Palatability
Pb	Lead
PFI	Perivisceral fat index
PUFA	Polyunsaturated Fatty Acids
RGR	Relative Growth Rate
SCPs	Single-cell proteins
Se	Selenium

SE	Season of sampling
SFA	Saturated fatty acids
SGR	Specific growth rate
SNK	Student-Newman-Keuls
SP	Species
SR	Survival rate
TAN	Total ammonia nitrogen
THC	Tetrahydrocannabinol
TP	Total phosphorus
TSA	Tryptose soya agar
TSB	Tryptose soya broth
TSS	Total suspended solids
TTO	Tea tree oil
UNICAM	University of Camerino
VETMEDUNI	University of Veterinary Medicine of Vienna
VSI	Viscerosomatic index
WG	Weight gain
WHO	World Health Organization
Zn	Zinc

LIST OF PUBLICATIONS DURING THE Ph TRIENNIUM

Felici, Alberto; Bilandžic, Nina; Magi, Gian Enrico; Iaffaldano, Nicolaia; Fiordelmondo, Elisa; Doti, Gerardo; Roncarati, Alessandra (2020). Evaluation of Long Sea Snail Hinia reticulata (Gastropod) from the Middle Adriatic Sea as a Possible Alternative for Human Consumption. Foods, 9(7), 905–. doi:10.3390/foods9070905.

Report "Valutazione del piano di interventi migliorativi realizzati dall'Azienda Agricolo Troticoltura Erede Rossi Trote nell'impianto di Sefro al fine di salvaguardare l'ambiente e tutelare il benessere animale". 28/03/2020.

Fiordelmondo E, Magi GE, Mariotti F, Bakiu R, Roncarati A. Improvement of the Water Quality in Rainbow Trout Farming by Means of the Feeding Type and Management over 10 Years (2009-2019). Animals (Basel). 2020 Sep 1;10(9):1541. doi: 10.3390/ani10091541.

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Fiordelmondo E, Roncarati A, Vincenzetti S, Pinzaru S. C, Felici A. Sterol and Mineral Profiles of the Common Sea Snail Hinia Reticulata and the Long Sea Snail Nassarius Mutabilis (Gastropods) Collected from the Middle Adriatic Sea. Curr Res Nutr Food Sci 2020; 8(3).

E-poster entitled “The importance of feeding strategies and water monitoring to obtain high quality meat in Rainbow trout production” presented at the Aquaculture Europe 2020 congress

Report: “Valutazione dello stato di benessere della trota iridea sulla base di indicatori ematochimici nelle ultime fasi del ciclo produttivo (raceways, vasca di pre-macellazione, vasca di stordimento”. 29/03/2021.

E-poster entitled “Effects of partial substitution of dietary protein sources with duckweed (Lemna sp.) meal on the Rainbow trout (Oncorhynchus mykiss) growth performances” presented at the Aquaculture Europe 2021 congress

Fiordelmondo, E.; Ceschin, S.; Magi, G.E.; Mariotti, F.; Iaffaldano, N.; Galosi, L.; Roncarati, A. Effects of Partial Substitution of Conventional Protein Sources with Duckweed (Lemna minor) Meal in the Feeding of Rainbow Trout (Oncorhynchus mykiss) on Growth Performances and the Quality Product. Plants 2022, 11, 1220. doi.org/10.3390.

Report: “Impatto del mangime sulla qualità delle acque di allevamento e sulle caratteristiche del filetto nell’alimentazione della trota iridea (Oncorhynchus mykiss). 13/10/2022.

Poster entitled “Use of duckweeds as vegetable protein source in the feeding of rainbow trout (Oncorhynchus mykiss)” presented at the PhD workshop in Life and Health Sciences, Camerino, 25-11-2022.

Fiordelmondo E. and Roncarati A. Impatto del mangime sulla qualità delle acque di allevamento e sulle caratteristiche del filetto nell’alimentazione della trota iridea (Oncorhynchus mykiss). Il Pesce 01/23, 40-51.

Manuscripts under review:

Submitted article to the Frontiers in Veterinary Science journal entitled “Effects of stress conditions on plasma parameters and gene expression in the skin mucus of farmed rainbow trout (*Oncorhynchus mykiss*)”

Manuscript in progress for publication:

Research article on the use of guar gum in the feeding of rainbow trout entitled “Trial on the use of guar protein concentrate as protein source in the feeding of rainbow trout (*Oncorhynchus mykiss*)

Research article on the use of fish by-products in fish feeding entitled “Effects of high substitution of conventional protein sources with fish proteins hydrolysates by-products, from rainbow trout processing, in the feeding for gilthead sea bream in pre-growing phase”.

