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EFFECT OF FUNCTIONAL FEED ADDITIVE ON SYMPTOMS REDUCTION OF ENTEROMYXOSIS IN GILTHEAD SEABREAM “SPARUS AURATA”.
(Field experiment)

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Master's degree in Aquaculture & Fisheries

(Field of Aquaculture)

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2020/2021

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Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam na listagem de referências incluída.

Universidade do Algarve, 30 de setembro de 2021

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Abstract:

Aquaculture is a fast-growing sector, very important for the food, nutrition, income, and livelihood of hundreds of millions of people. The demand for seafood is increasing and aquaculture plays an important role in satisfying that. The increasing number of fish farmed go at the same pace as the health and welfare problems especially because of the high density of the fish. One of the biggest sanitary problems for the gilthead seabream industry is *Enteromyxum leei*, a myxosporean parasite since the infection has a chronic course and is responsible for poor food conversion rate and difficulty to reach commercial size. Unfortunately, no treatments are authorized for this parasitosis, but a recent experimental study shows a good result using SANACORE® functional feed as a treatment for this parasitosis. This study aims to verify the efficacy and the feasibility of the functional feed as a possible treatment for *E. leei*. The trial was done in an important commercial facility of gilthead seabream in Tuscany (Italy) where enteromyxosis has been already diagnosed in the past. For this study, two different concentrations of SANACORE® are tested, respectively 0.5% and 0.7%. Prevalence and intensity of the parasite in the fish are evaluated using microscopy observation and molecular diagnosis (PCR) to combine qualitative and quantitative parasitological analyses comparing the groups under study. The weight and length of all the fish sampled were recorded and a biomass index was measured and compared along with the different fish groups. Unfortunately, the occurrence of a severe secondary bacterial infection influenced the evaluation of the efficacy of SANACORE's inclusion in fish feed. Concerning the comparison of the different diagnostic methods, the results showed that the qPCR is a more suitable method for the diagnosis of Enteromyxosis. Because of the possibility of a non-lethal technic to collect the samples (by rectal swab), it can be used as a valid screening test in a farm for newly introduced fish.

Keywords: Mediterranean Aquaculture, gilthead seabream, *Enteromyxum leei*, functional feed, sustainable aquaculture.

Resumo:

A aquacultura é um sector em rápido crescimento, muito importante para a alimentação, nutrição e sustento de centenas de milhões de pessoas. A procura dos produtos do mar tem crescido e a aquacultura tem tido um papel importante de modo a satisfazer as necessidades do mercado. O aumento do número de peixes cultivados contribui também para o aumento dos problemas associados à produção, especialmente devido à elevada densidade de peixe nos locais em que é cultivado. Um dos maiores problemas sanitários que a indústria de produção de dourada (*Sparus aurata*) enfrenta é *Enteromyxum leei,* um parasita mixosporiano, que causa infeções crónicas e é responsável por baixas taxas de conversão alimentar causando dificuldades em que os peixes atinjam os tamanhos comercializados. Infelizmente, ainda não há nenhum tratamento autorizado para esta parasitose, mas recentemente um estudo experimental demonstrou bons resultados através do uso da ração funcional SANACORE®. O objetivo deste estudo é verificar a eficácia e viabilidade do alimento funcional como possível tratamento para a *E. leei.* O ensaio foi realizado numa importante instalação comercial de Dourada na Toscana (Itália), onde enteromixose já tinha sido diagnosticada. Para este estudo, foram testadas duas concentrações diferentes de SANACORE®, 0,5% e 0,7%, respetivamente. A prevalência e intensidade do parasita nos peixes foi avaliada através de observações microscópicas e diagnósticos moleculares (PCR) para combinar análises parasitológicas qualitativas e quantitativas comparando os grupos em estudo. O peso e comprimento dos peixes amostrados foram registados e o índice de biomassa foi medido e comparado entre os diferentes grupos de peixes. Foi esperado um aumento da biomassa e redução da intensidade do parasita nos peixes tratados com ração funcional o que poderá ser uma boa oportunidade para o aquicultor reduzir os custos associados a baixas taxas de conversão alimentar.

Infelizmente, a ocorrência de uma severa segunda infeção influenciou a avaliação da eficácia da inclusão de SANACORE na ração para peixe. Relativamente à comparação dos diferentes métodos de diagnóstico, os resultados demonstraram que o qPCR é o método mais adequado para o diagnóstico de Enteromixosismo. Devido à possibilidade de uma técnica não letal para recolher as amostras (esfregaço retal), esta pode ser usada como um teste de triagem válido numa aquacultura para os peixes recém chegados.

Abbreviations:

ANOVA - analysis of variance

CTRL – control

Ct – threshold cycle

FCR – Feed conversion rate

K - Fulton's condition factor

L – Length

PCR - Polymerase chain reaction

qPCR – quantitative Polymerase chain reaction

SGR – Specific Growth Rate

W – weight

Contents

IMPORTANCE OF THE STUDY

This study takes place within the framework of the H2020 EU-funded Project PerformFISH - Integrating Innovative Approaches for Competitive and Sustainable Performance across the Mediterranean Aquaculture Value Chain (www.performfish.eu) coordinated by Katerina Moutou – University of Thessaly (Greece), in which UNIBO is partner. The planned field trial would aim to test on the field the effect of a functional feed additive on mitigation of the intestinal parasitic disease of *Sparus aurata* due to the myxozoan *Enteromyxum leei*, following the encouraging preliminary studies carried out in experimental conditions during the H2020 EU funded Project ParaFishControl - Advanced Tools and Research Strategies for Parasite Control in European farmed fish (www.parafishcontrol.eu). The results of the field trial will contribute to the improvement of knowledge on management and control strategies of one of the most impacting diseases of farmed gilthead seabream.

The purpose of this work is to evaluate the efficacy of the use of SANACORE® functional feed as a treatment for reduction of infection and related clinical signs induced by *E. leei* parasitosis in Gilthead seabream.

Specifically, this research wants to evaluate if it's possible to use a functional feed as SANACORE® as a treatment for *E. leei* infection in fish naturally infected in intensive fish farm conditions.

1. Introduction

1.1. Importance of aquaculture

Aquaculture is a fast-growing activity on all continents with an increased rate of around 7% per year, accounting for more than half of the fish used for human consumption (Engle et al., 2017). Is an important source of food, nutrition, income, and livelihood for hundreds of millions of people around the world.

According to the latest worldwide statistic of aquaculture compiled by FAO, the farming of aquatic animals in 2018 was dominated by finfish with 54.3 million tonnes (USD 139.7 billion) (FAO, 2020).

Mediterranean sea is a semi-enclosed sea, surrounded by 24 African, Asian and European countries and has a unique characteristic in terms of environmental, sociological, and economic features (Massa et al., 2017). In the Mediterranean area, there are different environmental, geomorphological, hydrogeological, and climate regions and for these reasons, different aquaculture systems and technologies develop and succeed.

The growth rate recorded in the period between 1997 and 2007 was 70% and the aquaculture sector in the Mediterranean region is expected to continue developing in parallel with the decline of the wild stock and the increasing of the demand for fish products for human consumption (Plan Bleu, 2014). In the MedTrends report, published by WWF in 2015, the Mediterranean aquaculture sector may grow by more than 100% by 2030 up to a total production exceeding 600,000 tonnes (Piante & Ody, 2015).

While the growth provides a rise in the sector value with additional jobs in the Mediterranean countries, it will be associated with environmental challenges especially regarding the biological interaction caused by the unintentional release of farmed organisms and the effluent discharge from aquaculture facilities (IUCN, 2007).

1.2. Gilthead seabream

Gilthead seabream belongs to the Sparidae family (Teleostei: Perciformes), which comprises 35 genera and 112 different marine fish species. The main distinctive features of the species are the silver-grey color with a large black blotch at the origin of the lateral line extending over

the upper margin of the opercula and the golden frontal bland between the eyes (Figure 1) (Pavlidis & Mylonas, 2011).

Gilthead seabream is found in temperate, subtropical, and tropical littoral waters and brackish inshore waters, commonly in seagrass beds and sandy or rocky bottoms. The species is eurythermal (tolerance range 5°-33° C) and euryhaline (tolerance range 3-70 ‰), thought its optimum temperature and salinity ranges for reproduction are 14°-18° C and 37-38 ‰ (Moretti et al., 1998).

Juveniles migrate towards shallower protected coastal areas in spring, living in waters of 30 m depth, while adults return to deeper waters during autumn, being found in depths up to 100-150 m. They are sedentary fish, rather solitary, though also forming aggregations during reproductive migrations. Gilthead seabream is mainly a carnivorous fish, feeding preferably on bivalve mollusks and small fish, cephalopods, or crustaceans, though also occasionally herbivorous. It is a protandrous hermaphrodite fish developing sexual maturity as male at 2 years of age (20-30 cm) and as female at 2-3 years (33-40 cm). Gilthead seabream is an excellent food fish and has been traditionally cultured extensively in coastal lagoons and saltwater ponds of the Mediterranean (FAO 2022).

During the early ’80s, artificial breeding was achieved and in turn, large-scale production of juveniles started. The species adapted quickly to intense rearing conditions and is nowadays cultured in cages, ponds, and tanks. From the hatching, it takes between 18 and 24 months for gilthead seabreams to reach a 400 g size and commercial sizes range from 250 g to 1,500 g (Moretti et al., 1998).

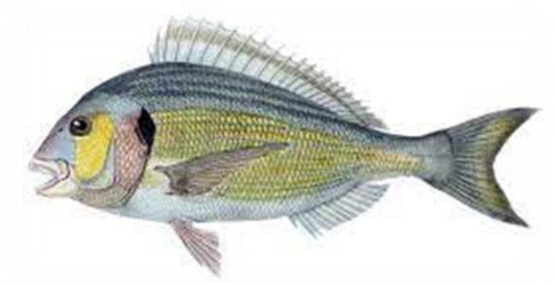


Figure 1:Gilthead seabream (Sparus aurata) draw by FishEthoBase (http://fishethobase.net/db/49/)

1.3. Parasitosis in Gilthead Seabream

Among the problems that arise from these types of production, the occurrence of transmissible diseases, comes with great importance.

Parasitic pathologies have acquired increasing importance among the factors affecting animal welfare and limiting aquaculture productivity (Piazzon et al., 2017; Cuadrado et al., 2008; Fioravanti et al., 2006).

Intensification of marine fish aquaculture has favored the development and severity of parasitic infections which can be further worsened by an immunosuppressed status of the fish due to the environment or dietary factors (Fioravanti et al., 2006). Culture procedures influence greatly the type and severity of the disease. The structural characteristic of the facilities whether inland, inshore, offshore floating cages or submersible cages, are commonly decisive for the onset of the infections and the appearance primary and opportunistic pathogens. Related factors involved are fish density, water renewal rate, proximity to the seabed, water temperature, and water salinity (Fioravanti et al., 2006).

The main detrimental consequences caused by parasites on cultured fish are:

- high mortalities during outbreaks;
- chronic mortalities throughout the life cycle;
- decreased growth performance;
- increased susceptibility to secondary infections.

Several parasites with different levels of pathogenicity have been reported in gilthead seabream as shown in the table below (Table 1).

*Table 1 : Major parasites affecting gilthead seabream (Sparus aurata) *Direct ** Indirect* (Muniesa et al., 2020).

Etiological agent	***Disease***	***Site of infection***	***Transmission***
Amyloodinium ocellatum (Dinoflagellata)	Amyloodiniosis (velvet disease)	Skin, gills	D*
Cryptocaryon irritans (Ciliophora)	Marine white spot disease	Skin, gills	D*
Eimeria spp. (Apicomplexa)	Coccidiosis	Gut	D*
Enterospora nucleophila (Microsporidia)	Microsporidiosis	Muscle, gut	D*
Enteromyxum leei (Myxozoa)	**Enteromyxosis or "razor blade syndrome"**	**Gut, gall bladder, liver**	**D*, (I**)**
Sphaerospora sparis (Myxozoa)	Renal Sphaerosporosis	Kidney	I**
Sparicotyle chrysophrii (Monogenea)	Sparicotylosis	Gills	D*
Cardicola aurata (Digenea)	Blood flukes	Gills (eggs), Heart (adults)	I**
Isopoda e Copepoda (Crustacea)	Crustaceans infections	Skin, mouth, gills	D*

Among these, some diseases caused by myxozoan parasites have taken on increasing importance, causing considerable economic losses (Feist et al., 2008; Fioravanti et al., 2006). In particular, the enteric parasite *Enteromyxum leei*, which already in 1995 was described by Le Breton and Marques as a future threat to marine farms in the Mediterranean (Le Breton & Marques, 1995), and today represents a pathogen of primary importance in the intensive farming systems of the Sparids, gilthead seabream and sharpsnout seabream overall, mainly because its direct transmission by the fecal-oral route and the lack of effective and authorized therapeutic devices.