SCIENTIFIC ACTIVITIES AND TRAINING DURING THE PhD TRIENNIUM

18th - 2th1 February 2020 – Aquafarm Pordenone (UD), Italy.

19th September 2020 – Corso di formazione "Vaccinazione, terapie innovative e qualità dei prodotti ittici". Ostuni (BR), Italy.

30th October – 3rd November 2020 – Poster presentation :"The importance of biosafety in obtaining antibiotic free rainbow trout production". 6th World One Health Congress, Berlave (live streaming), Belgium.

8th February 2021 - Oral scientific presentation: "Valorizzazione dei fish-by products per l'alimentazione degli organismi acquatici". ASPA webinar.

12th – 15th April 2021 – Poster presentation: "The importance of feeding strategy and water monitoring to obtain high quality meat in rainbow trout production". Aquaculture Europe 2020 online "Creating an Optimum Environment".

20th – 21th April 2021- Poster presentation: "Quality traits of fillets of rainbow trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata) assessed by n-3LCPUFA

content and “Antibiotic free” status ”. Congress: 5th International Symposium "Dietary Fat and Health", Frankfurt.

From March to July 2021 and from November 2021 to January 2022, for a total of 9 months: Research training at Clinical Division of Fish Medicine, University of Veterinary Medicine, Vienna, Austria.

21-25 June 2021: 4° European Summer School in Nutrigenomic, University of Camerino (UNICAM)

1st – 6th August 2022 – ASPA Summer School: Protein transition in Animal Feeding. Dipartimento delle Scienza Agrarie e Forestali, Università della Tuscia, Pievetesino (TN), Italy.

5th – 9th June 2022 – Poster presentation: “Effects of high substitution of conventional protein sources with hydrolysed fish proteins in feeding for gilthead sea bream in pre-growing phase”. XX International Symposium on Fish Nutrition and Feeding towards Precision Fish Nutrition and Feeding. Sorrento, Italy.

Anno accademico 2021/2022: Corso di Perfezionamento in "Aspetti Molecolari della Nutrizione: dalla Nutrigenomica alla Nutrizione Funzionale". Scuola di Scienze del Farmaco e dei Prodotti della Salute, Università degli studi di Camerino (UNICAM), Italy.

PROPOSED RESEARCH ACTIVITIES with the University of Veterinary Medicine of Vienna (VETMEDUNI, Austria), Department for Farm Animals and Veterinary Public Health, Clinical Division of Fish Medicine

Training activities and collaboration

The study on rainbow trout skin mucus was performed in collaboration with the University of Veterinary Medicine of Vienna (VETMEDUNI, Austria), Department for Farm Animals and Veterinary Public Health, Clinical Division of Fish Medicine, under the supervision of the Head of the Clinical Division of Fish Medicine, Prof. Monsour El-Matbouli. In that Institute a total of seven moths were spent with high professional activities in different unit with the scope to acquire professional techniques and experience. The clinical unit object of the trial was: RNA- and DNA-laboratories, dissection unit, diagnostic unit, bacteriology unit, fish house. The first part of the mobility was dedicated to personal acclimatization and getting familiar with procedures, helping colleagues with research activities, working in the experimental fish house. In a second time, a personal project on the study of the properties of skin mucus in rainbow trout welfare was proposed to the Head of the Clinical Division of Fish Medicine, Prof. Monsour El-Matbouli, and accepted to be conducted in his Department, making their laboratories and materials available. After that, the study was completely conducted at the University of Veterinary Medicine of Vienna (VETMEDUNI, Austria), Department for Farm Animals and Veterinary Public Health, Clinical Division of Fish Medicine, staying in the university accommodation and participating overall in night and public holidays shifts to take care on animals. All the PhD activities were carried out under the supervision of Dott. Mona Saleh and the laboratory manager Adina Friedl, attending the DNA- and the RNA- laboratories and the microbiology laboratory to conduct activities

related to the identification and the isolation of genetic sequences for the DNA- and the RNA- laboratories, and to perform bacteria isolation related to the microbiology laboratory.