1.4. Myxosporean

Myxosporean being common parasites affecting intensively reared fish in the Mediterranean Sea, induce a broad spectrum of diseases depending on parasite species, host sensitivity,

environmental and feeding conditions (Rigos & Katharios.,2010; Sitjà-Bobadilla et al., 2007; Golomazou et al.,2006; Rigos et al., 1999). Most of them are not highly pathogenic for their host but in some cases, cause important disease, and severe economic loss in aquaculture.

Myxozoas are multicellular and spore-forming endoparasites that infect mainly marine and freshwater fish. Commonly, their life cycle includes two hosts: an invertebrate definitive host and a vertebrate intermediate host (Yokoyama & Shirakashi, 2007). Direct transmission is also reported for some marine species such as species belonging to the genus *Enteromyxum* (Gómez et al., 2014).

Enteromyxum leei was first described from tank-reared gilthead seabream and associated with mortality outbreaks (Diamant et al., 1994). Thereafter, mortality and morbidity caused by the parasite in aquaculture facilities were reported on many occasions for gilthead seabream (Palenzuela et al., 2020; Davey et al., 2011; Sitjà-Bobadilla et al., 2008; Cuesta et al., 2006; Fioravanti et al., 2006). Other marine and freshwater fish, like zebrafish (*Danio rerio)*, tiger barb (*Puntius tetrazona*) are also susceptible (Sitjà-Bobadilla et al., 2007; Golomazou et al., 2006) convening this parasite a very important disease.

Enteromyxum leei infect gilthead seabream (*Sparus aurata*) among other sparids and up more than 60 different species (Figure 2) and its definitive host remain unknown (Golomazou et al., 2006). In literature is already known how big is the problem of *E. leei* for intensive farming of seabream in terms of fish mortality and economic losses.

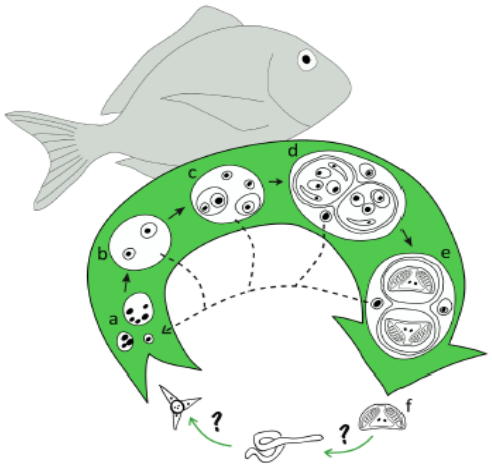


Figure 2 :Life cycle of Enteromyxum leei in Gilthead Seabream "Sparus aurata". In the intestinal epithelium of the fish, proliferative (a-c) and sporogonic (d-f) development occurs. Stages a-e are responsible for the invasion and dispersion within the fish, as well as for transmission to other fish, the definitive host is unknown (from Estensoro et al., 2013*).*

1.5. Enteromyxosis in seabream

Enteromyxosis in gilthead seabream is a chronic disease, evident in land-based facilities but sometimes undetected in cages. The parasite induces severe catarrhal enteritis, with direct mortality (rate up to 20%), and also causing weight loss, poor conversion rates, delayed growth, and reduced marketability. The most evident clinical sign is severe emaciation which means in seabream the typical knife-edge body shape, sometimes accompanied by distended abdomen, accumulation of ascitic fluid, and bile (Figure 3).

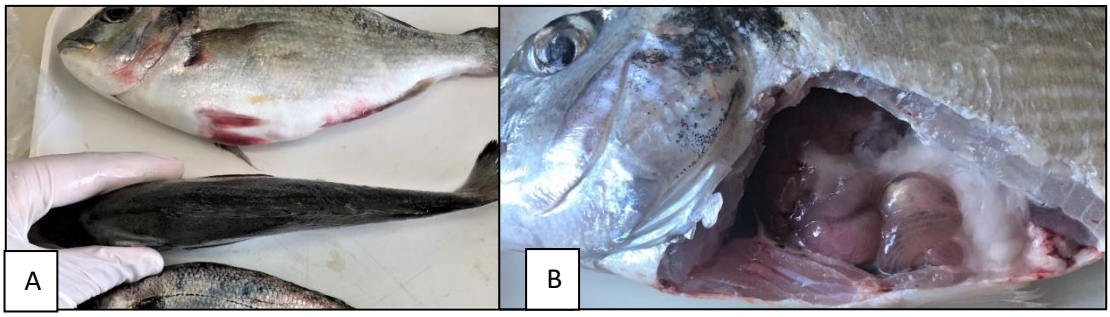


Figure 3: General finding in Gilthead seabream affected by E. leei. a) Distended abdomen caused by ascites and the typical knife-edge; b) Gilthead seabream enteritis caused by E. leei. Photo Mirko Pierantozzi.

The infection in gilthead seabream is usually restricted to the intestine, following a directional pattern from the rectum towards the anterior intestine and pyloric caeca. At the early stages of the infection, the parasite presents a random distribution in localized foci. In heavily infected fish, parasite stages have occasionally been observed in the urinary bladder, gall bladder epithelium, intrahepatic biliary ducts, and the gastric lumen (Cuadrado et al., 2008; Fleurance et al., 2008; Diamant et al., 1994).

Macroscopically, infected intestine shows fragile and semitransparent walls, focal congestion, and luminal mucus liquid. Histopathology of Enteromyxosis in gilthead seabream shows intestinal epithelia invaded and disorganized by parasite stages leading to the desquamation of the epithelial layer and subsequent disruption of the epithelial barrier and impairment of the absorptive intestinal function (Estensoro et al., 2013; Alvarez-Pellitero et al., 2008).

E. leei stages stay in the paracellular spaces between enterocytes, extend the cytoplasmatic projection and present a convoluted surface to increase their absorptive area, thus enhancing nutrition from host cells (Cuadrado et al., 2008). The inflammatory response induced by the

host includes hypertrophied submucosae infiltrated by numerous immune cells (Fleurance et al., 2008).

Infected gilthead seabream exhibits a cachectic syndrome as a result of the numerous systemic and local impacts of the parasite on the fish physiology. The heavy weight loss is not only a consequence of the damaged intestinal tissue but also attributed to anorexia, intestinal osmoregulatory failure, anemia, and the metabolic cost of the immune response itself (Sitja-Bobadilla,A, 2012).

Among the factor influencing enteromyxosis, water temperature is determinant for its onset, since the development of the parasite is suppressed below 15°C. However, the infection can remain latent during the cooler period. This fact is very important because a false-negative subject (during winter) could be a source of the parasite when the temperature rises. (Golomazou et al., 2014). The optimal temperature for developing enteromyxosis clinical signs in Gilthead seabream, ranges from 18°C to 22°C (Estensoro et al., 2010).

Another factor involved in the fish susceptibility to enteromyxosis is the diet. It has been suggested, that the proliferation of the parasite is favored in fish fed a diet with excessive fat content (Rigos et al., 1999). The diet plays an important role in maintaining intestinal integrity and functionality, including its microflora, which are essential keys for an effective intestinal epithelial barrier and immune response against a pathogen (Sitjà-Bobadilla et al., 2016; Fekete & Kellems, 2015).

Enteromyxosis does not have specific clinical signs and for this reason, it cannot be diagnosed directly from a clinical approach (Gómez et al., 2014). Confirmatory diagnosis usually is made by detection of parasite stages in infected intestinal smears, fresh or stained, histological sections, or by molecular methodology (Sitja-Bobadilla, 2012). Early detection is crucial for the isolation or sacrifice of infected fish stocks, the direct fish to fish transmission makes an important role in the spreading of the disease in cultured stocks and hampers its prevention and control.

There are no approved antiparasitic chemotherapeutics treatments against *Enteromyxum* spp. or any other myxosporean. Against *E. leei* some coccidiostats were tested with relative success (Sánchez-García et al., 2014; Sitja-Bobadilla, 2012.; Davey et al., 2011). The

combination of Salinomycin and Amprolium significantly reduce prevalence, intensity, and mortality in *E. leei* infected sharpsnout seabream without apparent toxic effects (Golomazou, 2006).

However, empirical evidence in the farms and recent laboratory-controlled experiments have shown promising results in the control of enteromyxosis in gilthead seabream, *Sparus aurata* using some functional feed additives (Palenzuela et al., 2020; Piazzon et al., 2017).

1.6. Functional feed

Because of the lack of effective and/or authorized anti-parasitic treatments, the nutraceutical approach is raising interest in the aquaculture industry and represents a great opportunity, responding to the lack of therapeutic tools for the management of parasitic problem (Palenzuela et al., 2020).

The goal of functional feed is to promote the growth and the health of farmed organisms, improve their immune system and induce physiological benefits beyond traditional feeds. The nature and characteristics of the feed additives are diverse and their application into diet formulations targets a specific purpose. Some additives, such as acidifiers or exogenous enzymes are used to improve the animal's performance, counteracting the negative effect of antinutrients, or improving the digestibility of feed nutrients (Encarnação, 2016).

The concept of functional aquafeeds represents an emerging new pattern to develop diets for fish and crustaceans. Feed transformation into biomass gain is a process that starts in the digestive system of the animal. As such, its health and its functionality correlate directly with the economic results of the farmer.

It is well known that the gastrointestinal tract is responsive and sensitive to a wide range of stressors. Some of the more common features are degeneration of the intestine mucosa and disturbance /alteration of its barrier function and uptake mechanism. Closely connected with the state of health of the gut is a well-balanced intestinal microflora, which helps the digestive and absorptive process and protects the host against invading pathogens. Several studies have also shown that different feed ingredients and changes in diet composition can affect the gut structure and microbiota balance influencing digestive and absorption functions (Ringø et al., 2007; Bendiksen et al., 2000).

Alteration of the intestinal microbiota composition and consequent reduction of protective gut microflora may contribute to pathogenesis in the gut. Management of the gut flora is, therefore, an important issue to achieve good feed efficiency, animal growth, and animal health. A sustainable way to manage gut microflora and fish performance can be the use of nutraceuticals or functional foods as feed supplementing, modulating health, and performance of farmed animals (Ringø et al., 2007).

There are several options available to manage and regulate the fish gut environment, which includes the use of probiotics, prebiotics, immune-stimulant, phytogenic substance, and organic acids. Natural compounds with anti-parasitic activity can directly affect gut parasites or indirectly affect ectoparasites through their effects on blood or the composition and quantity of mucus (Henry et al., 2020; Glencross, 2016; Coutteau, 2014).

2. Objectives

Parasitic enteritis caused by the myxosporean *Enteromyxum leei* in gilthead seabream is among the most important diseases with pathological impact in marine Mediterranean pisciculture.

E. leei threatens the fish health status and causes important economic losses to the aquaculture industry. The control of enteromyxosis is difficult due to its horizontal fish-to-fish transmission and the lack of effective preventive as well as therapeutic treatments. The host range of *E. leei* poses many reservoirs able to transmit the disease, including numerous other fish species (marine and freshwater species) and potential invertebrate hosts.

Scarce studies exist so far regarding the effect of functional feed additives on the gilthead seabream *Sparus aurata* resistance to enteromyxosis. The recent study of Palenzuela et al. (2020) showed the possibility to use a functional feed as 'SANACORE®' as a preventive treatment against *E. leei* in Gilthead seabream. In this work, they used two different concentrations of functional feed 0.2-0.4% and were demonstrated efficacy especially in the group with higher doses, to reduce the prevalence and intensity of *E. leei* infection.

The present work aims to evaluate the efficacy of SANACORE® functional feed, at different concentrations of inclusion, as a therapeutic treatment for *E. leei* in Gilthead seabream.

Specific objectives of this thesis are:

- Evaluation of the efficacy of SANACORE® in the reduction of intensity and prevalence of *E. leei* in infected Gilthead seabream.
- Evaluation of the effect of SANACORE® in the Specific Growth Rate in Gilthead seabream.
- Comparison of different diagnostic methods for the detection of *E. leei.*
  Microscopical evaluation of fresh intestinal smear ***vs*** Molecular methodology ***vs*** Histology.

3. Material and methods

3.1. Farm and Fish

The study was performed in a commercial fish farm located in the Orbetello area, where Enteromyxosis has been diagnosed in the past and is still present on the farm. The lagoon of Orbetello, located on the southern coast of Tuscany, is one of the brackish wetlands still preserved in Italy and covers a total area of 25.25 km^2 (Figure 4). The majority of marine products in Tuscany are reared in land-based fish farms, using intensive techniques, the southern coast of Tuscany, have the greatest concentration of these farms.

The Orbetello intensive aquaculture began in the middle of the 1970s and grew during the 1990s, producing at that time nearly 30% of the Italian seabream and seabass (Thomson & Venzi, 2005).

Figure 4 : Aerial view of Orbetello lagoon (from: https://siviaggia.it).

In this fish farm, water is pumped from the ground at a constant temperature of 20 °C and salinity in the range of 17-20 ‰. Water is partially recirculated and aerated by mechanical agitation of water and direct supply of liquid oxygen.

Seabream is produced in 600 m^3 concrete tanks, with a fish density of approximately 30 (up to 40) Kg/m^3 (Figure 5; Figure 6). These are perfect conditions for the survival of *Enteromyxum leei.*

Figure 5: General view of the fish farm. Photo Mirko Pierantozzi.

Figure 6: Land-based tank with a mechanical aerator. Photo Mirko Pierantozzi.

For this study, we incorporate SANACORE® in the fish feed, at 0.5% and 0.7%, as a method to control Enteromyxosis impact on gilthead seabream production.

Previously to the feed experiment, a survey on the *E. leei* infestation status on 8 tanks of the farm, was performed. In January 2021, 10 fish from each tank were sacrificed and examined for the presence of *E. leei*, both by microscopical examination and qPCR test. The 3 tanks with the higher positivity for *E. leei*, were chosen for the feed trial.

During 105 days, from 6/03/2021 to 19/06/2021, 300 g mean weight gilthead seabream from each of the chosen tanks, received a diet with different levels of SANACORE® incorporation. Fish were fed with the daily amount suggested by the manufacturer. Group A receives basal control feed (diet A) with no SANACORE®, group B (diet B) the same feed with the inclusion of 0.5% of SANACORE®, and Group C with 0.7% of SANACORE® (diet C). (Sanacore® % = SANACORE® weight / feed weight)

The study was carried out with Gilthead seabream (*Sparus aurata*) by three partners involved in PerformFISH project. The thesis author was involved by the partner UNIBO (University of Bologna) for sample collection and analysis.

3.2. Functional feed plan

SANACORE® is a mix of organic acids, inactivated yeast, and yeast extract (*Saccharomyces cerevisiae*) with herbal extracts on a mineral carrier.
SANACORE® additive was mixed every day with the traditional feed directly in the fish farm using a feed mixer. For the mix, a vegetable oil coating was used to permit SANACORE® powder to remain attached to the food pellet (Figure 7).

Figure 7: A) SANACORE® powder; B) Feed pellets after SANACORE® inclusion. Photo Mirko Pierantozzi.

As suggested by the feed additive supplier, during the first 15 days of the trial, an "attack dose" was used by administering a double dose of SANACORE® (1.0% and 1.4% incorporation respectively for group B and C). For the following experimental days, fish groups were fed with the experimental supplement doses (0.5% and 0.7%).

3.3. Sampling plan

On days 15 (T1), 45 (T2), 75 (T3) and 105 (T4), 30 fish from each group were randomly sampled, using a hand net. Immediately after being caught, fish were euthanized by anesthetic overexposure, using clove oil at the dose of 0.005 mL/500 mL. Total weight and total length were registered. Ten fish were immediately subjected to the microscopical examination of intestinal smears, looking for the presence or absence of *E. leei*. From the same fish it was collected a rectal swab for qPCR and a portion of intestine, for histology. The remaining 20 sampled fish/tank were transported to the University labs and subjected to microscopical observation and rectal swab for molecular analysis.

Dead fish were collected every morning by the workers in each tank and mortality rate was calculated. The percentage of mortality was obtained counting the total number of dead fish in that period on the total number of the fish in that tank.

Daily food intake was recorded, and the performance index specific growth rate (SGR), feed conversion rate (FCR), and Fulton's condition factor (K) were calculated.

The specific growth rate (SGR) was calculated for each group following the formula:

$$Specific\ growth\ rate\ (SGR) = \frac{Ln\ (Final\ weight\ (g)) - Ln\ (Initial\ weigth\ (g))}{Culture\ days} \times 100$$

Feed conversion rate (FCR) was calculated following the formula:

$$FCR = \frac{feed\ intake\ (g)}{weight\ gain\ (g)}$$

Fulton's condition factor (K) was calculated with the formula given below:

$$K = \frac{W \times 100}{L^3}$$

Where, W=weight of fish (g), L=Length of fish (cm).

Sampling activities were scheduled on monthly basis except for the T1 sample, taken after 15 days of double doses ("attack dose") administration (Figure 8).

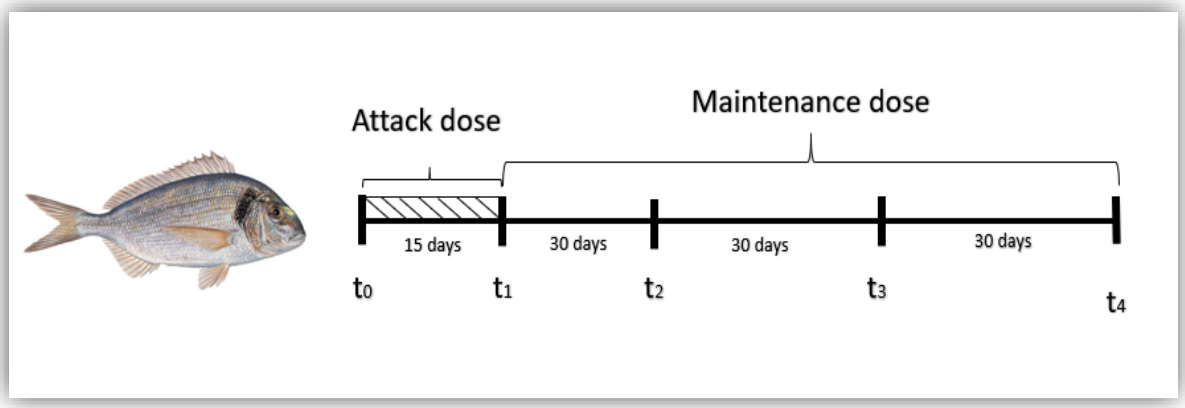


Figure 8: Sampling timeline applied to the experiment. On each sample date (T1, T2, T3, T4), 30 fish from each group of Gilthead seabream "Sparus aurata", were collected for Enteromyxum leei analysis.

3.4. Laboratory analysis

For the diagnosis of *E. leei,* three different methods were performed and compared: microscopical observation of intestinal smear, microscopical observation of histological preparations of intestine (proximal, medial, and distal part), and molecular analysis (qPCR).

3.4.1. Microscopical observation - smear

For the microscopical smear analysis, the anus was squeezed, and a little drop of intestinal content was collected on a slide and covered with a coverslip. The observation was performed

with a light microscope. For each fish, 100 random observations per slide (400X magnification) were performed, following an imaginary line as in Figure 9.

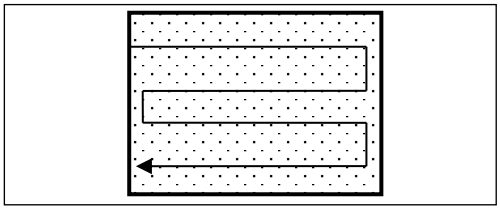


Figure 9: Representation of the method used for the random observation for the detection of parasite in intestinal smear.

Only sporoblast and mature spore were counted (Figure 10). The final total number of the parasite counted in 100 random field observation represent the intensity value of infection for that fish. Fish considered positive when at least 1 sporoblast or mature spore was found.

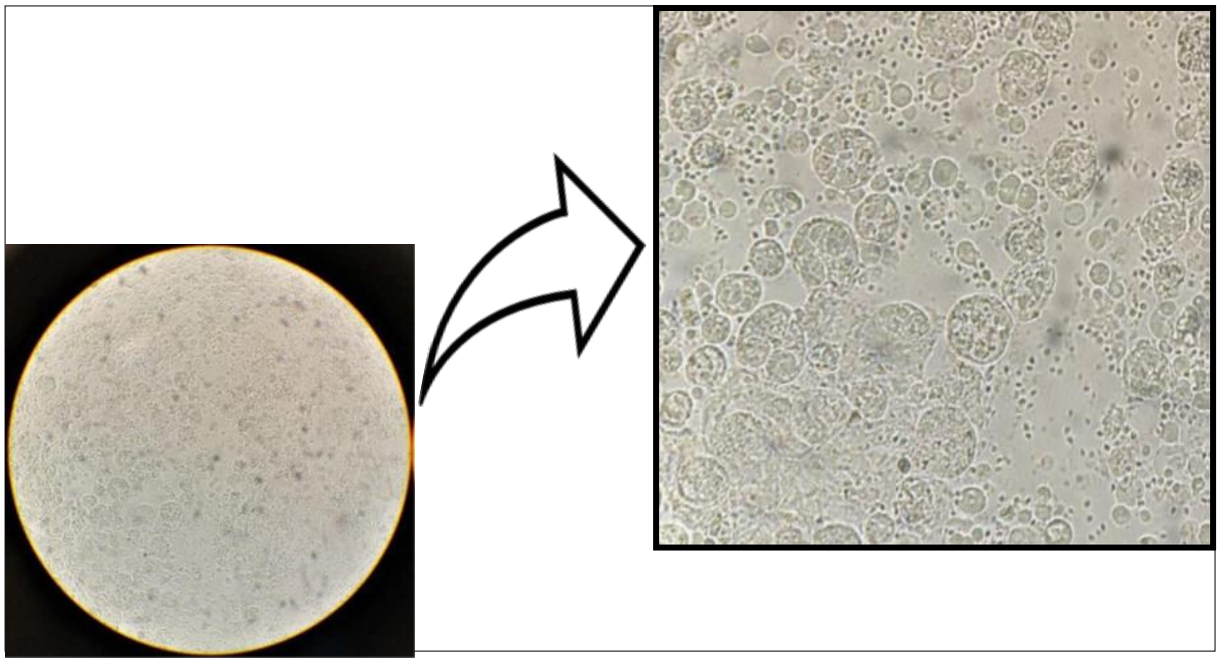


Figure 10: Microscopical observation of a fresh drop, squeezed from individual Gilthead seabream anus, to identify the presence of E. leei sporoblast and mature spores.at 400X magnification.

3.4.2. qPCR analysis

Rectal swabs were taken from each fish (30 samples per tank), in total 90 swabs per sampling. The rectal samples were obtained by probing the rectum with a cotton swab and preserved at -20° until further use. For molecular analysis, all the swabs were subjected to DNA extraction by a commercial kit (PureLink™ Genomic DNA Mini Kit, Life Technologies) followed by species-specific Sybr green qPCR targeting 128 bp of the 18S rDNA. Only data from reactions with

standard curves within an efficiency range (E = 0.85–1.1), and R2> 0.99 will be accepted. Samples with Ct < 38 will be considered positive (Palenzuela et al., 2020).

3.4.3. Histological analysis

At each sampling, from 10 fish, the whole intestine was collected and preserved in 10% buffered formalin. At the lab, the intestine was divided into three parts (proximal, medial, and distal), processed, and stained with Haematoxylin Eosin then checked under light microscopy to detect spores or developmental stages of *E. leei,* together with histopathological changes due to the presence of the parasite. Fish was considered positive to infection when parasite was found at least in one intestinal segment.

During dissection kidney, liver and spleen were visualized and if some macroscopical alterations were detected, they were collected. A microscopical observation of smear was done first and then the samples were preserved in 10% buffer formalin. All the fish with organs alteration are counted and the percentage of altered fish per tank was done.

3.5 Data analysis

All statistical analyses were performed using the software R version 4.0.5. (Released on 2021-03-31). We considered statistical differences significant for $p<0.05$. Differences in growth performance, (specific growth rate, conditions factor, weight, and length differences among groups), mortality, and *E. leei* intensity due to treatments were evaluated using two-way analysis of variance (two-way ANOVA) after assessing equality of variances by Levene's test. Post hoc multiple comparisons were carried out using Tukey's test.