During my permanence in the University of Veterinary Medicine of Vienna, I helped my colleagues (PhD students and researchers in VETMEDUNI) with their experiments. In particular, the first 3 months were spent in the so called "fish lab" performing fish dissections and necrsoscopies under the supervision of Prof. Eva Lewisch. The labs of the University of Veterinary Medicine of Vienna is the National reference laboratory for notifiable infectious diseases.

Furthermore, during my permanence in VETMEDUNI I was employed in the routine house fish keeping in indoor and outdoor tanks, both located in a specific and circumscribed area. The routine activities, holidays days included, consisted of checking the fish healt status, removing the dead fish, clining tahnks, feeding, giving medical treatments. All the activities were recorded in a dedicated book.

Project proposal on skin mucus in rainbow trout welfare

Premise of the project

In aquaculture it is now a day proven that the improvement of fish welfare is an essential factor to prevent diseases and improve non-specific immune response, with consequent improvement of farming and growth fish performances. It is also one of the main principle of the antibiotic free fish production. In fact, in the last few years the prevention of fish diseases has come a priority in order to avoid the use of antimicrobials to optimize human health.

Considering the rainbow trout species as one of the main fish species reared by aquaculture techniques, mucus surfaces, and skin mucus firstly, provide trout with the first line of

defences against threats and pathogens come from the aquatic environment. In particular, skin mucus can be used as a sample to check the health in farming trout, and its collection is possible with a minimally invasive procedure. Furthermore, the study of skin mucus allows not only to acquire information on the healthy status of fish but also on the aquatic environmental conditions in which they live (Cabillon NAR and Lazado CC, 2019), optimizing the knowledge on water quality.

In this context, the present PhD project had the aim to find strategies to increase the non-specific immune defences of farming fish avoiding the use of drug, in particular antibiotics, in order to obtain a high nutritional quality products for human consumption. Considering that animal health is crucial to improve food and nutrition security on one side and Public Health on the other side, fish welfare is an essential factor to improve and the constantly interaction between fish surface and aquatic environment must be considered. Since skin mucus has important defence functions, the current research on fish welfare is moving to analyse the different characteristic of skin mucus from fish living in different conditions.

Description of the projectual research activities

The purpose of this research is to go into detail and better show the topic of skin mucus characterization in order to analyse variations between healthy and stressed trout. Initial experiments were performed in Italy on the response of rainbow trout to stress factors by evaluating the blood concentration of cortisol and blood chemistry parameters (Castro et al., 2011; Sadul B and Geffroy B, 2019). As a corollary to these results, to study fish welfare this project aims to investigate the variation of skin mucus microbiota and skin mucus immune parameters between fish from the last raceway of the farm, as “healthy group”, and fish after stunning, as “stressed group”, taken from the slaughter line after stunning and before killing.

Fish are handled in these last stages before slaughter, and it could cause a stressful condition with a reduction in the efficiency of the innate immune system (Janeway et al., 2001; Gomez et al., 2013).

This research project concerns variation of skin mucus parameters in stressed rainbow trout, compared to healthy fish.

Letter of evaluation of Prof. El-Matbouli (Head of Department for Farm Animals and Veterinary Public Health, Clinical Division of Fish Medicine)

Department for Farm Animals and Veterinary Public Health

Clinical Division of Fish Medicine
Head: Prof. Dr. Mansour El-Matbouli

University of Veterinary Medicine Vienna, Austria

Vienna, 03.02.2022

Letter of Recommendation

To whom it may concern

To Whom It May Concern

I am very pleased to write this recommendation for **Dr. Elisa Fiordelmondo** as I am very grateful for her contributions to our Fish Clinic at the University of Veterinary Medicine and very confident that she has the intelligence, work ethic, and communications skills to add value wherever she works as Fish Health specialist. She was employed in our Fish Clinic and was involved in our routine diagnostic and in the research projects. She was also working intensive on the optimization of the methodologies connected to her PhD project in One Health Science. In particular, she conducted activities on animal house-keeping, animal car and animal cleaning during weekdays and public holidays. She acquired excellent skills and very good practices on fish pathology, molecular genetics and all research related to Fish Medicine. Furthermore, she conducted high performing procedures and activities on bacteriology, MALDI-TOF and PCR- evaluations, with a particular attendance on RNA- and DNA-laboratories.