4. Results

4.1. Growth performance

A parametric index of growth was calculated. At the end of the trial, with statistically significant difference (*p<0.05*), the average of individual wet weight (Table 2) was higher in Group B (0.5% additive inclusion), followed by group A (control group) and Group C (0.7% additive inclusion).

Similar results for the fish length (Table 2). Group B has an average of 30.46 cm, followed by Group A with 28.66 cm and Group C 27.46 cm with a *p* value < 0.05. Tukey's test performed shows a significant difference between group B-C and A-C.

Table 2: Mean and SD of wet weight (g) and length (cm) at the beginning and at the end of the trial of the different groups of Gilthead seabream "Sparus aurata". Length and weight at the end of the trial were significant higher (p<0,05) in group B (0,5% additive inclusion) followed by Group A (control Group) and Group C (0,07% additive inclusion).

	Initial wet weight (g)	*SD*	*Initial length (cm)*	*SD*	*Wet weight (g)*	*SD*	*Length (cm)*	*SD*
Group A	315.33	± 78.31	26.54	± 1.99	400.86	± 115.45	28.66	± 2.24
Group B	277.56	± 65.01	26.21	± 1.76	450.96	± 91.26	30.46	± 1.55
Group C	303.76	± 51.60	26.96	± 1.55	332.03	± 100.19	27.46	± 2.30

Specific growth rate (SGR) was calculated for each group and each time of sampling. There is no correlation between the variance of SGR among time and the prevalence of infected fish. At the end of the trial, SGR was higher in Group B with a value of 0.49, followed by Group A with 0.24 and Group C 0.09 (Figure 11).

SGR was calculated also for each time of sampling (Figure 12). No significant differences among groups were found (*P value > 0.05*).

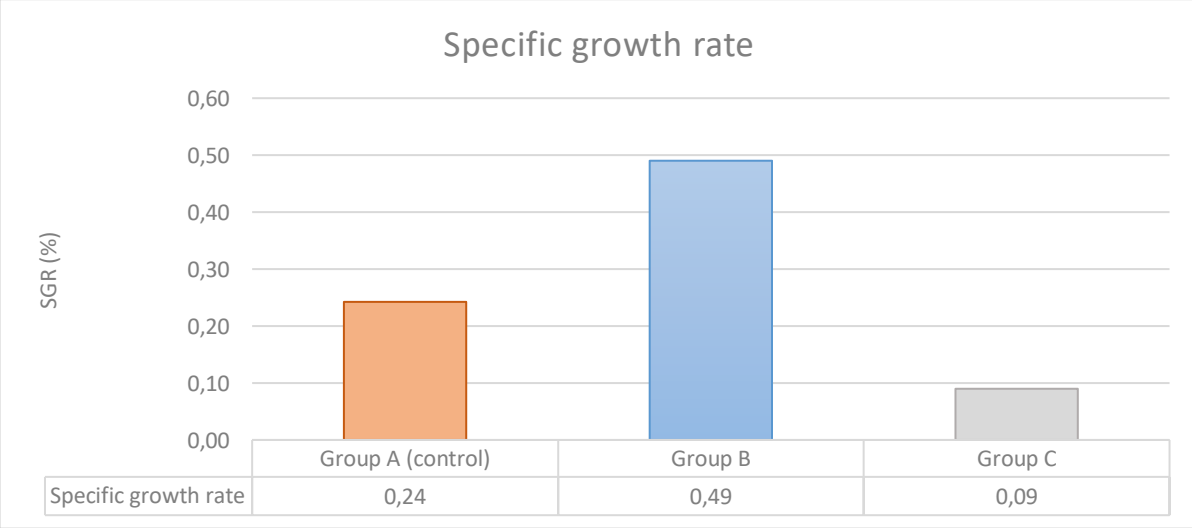
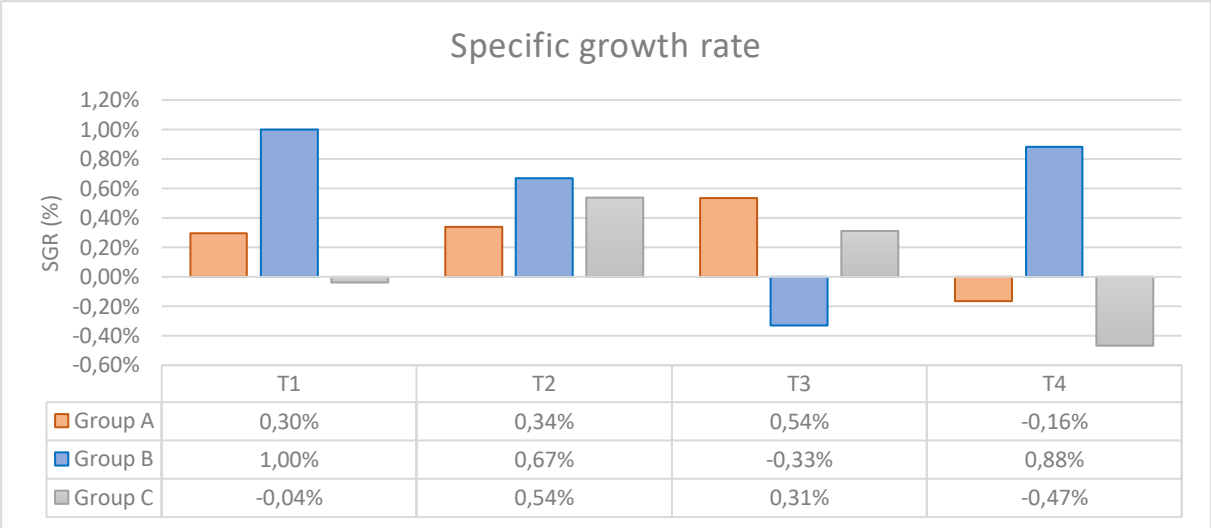


Figure 11: Specific growth rate (SGR) was calculated per experimental group of Gilthead seabream's tank and is represented as the mean. Group B (treatment with 0.5 % of functional feed addiction) have the higher SGR with 0.49, followed by control group A with a value of 0.24 and group C (treatment with 0.7% of functional feed) with a value of 0.09.



	T1	T2	T3	T4
Group A	0,30%	0,34%	0,54%	-0,16%
Group B	1,00%	0,67%	-0,33%	0,88%
Group C	-0,04%	0,54%	0,31%	-0,47%

Figure 12: Specific growth rate (SGR) for each time of sampling for each experimental group of Gilthead seabream. A negative value can be appreciated for group C (0.7 functional feed addiction) for T1 and T4, group B (0.5% functional feed addiction) for T3, and group A (control group) for T4. No correlation between SGR variation and parasite infection were detected.

The Fulton's condition factor (K) was calculated and at the end of the trial Group A have a K value of 1.70, Group B and Group C have respectively 1.59 and 1.60 (Figure 13).

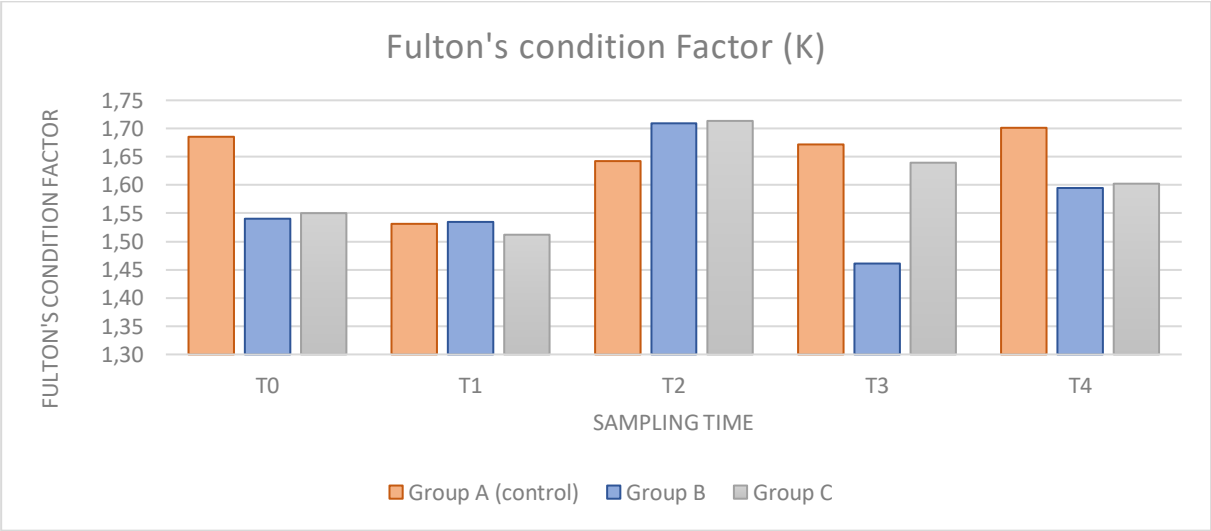


Figure 13: Fulton's condition factor calculated for each group of Gilthead seabream "Sparus aurata" in each sampling time, data are represented as the group (all fish from tanks) mean.

Feed conversion rate (FCR) has been calculated for each group at each time of sampling; the most negative value is on group C at T1 with a value of -11.07 while the most positive value in Group A at T1 with a value of 2.2 (Figure 14).

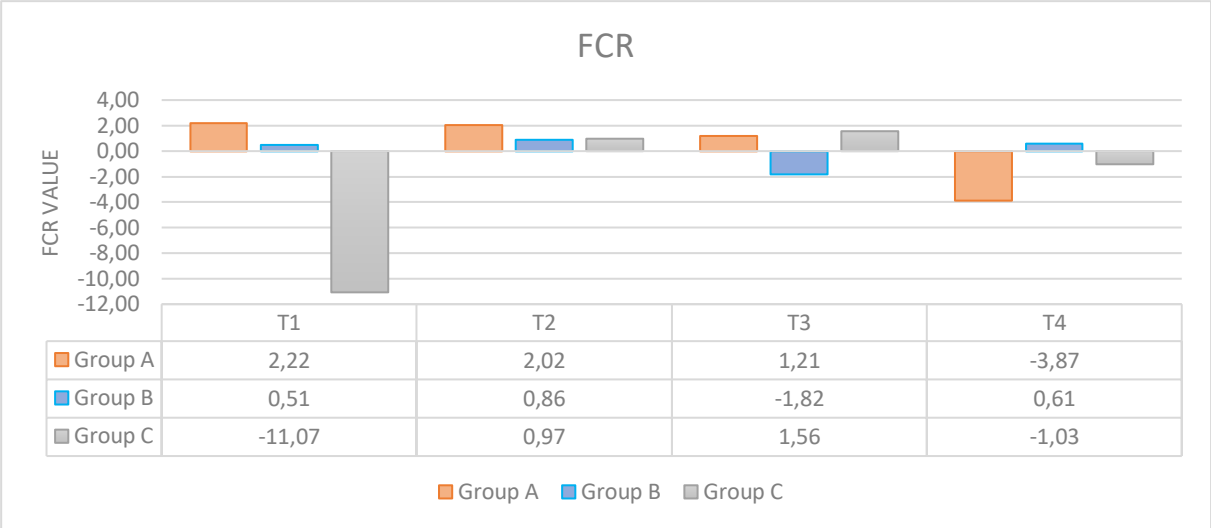


Figure 14: :Feed conversion rate (FCR) calculated for each group of Gilthead seabream "Sparus aurata" in each sampling time, data are represented as the group mean (all fish from tanks).

4.2. Mortality rate

Lowest mortality was detected in the control group (group A). At the end of the trial, the mortality rate has a statistical significance ($p<0.05$) for 1.28% for group A, 3.11% for group B and, 3.24%for group C.

Interestingly during the fish dissection spleen and kidney showed alterations, further analysis reveals a bacterial origin (Figure 15,Figure 16).

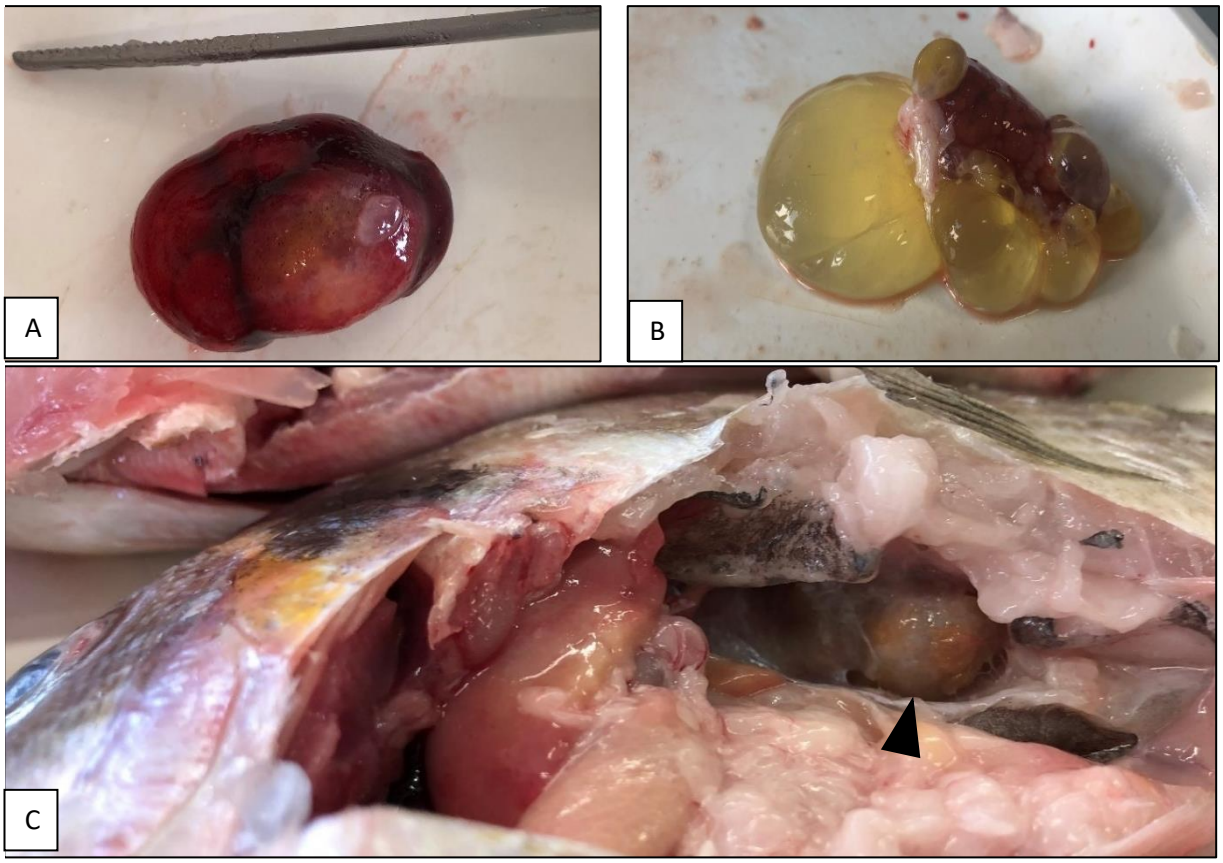


Figure 15: Different finding in the necroscopy of Gilthead seabream "Sparus aurata": in the image A and B there are different type of spleen alteration with several confluent cysts, sometime filled by yellowish liquid. In image C an alteration in the kidney, is evident with a nodular formation (arrow). Photo Mirko Pierantozzi.

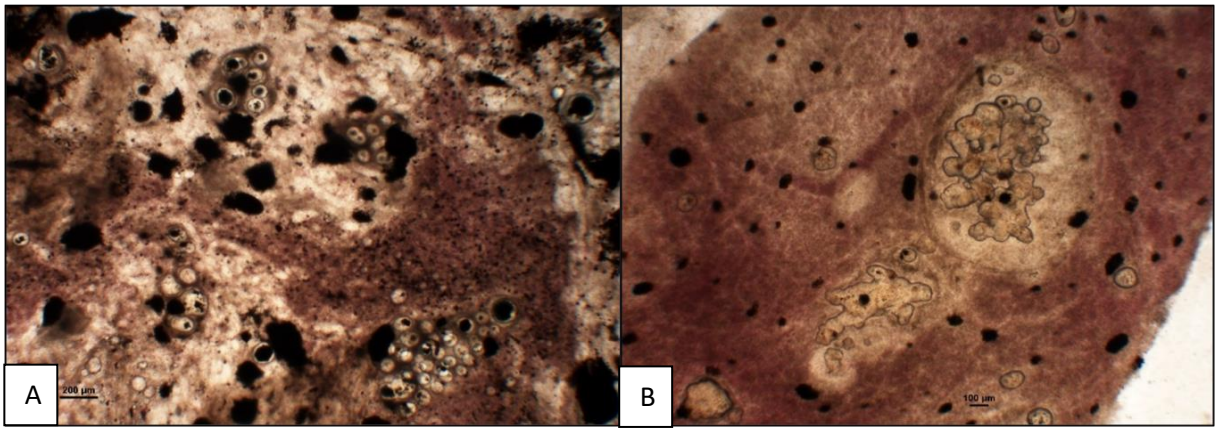


Figure 16: Fresh mount of kidney (Figure A, scale bar 200 µm) and spleen (Figure B, scale bar 100 µm) of Gilthead seabream "Sparus aurata" with diffuse granulomas. Photo Mirko Pierantozzi.

A comparison between fish mortality and spleen and kidney alteration was carried out. Statistical analysis (Spearman's correlation test) shows a moderate/strong correlation with a 0.7 value ($p<0.05$). There is no correlation between the rising of mortality and prevalence of Enteromyxosis in the tank (Figure 17; Figure 18; Figure 19).

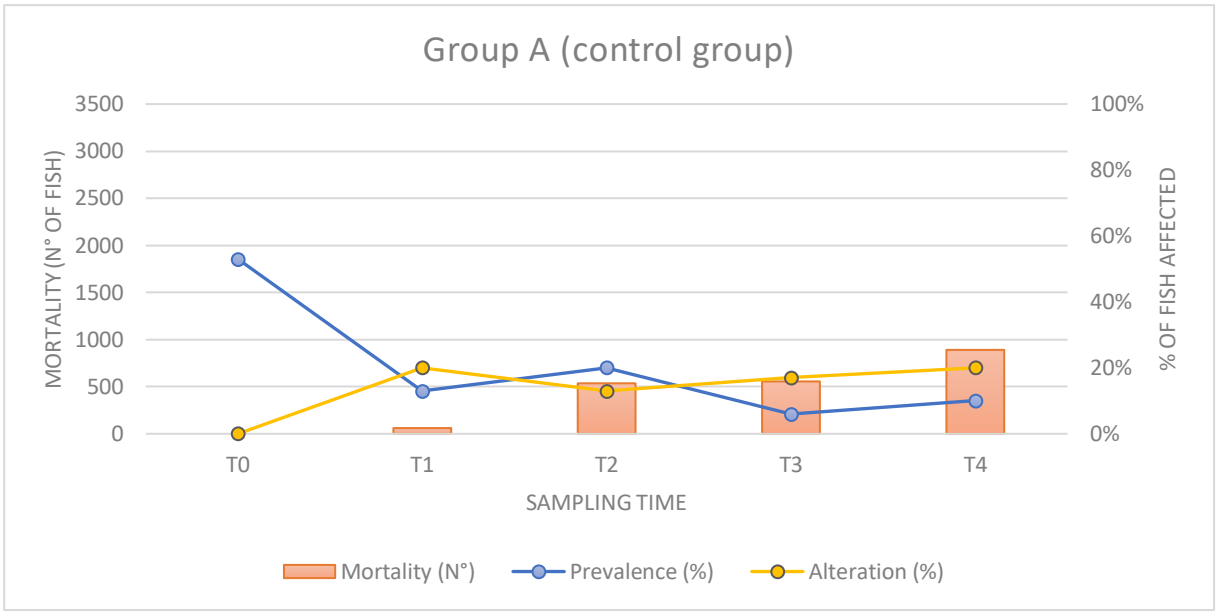


Figure 17: Group A (control group) comparison between Gilthead seabream's mortality (number of fish died in that period) and percentage of fish affected by E. leei (blue line) and spleen / kidney alteration (yellow line) along 105 days of feeding died without SANACORE® supplementation.

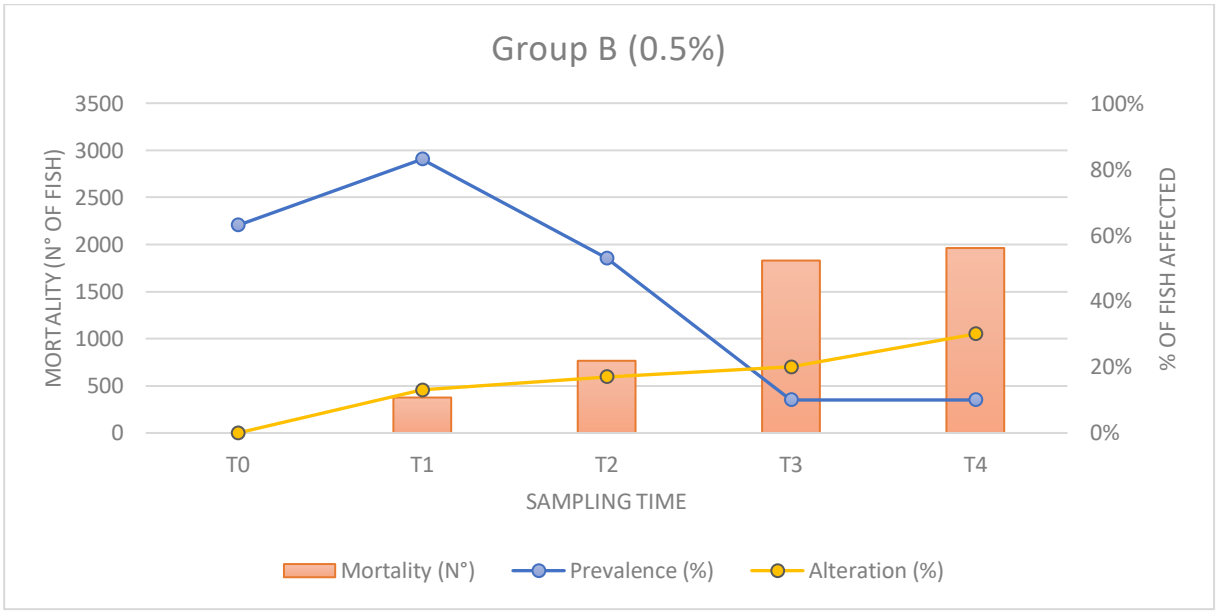


Figure 18: Group B (0.5% feed additive inclusion) comparison between Gilthead seabream's mortality (number of fish died in that period) and percentage of fish affected by E. leei (blue line) and spleen / kidney alteration (yellow line) along 105 days of feeding died with 0.5% of SANACORE® supplementation.

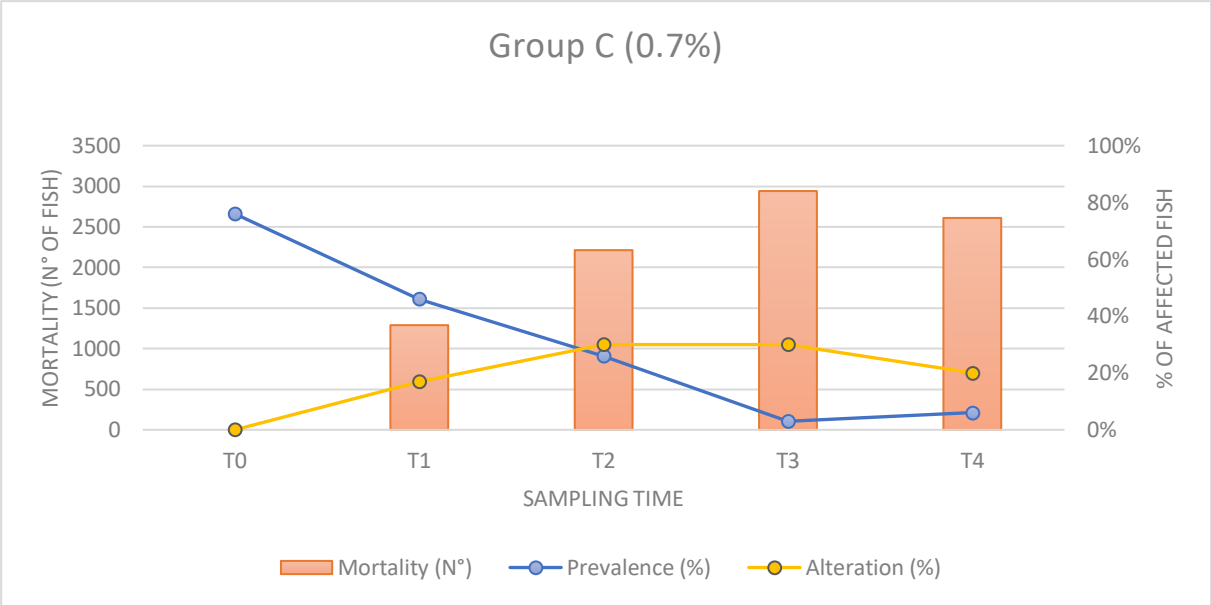


Figure 19: Group C (0.7% feed additive inclusion) comparison between Gilthead seabream's mortality (number of fish died in that period) and percentage of fish affected by E. leei (blue line) and spleen / kidney alteration (yellow line) along 105 days of feeding died with 0.7% of SANACORE® supplementation.