During her research stay in our clinic she has demonstrated excellent organizational and relational skills and a strong aptitude for working in a team, as well as in perfect autonomy. She has always been punctual, dedicated to work, reserved and attentive. She showed great learning and problem solving skills, organized and reliable. She is flexible and welling to work on any practice proposed to her. She was always ready and welcoming to volunteer to assist colleges in other projects.

I highly recommend Dr. Elisa Fiordelmondo as a candidate for employment as excellent veterinarian.

Sincerely,

(Univ.-Prof. Dr. Dr. M. El-Matbouli)

A-1210 Wien, Veterinärplatz 1
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ATTACHED CHAPTER: Summary of the thesis in Nutrigenomics "Corso di Perfezionamento in Aspetti Molecolari della Nutrizione: dalla Nutrogenomica alla Nutrizione Funzione"

USE OF DUCKWEEDS (LEMNA spp.) AS AN ALTERNATIVE VEGETABLE PROTEINS

On the topic of Nutrigenomics different papers in bibliography show that there is an interaction between nutrition and genotype when a critical component of the diet such as proteins is present in a voluminous vegetative state. This is the case of fresh duckweeds, a group of plants that shows a global constant increasing interest for its organoleptic characteristic and its possible applications.

1. Introduction

The world population had reached 7.3 billion in mid-2015 and it seems to reach 9.7 billion in 2050 (United Nations, 2015). Considering the growing standard of living in the developing countries, it is expected at the same time that the global population increase will require a constantly higher demand of food products from animal origin, especially in relation to protein sources. It seems therefore inevitable to explore alternative sources of protein. […]

2. Chemical composition of duckweeds

In the world there are about 40 different species of duckweeds, and the main ones are of the genus *Lemna, Wolffia, Wolffiella* and *Spirodella* (Les et al., 2002). These plants have few common characteristics in their proximate composition but the most important and common aspect is the good protein content, that ranges between 6.8% and 45.0% in dry matter (Landolt E and Kandeler R, 1987) and a crude protein content between 35% and 43% when the plants grow under ideal conditions (Leng et al., 1995). So, duckweeds are a very good

source of proteins. Moreover, they are rich in both macro and microelements, and vitamins, especially group A and group B. On the other hand the level of fat is very low, only 5% and up to 15% in dry matter, with a polyunsaturated fat content of about 5% depending on the species involved (Leng et al., 1995). The proximal composition of the plant varies according to the species considered. […]

The species *Lemna minor* appears to be one of the most common duckweeds used to make food and feed (Yahaya et al., 2022) and one of the best ones to be used in human and animal nutrition for its organoleptic characteristics. *Lemna minor* is a good source of essential (39.20%) and not-essential (53.64%) amino acids, poly-unsaturated fatty acids (largely a-linolenic acid at around 41-47% and linoleic acid at 17-18%), followed by saturated fatty acids (23-26%), b-carotene, and xanthophylls (Chakrabarti et al., 2018).

3. Duckweeds in animal feed

Vegetables are well known as functional food for their pigments and phenols contents. They show few properties, mainly anti-inflammatory, antioxidant, probiotic, and immunostimulating. For these properties, vegetables are used also for animals feeding, aquaculture included. Now a day aquaculture is a constantly growing sector, which therefore needs the employ of all the scientific world to support the increasing demand of fish products in the planet, without missing the concepts of sustainability, circular economy and food safety. Sustainable aquaculture concept involves environment, community, business, and farm management practices. In particular, in aquaculture one of the main problem is finding new raw materials to be used in fish feeding. Reducing the dependence of aquafeed on marine sources has been a goal in European projects for 25 years. So now it is necessary that a relevant production of feed ingredients is replaced by plant-based sources. But, the increasing feed demand and the competition of plants crops for traditional and novel uses

intensify the search for sustainable sources. Solution deals with new products produced in high efficient system are necessary. In this context, duckweeds should be used to replace commercial fishmeal that is currently used in aquaculture. Duckweeds have been used in the Asian continent in primary production systems for hundreds of years to produce animal feed (Leng et al., 1999). In particular Lemna spp. looks to be a good potential ingredient to replace fishmeal for different reasons. A part its biochemical composition, already discussed and that makes it a good protein source, the low fibre content has a beneficial impact on digestibility (Chakrabarti et al., 2018). Concerning this aspect, digestibility tests of duckweeds were conducted in different fish species, and in carps and tilapia showed promising results (Hassan MR and Chakrabarti R, 2009). Going more in deep, Sharma et al. (2016) reported that the protein content in *Lemna minor* was 39.75 ± 0.47% and that digestibility of this plant protein for (*rohu Labeo rohita*) and common carp (*Cyprinus carpio*) was high as determined by an in vitro digestibility study.