4.3. Parasitological examination and diagnosis of enteromyxosis

The prevalence of infection, based on microscopical observations of intestinal smears, during the whole period was calculated (Table 3), although these differences did not reach statistical significance.

Table 3: Gilthead seabream (Sparus aurata) Enteromyxum leei infection, based on microscopical observations of rectal smear, along 105 days of feeding diets with different supplementation of SANACORE®, on an operating fish farm.

Prevalence %	***Treatment A (control)***	***Treatment B (0.5%)***	***Treatment C (0.7%)***
T0	53.33%	63.33%	76.67%
T1	13.33%	83.33%	46.67%
T2	20.00%	53.33%	26.67%
T3	6.67%	10.00%	3.33%
T4	***10.00%***	***10.00%***	***6.67%***

Each group at the end of the trial showed a reduction in the number of *E. leei* stages counted with microscopical smear observation (Figure 20).

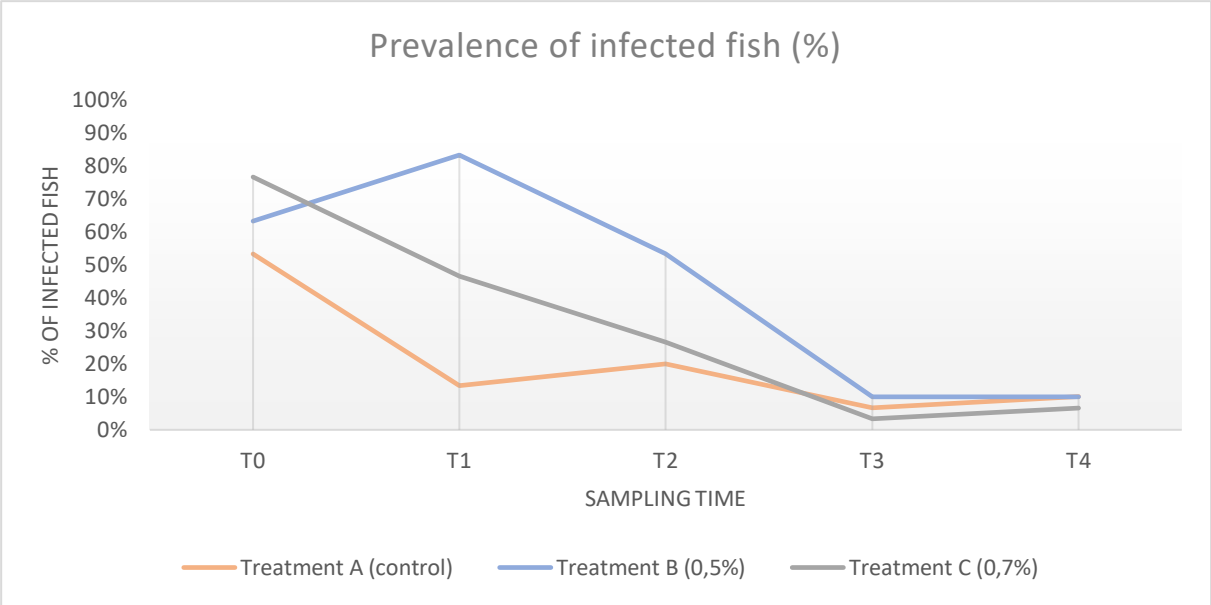


Figure 20: Comparison of E. leei microscopic prevalence(%) in each group of Gilthead seabream "Sparus aurata" at different sampling times. The prevalence was counted by the microscopical observation of fresh intestinal smear of 30 fish per tank. The prevalence of E. leei is reduced in each group at the end of the trial.

Comparing the mean value of the number of parasites counted in the intestinal smear and qPCR ct value we can observe a different trend line, which means the number of parasites counted with microscopic observation decrease, and the mean number of ct value (threshold cycle) increase (Pearson's correlation test -0.634), (Figure 21-Figure 22-Figure 23).

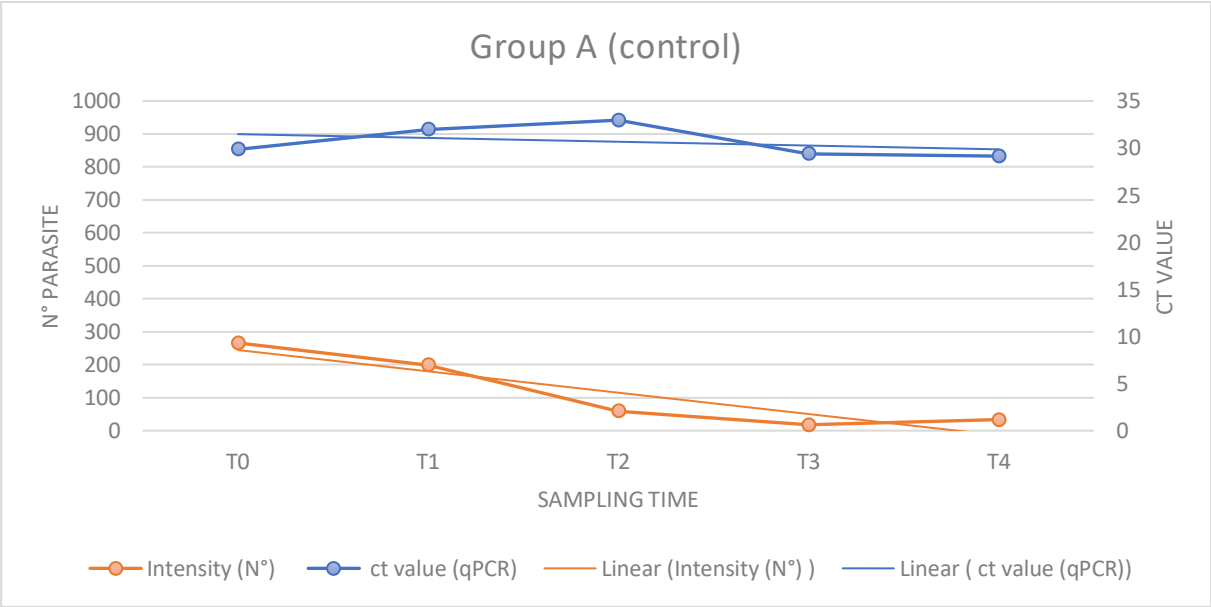


Figure 21: Group A (control group) comparison of the mean intensity of E. leei counted by microscopic observation of fresh intestinal smear in 100 field observation and mean of the ct value of qPCR along 105 days of feeding diet without SANACORE® additive supplementation in experimental group of Gilthead seabream.

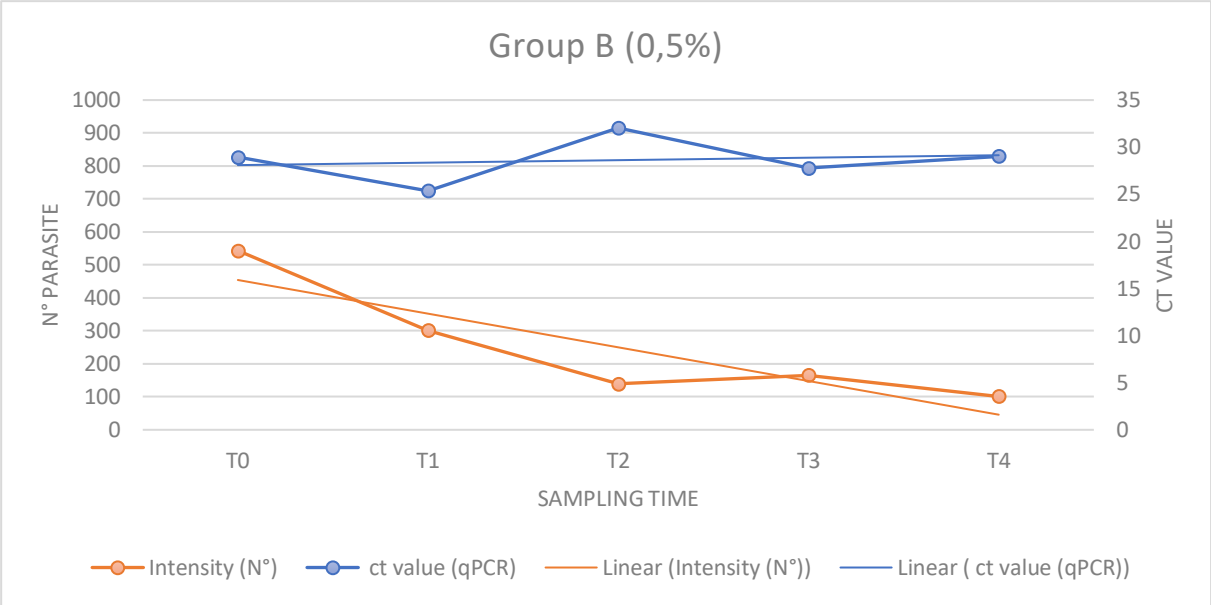


Figure 22: Group B (0.5% feed additive inclusion) comparison of the mean intensity of E. leei counted by microscopic observation of fresh intestinal smear in 100 field observation and mean of the ct value of qPCR along 105 days of feeding diet with 0.5% of SANACORE® supplementation in experimental group of Gilthead seabream.

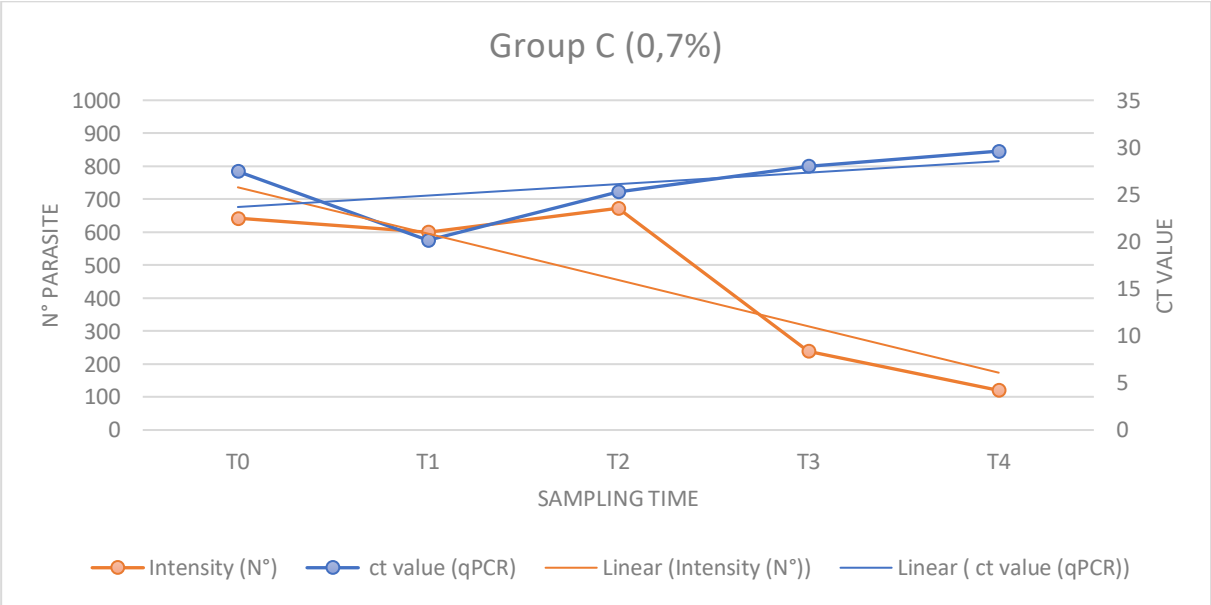


Figure 23: Group C (0.7% feed additive inclusion) comparison of the mean intensity of E. leei counted by microscopic observation of fresh intestinal smear in 100 field observation and mean of the ct value of qPCR along 105 days of feeding diet with 0.7% of SANACORE® supplementation in experimental group of Gilthead seabream.