By virtue of all the mentioned properties, duckweeds see the interest of the scientific world grow more and more, and a numerous amount of trials and studies have been conducted by scientific teams all over the world in order to investigate its possible applications in different species. In an interesting study published in 2022, our research group of the University of Camerino conducted a trial with the aim to investigate the use of a local duckweed, recognised as *Lemna minor*, collected in not contaminated waters, as protein source in partial substitution of the conventional feedstuffs, which are fish meal and soybean meal, in rainbow trout (*Oncorhynchus mykiss*) fattening farm to find out how the plant could be used in rainbow trout feeding (Fiordelmondo et al., 2022). Different percentage of substitution were tested, and results showed that the higher replacement was at 20% of substitution instead of standard protein sources without negative consequences in the growth performance of fish and in the quality of the fillet meat. Not adverse effects were observed in fish body weight,

weight gain, and final length, and in the fish fillet proximate composition of moisture, protein, fat, ash did not show notable differences between fish fed with 20% of *Lemna minor* substitution and fish fed with the control diet, represented by a standard diet based on fish meal and soybean meal as protein sources.

Duckweeds see also a very different applications in the world, and they were also largely used for feeding ruminants (Leng et al., 1995; Huque et al., 1996), pigs (Due et al., 1998; Mwale M and Gwaze FR, 2013) and poultry (Haustain et al., 1994; Samnang H, 1999), and for making pet foods (Brown et al., 2013; Yan et al., 2013). Because the inclusion of duckweeds in animal feed is effectively an additional intake of proteins, these plants were added in feed in different animal species, and many trials were conducted during the last thirty years. For example, in 1998 Du and colleagues founded that in pigs the 5% of fresh duckweeds providing in the diet as dry matter looked to have a positive effect on live-weight gain. Some years later, in 2001, Gutierrez proved that the use of 10% of duckweeds in the diet for growing pigs is as a viable option. In poultry farming, already in 1994 Haustain declared that duckweeds used at levels up to 15% in the diets of broiler chickens could represent an important protein source for poultry feed in developing countries, where soybean meal or fishmeal are not available. Some years later, in 1999 Samnang claimed that offering small amounts of fresh duckweeds (30-40 g/day) to chickens improved their growth rate. Therefore, in conclusion, duckweeds could be used in the formulation of balanced diets for broiler, and this was confirmed also by other authors (Ahammad et al., 2003, Khang NTK, 2003, Mwale et al., 2013). Also in ruminants experimental feed trials were conducted, showed that the combination of crop residues and fresh duckweeds provided a balance of nutrients able to optimising rumen microbial fermentative capacity (Leng 1995). Moreover, dry matter and crude protein of the available duckweeds were highly degradable in the rumen (Huque et al., 1996).

4. Duckweeds in human nutrition

Duckweeds are used in human food oriental traditions, in particular in Thailand, Vietnam and Laos. In some areas in the world they are widely used in folk medicine for its cholagogic, carminative, expectorant, diaphoretic, diuretic and anti-carcinogenic properties, and more against bronchitis, liver disease, rheumatoid arthritis and gout (Sonta et al., 2018). Over the years, different populations in the world have used duckweeds as a medicinal plant, and nowadays it is also considered a superfood. In fact, a new trend is an oriental superfood called Mankai. It is a sort of vegetarian burger made with duckweeds characterised by high content of nutritional values, and protein in particular. The Mankai lentil is not only an excellent protein supply, but it is rich in other nutrients: polyphenols, essential amino acids, dietary fibres, vitamin A, vitamins B, iron, folic acid and zinc. In some areas of the world Mankai is called "vegetarian balls" for the protein dose similar to the meat's one. In addiction it helps to regularize the level of blood sugar after carbohydrate intake. The integration of Mankai into human diet would reduce the meat consumption, and all the negative impact of that on the planet, carbon print firstly (Diotallevi et al., 2021; Meir et al., 2021; https://www.forbes.com/health/body/what-is-mankai/). Mankai is just an example to show how duckweeds are employed in the food system to made novel foods, alternative to meat meals. As known, to ensure that new products in the food market are successful accepted and it is purchased and appreciated by consumers, the best strategy to adopt is producing foods yet well known by final consumers, who must be also informed on the beneficial effects of the new food products to encourage their consumption (Beukelaar et al., 2019). A curious example of the use of duckweeds in a common food is an ice cream made with the inclusion of 2% of dried *Lemna minor* (Yahaya et al., 2022). This novel product was subjected to nutritional analysis, showing that the ice cream with the inclusion of *Lemna minor* as an additional ingredient had significantly increased the content of protein, fibre and ash respect

to the ice cream without it; moreover, fat content was reduced in the ice cream with *Lemna minor* respected the control (no inclusion of *Lemna minor*).