4.4. Histological examination

At histology, the intestines of fish affected by Enteromyxosis showed the presence of a huge number of intramucosal developmental stages of *E. leei* associated with the presence of extensive necrotic areas of the lamina propria, and inflammatory infiltrate of eosinophilic granular cells (Figure 24,Figure 25). All the positive fish showed the presence of *E. leei* developmental stages at least in the terminal intestine (rectal ampulla).

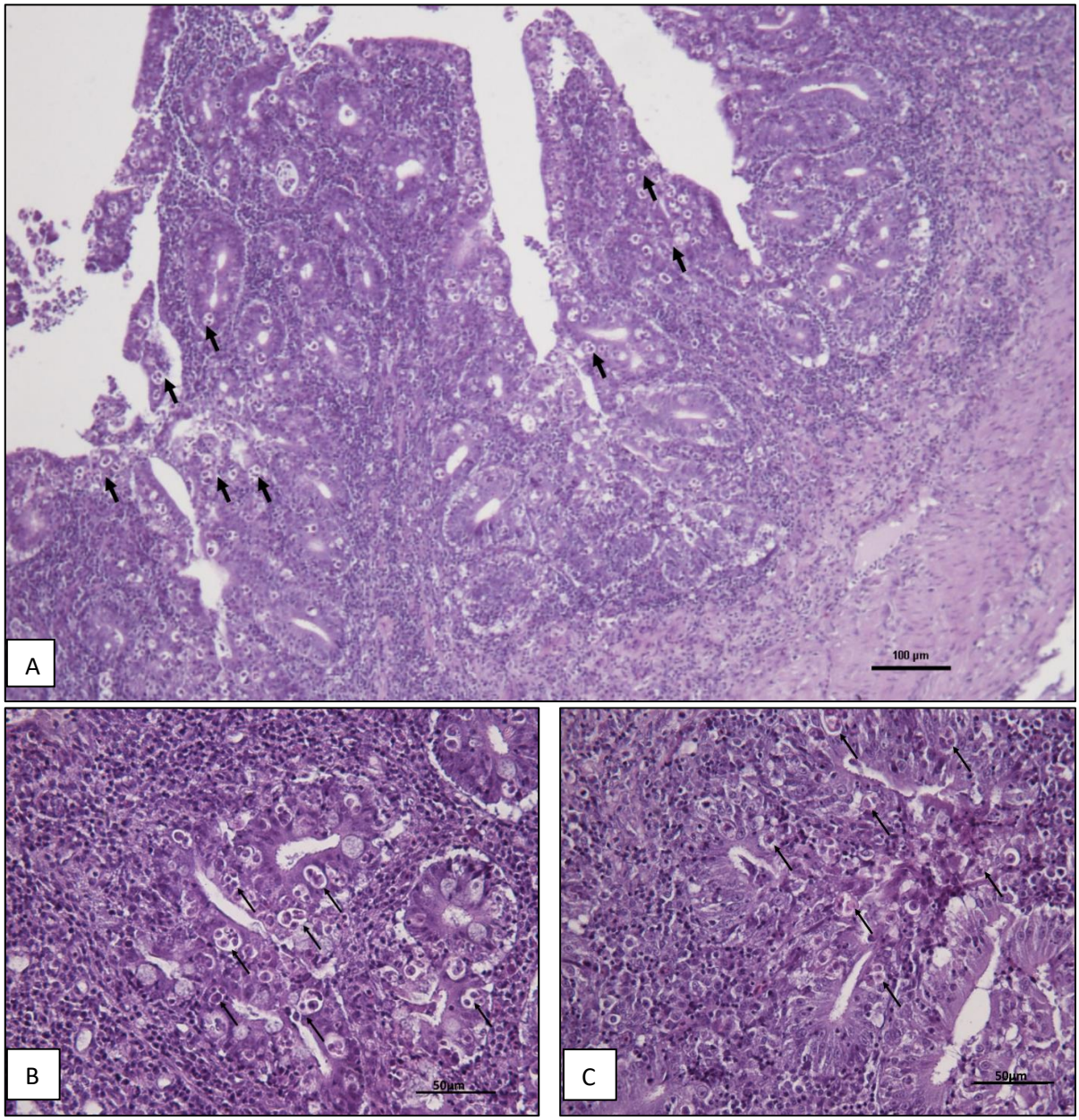


Figure 24: Intestine of Gilthead seabream "Sparus aurata": massive E. leei infection with several intramucosal developmental stages of the parasite (arrows) (Figure A scale bar 100µm, Figure B and Figure C, scale bar 50 µm) (H&E). Photo Mirko Pierantozzi.

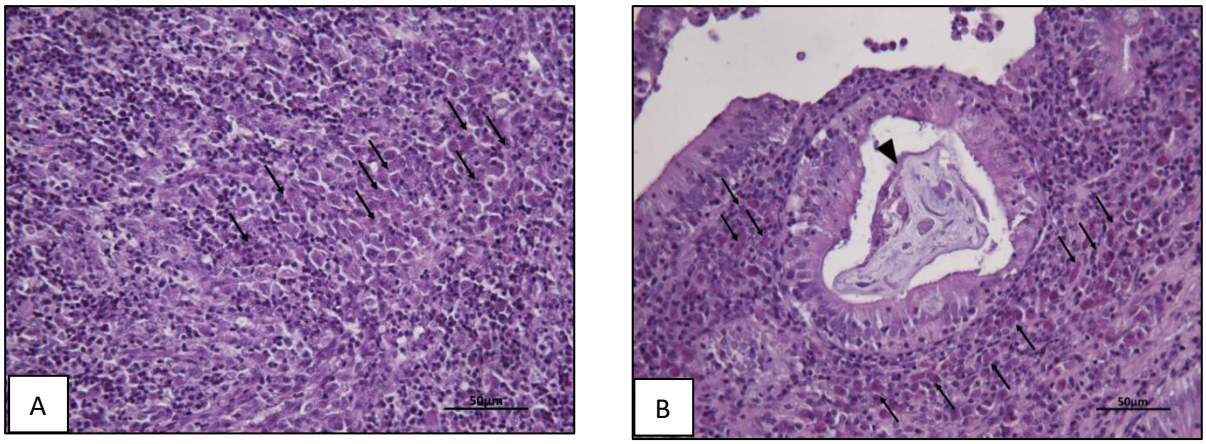


Figure 25: Intestine Gilthead seabream "Sparus aurata": massive eosinophilic granular cells infiltration in the lamina propria (arrows) (Figure A, scale bar 50µm); the presence of necrotic material in a tubule (arrowhead) with infiltrating of eosinophilic granular cells (arrows) (Figure B scale bar 50µm) (H&E). Photo Mirko Pierantozzi.

In areas where *E. leei* plasmodia are evident, the inflammatory infiltrate is usually less severe than other parts, in which other inflammation processes could be involved worsening the whole pathological context. Vascular congestion is also observed.

In the transverse sections, the lamina propria is thickened and infiltrated (Figure 26) by lymphocytes, macrophages, and eosinophilic granular cells and necrosis of the epithelium of the tubules with goblet cells hyperplasia (Figure 27).

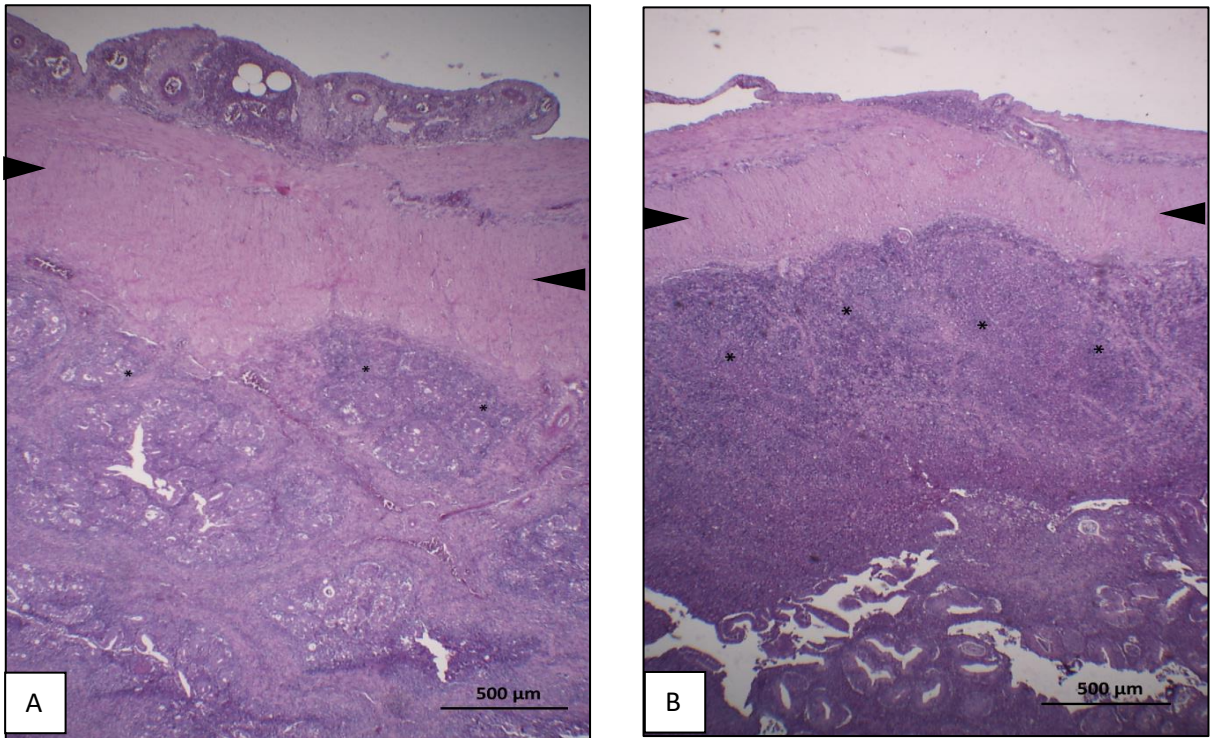


Figure 26: Intestine of Gilthead seabream "Sparus aurata": severe thickening of the intestinal wall (arrowhead) and massive inflammatory infiltration (*) (Figure A and Figure B, scale bar 500µm) (H&E). Photo Mirko Pierantozzi

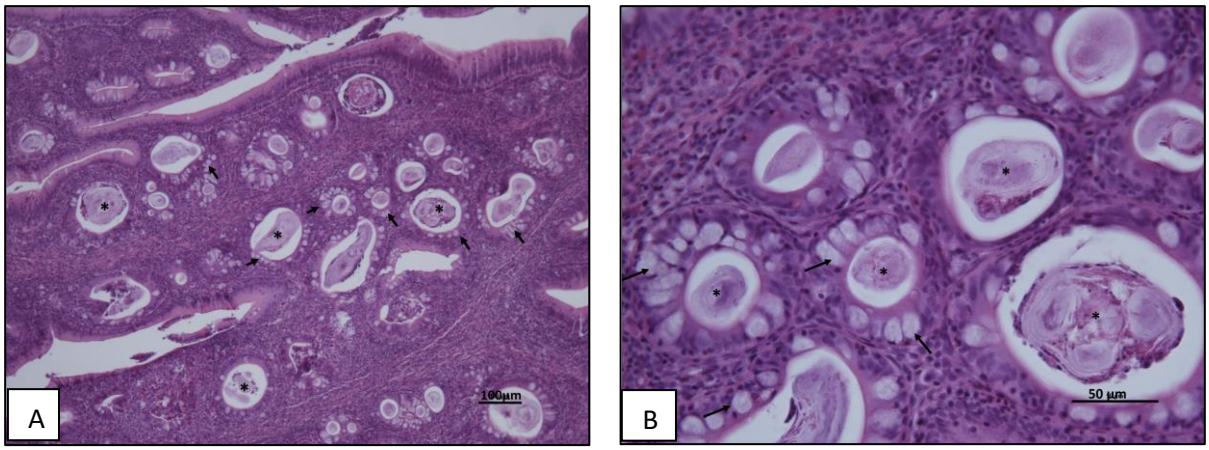


Figure 27: Gilthead seabream "Sparus aurata" Intestinal lesions observed in the mucosa at the basis of villa with presence of mucus and epithelial debris embedded on it (Figure A). Goblet cells hyperplasia (arrows) was also observed (Figure B).(Figure A scale bar 100 µm, Figure B scale bar 50µm) (H&E). Photo Mirko Pierantozzi.