5. Sustainability of duckweeds production

The application of duckweeds as potential ingredient in food and feed requires a continuous and constant production, and it must be performed on a sustainable way. Duckweed grows on water with relatively high levels of sodium, potassium and nitrogen (Chakrabarti et al., 2018), and its spontaneous growth is common in lagoon basins, streams and natural ponds. By absorbing these compounds, duckweeds act as a chemical filter of the aquatic environment in which the plant grows. That means that to be used in the human food industries its growth required clean and quality water and controlled aquatic environment. To make possible the use of duckweeds in human and animal nutrition at a global level, it is necessary to create and implement a large-scale cultivation. Regard that supposition, it is inevitable not thinking about some practical considerations and answer to some questions. Regardless of the scope, if it is a low (5%) or high (20% and more) duckweeds intake in food and feed, as a supplementation in recipes or as meat substitution, how many kilos of duckweeds are necessary and must be produced to support the productions of novel food and feed? Which volume of water, clean and not contaminated, is required? What about the outlet water? How much big ponds or tanks must be? Do they need a cleaning treatment with chemical substances? Finally, is this cultivation really sustainable? The best scope to reach in the next future should be making the cultivation and the extraction process of duckweeds in a low impact way, environmentally sensitive aqua farms, using no pesticides and recycling the most quantity of water as possible. Furthermore, a safety protocol needs to be established to produce high nutritional food with free food-borne microorganisms.

6. Future applications in animal feed and human nutrition

The demand for animal products, especially in developing countries, will increase significantly due to population growth and urbanization, and the levels of consumption of animal products are constantly growing. For these reasons, in farming realities new feeding strategies are necessary to cope the constantly growing demand of animal products, and in the food industries developing innovative technologies to produce most sustainable plant-based protein is required. In this prospect, duckweeds look to be a good candidate thanks to several attributes which include ease of establishment, adaptation to a wide range of agro-ecological zones, minimal carbon footprint and high nutrient composition. All these elements make this plant the ideal novel ingredient in human food and animal feed. Beyond their proximate composition and the potential application in food and feed industries, duckweeds are already appreciated also for other types of qualities. The main one is the ability to reduce the concentrations of nutrients in water, absorbing in particular nitrogen compounds (Ceschin et al., 2020). The great potential of removing mineral contaminants from wastewater (from sewage treatment plants, intensive livestock industries or from intensive irrigation crop production) increases the interest of scholars and scientists on the possible applications of duckweeds in the next future. Due to their excellent ability to absorb nutrients and produce at the same time a good quantity of protein biomass, duckweed ponds should be taken into consideration as a potential cleaning and filtering wastewater activity. As a support to this consideration, in Brazil duckweed ponds have been successfully used in swine waste polishing using *Landoltia punctata* (Mohedano et al., 2012). Also other authors recently suggested that these plants should be used as purifying plants in the next future for biological waste water treatment, because they adsorb ammonia from the water, specially nitrates (Sonta et al., 2019). And just for this particular skill, early in 1995 Leng and colleagues suggested that ionized ammonia (NH^{+4}) should be used as preferable nitrogenous substrate for *Lemna*

minor culture in order to produce this plant in a big scale. Finally, in Polish duckweeds were yet used from years for a complete different issue: the production of a biomass to obtain an alternative substrate for the production of renewable energy (Czerpak R and Piotrowska A, 2005).

Concerning applications in human health, Czerpak and colleagues in 2005 suggest that *Wolffia arrhiza* can be taken into consideration in scientific researches on biotechnology in order to obtain important biologically active compounds for dietetics, phytotherapy and phytocosmetics. Furthermore, for its antioxidant activity, duckweeds should be potentially used to protect human cells from oxidative damage and inhibit cancer growth (Yahaya et al., 2022).