In the spleen, histological analysis revealed and confirm the presence of severe granulomatous lesions with the presence of Ziehl-Neelsen positive bacterial aggregates within (Figure 28). The structure of splenic parenchyma was in the worst cases totally altered (Figure 28).

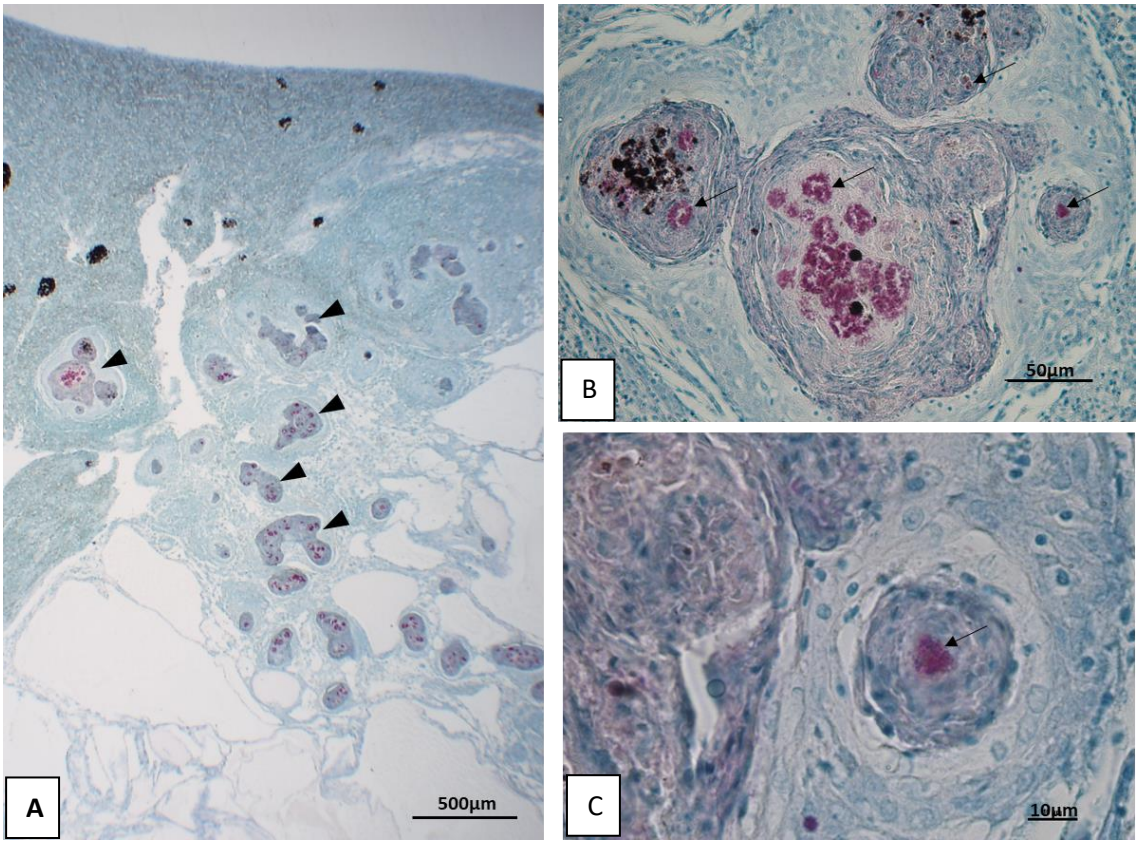


Figure 28: Spleen of Gilthead seabream "Sparus aurata": granulomatous lesions with Acid-Alcohol Resistant Bacteria (arrows). (Figure A scale bar 500µm, Figure B scale bar 50µm, Figure C scale bar 10µm) (Z-N stain) Photo Mirko Pierantozzi.

A comparison between histology examination and qPCR has been done. The average qPCR ct value of fish positive and negative at the histology has been calculated with minimum and maximum values (Table 4).

The qPCR mean have a difference significant with a *p value* <0.05 (one-way anova).

Table 4: Minimum, maximum and mean of qPCR ct value calculated for fish positive or negative at histology examination.

Histology	*Minimum ct value*	*Maximum ct value*	*Average qPCR ct value*	*S.D.*
+	15.85	31.54	22.42	2.77
-	19.35	37.48	33.74	4.12

5. Discussion

Enteromyxum leei infection is a very important disease able to cause severe mortality in different cultured fish species. Weight loss, arrested growth, and anorexia are one of the most obvious effects of the infection in gilthead seabream (Ronza et al., 2020; Estensoro et al., 2013; Alvarez-Pellitero et al., 2008).

Following the work of Palenzuela (Palenzuela et al., 2020), we used the functional feed "Sanacore®" in order to test their efficacy against *E. leei* in Gilthead seabream on growing facilities.

Unlike the previous research, during our study, we used 2 different and higher concentrations (0.5%, 0.7%) of functional feed in the commercial diet. Moreover, as recommended by the feed additive supplier, a double dose (attack dose of 1.0%-1.4%) have been used in the first 15 days of the trial. Despite the use of higher doses compare to Palenzuela et al. (2020), our results do not confirm the efficacy of the Sanacore® additive in reducing the negative impact of the parasites on treated fish.

Unfortunately, during the first period of the trial (T1) unexpected fish mortality in all the tanks under study has been observed. The necropsy showed the presence of white nodules and cysts in the kidney and spleen associated with an enlargement of the latter. Microscopical observation of smear and histological section stained with Ziehl-Neelsen allowed the detection of acid-fast bacteria referable to Nontuberculous Mycobacteria (NTM). We can hypothesize that the reduced effect of the functional feed on *E. leei* could be due to the

presence of these bacteria, able to cause a chronic disease that probably can lead to a drop in the immune response against the parasite.
The mortality rate increase during the whole trial period and the mean values were statistically significantly higher in Group C (3.24%) and Group B (3.11%) compared to Group A (control group) with a percentage of 1.28%.

A correlation test between mortality rate and macroscopical alteration finding during necropsy, showed a value of 0.64 indicating a good positive relationship between them.
We didn't find any significant statistical evidence of a correlation between mortality rate and *E. leei* intensity of infection. In fact, the lowering prevalence values observed in all groups under study, included the control ones, could be explained by a low effect of the functional feed against *E. leei*, at least in this trial.

This study represents the first field work trial carried out in a fish farm historically positive for *E. leei,* we have no data to compare in order to understand the reasons for the very low effect of the feed additive. Indeed, the only possible comparison is with the results of Palenzuela et al. (2020) that used the SANACORE® additive in experimentally infected fish, under laboratory conditions. The authors obtained interesting results but probably not completely applicable in field conditions, that are affected by several variables, including the coinfections with bacteria as in our case, as stated by Palenzuela et al. (2020).

Regarding the growth performance index, SGR is higher in group B with the inclusion of 0.5% of the functional feed with a value of 0.49, the control group has a value of 0.24, and group C with 0.7% functional feed inclusion has a value of 0.09. Statistical significance has the length (cm) and wet weight (g) of the fish at the end of the trial, group B with 0.5% additive inclusion have the higher value of length and weight followed by the control group and Group C with 0.7% additive inclusion. These results can be influenced by the bacteriosis outbreaks during the trial, the high mortality rate observed in group C can be responsible for the lower growth of the fish.

Concerning the comparisons between the three diagnostic methods used, the results obtained showed the qPCR is the more suitable method for the diagnosis of enteromyxosis. In particular, the microscopical observation showed some limits directly connected to the experience of the operator to recognize the stages of *E. leei* in the wet mount, moreover, is a

time-consuming technique. For these reasons counting the parasites under the light microscope could underestimate the intensity of infection. In fact, if we compare the cycle threshold (ct) values obtained with the qPCR we can see that some samples were negative microscopically but positive at the former method; the ct values increase at decreasing of the intensity of infection, meaning that maybe the developmental stages are not visible as few, but the DNA is still detectable. The comparisons of the qPCR with the histology showed an interesting result. We noticed that at ct ≥ 31 the histological section didn't show any developmental stages of the parasites, even if the accepted value to consider one sample positive in <38. For these reasons, we have to take carefully the histological results if used to diagnose enteromyxosis. In conclusion, we can state that, depending on the aims of the diagnosis, we have to carefully evaluate which one use. Based on the results obtained during the trial, we could suggest the use of qPCR for the screening of the newly introduced fish, to be sampled by a non-lethal method (rectal swab), the histology only for establish the lesion due to *E. leei* and the microscopical observation only in the lab.

6. Conclusion

The results obtained during the field trial, on the use of functional feed as mitigating agent on parasitic infections, in particular Enteromyxosis by *Enteromyxum leei*, we can conclude that a correct evaluation of its efficacy is not easily confirmed, when working in production conditions. The interaction of both biotic and abiotic factors could play a role in influencing the functional feed intake and its action on target parasitic diseases. As also stated in the paper by Palenzuela et al. (2020) laboratory-controlled experimental infection cannot be compared with the much more complex farming settings. Experimental follow up from the present study results, would be necessary to obtain a more robust set of data from field production conditions.

In this regard, the results showed in the present study, suffered the occurrence of secondary bacterial infections probably able to heavily influence the course of the Enteromyxosis in the three tanks under study. Further trials set up with a number of variables as lower as possible will help to better interpret the real contribute on diseases mitigation of functional feeds, the latter representing a very interesting and promising approach for the future of fish farming management.

The comparison of the different diagnostic methods used shows qPCR as an important tool in diagnosis. The possibility to use a non-lethal sample (rectal swab), the high sensitivity, and the relatively fast results of this diagnostic method, make it the perfect screening method for the new fish on the farm.

7. References

Alvarez-Pellitero, P., Palenzuela, O., & Sitjà-Bobadilla, A. (2008). Histopathology and cellular response in Enteromyxum leei (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). Parasitology International, 57(2), 110–120.

Bendiksen, H. R., Wesmajervi, M. S., Olsen, R. E., Jansen, P. A., & Mikkelsen, H. (2000). Lactic acid bacteria associated with the digestive tract of Atlantic salmon *(Salmo salar L .).* Journal of Applied Microbiology, 89(2), 317-22.

Breton, A. Le, & Marques, A. (1995). Occurrence of an histozoic Myxidium infection in two marine cultured species: *Puntazzo puntazzo C*. and *Pagrus major*. Bulletin of The European Association of Fish Pathologists, 15(6), 210–212.

Coutteau, P. (2014). Functional feed additives to prevent disease in farmed shrimp. Aquafeed: Advances in Processing & Formulation, 7(4), 24–27.

Cuadrado, M., Marques, A., Diamant, A., Sitjá-Bobadilla, A., Palenzuela, O. R., Álvarez-Pellitero, P., Padrós, F., & Crespo, S. (2008). Ultrastructure of *Enteromyxum leei* (Diamant, Lom, & Dykova´, 1994) (Myxozoa), an Enteric Parasite Infecting Gilthead Sea Bream (*Sparus aurata*) and Sharpsnout Sea Bream *(Diplodus puntazzo)*. The Journal of Eukaryotic Microbiology 55(3), 178-184.

Cuesta, A., Muñoz, P., Rodríguez, A., Salinas, I., Sitjà-Bobadilla, A., Álvarez-Pellitero, P., Esteban, M. A., & Meseguer, J. (2006). Gilthead seabream (*Sparus aurata L*.) innate defence against the parasite *Enteromyxum leei* (Myxozoa). Parasitology,132(Pt 1):95-104.

Davey, G. C., Calduch-Giner, J. A., Houeix, B., Talbot, A., Sitjà-Bobadilla, A., Prunet, P., Pérez-Sánchez, J., & Cairns, M. T. (2011). Molecular profiling of the gilthead sea bream (*Sparus aurata L*.) response to chronic exposure to the myxosporean parasite *Enteromyxum leei.* Molecular Immunology, 48(15–16), 2102–2112.

Diamant, A., Lom, J., & Dykova, I. (1994). *Myxidium leei* n.sp., a pathogenic myxosporean of cultured sea bream *Sparus aurata*. Diseases of Aquatic Organisms, 20(2), 137–141.

Encarnação, P. (2016). Functional feed additives in aquaculture feeds. In *Aquafeed Formulation* (pp. 217–237). Elsevier Inc.

Engle, C., D'Abramo, L. & Slater, M. J. (2017). Global Aquaculture 2050, Journal of the World Aquaculture Society, 48 (1), 3-6

Estensoro, I., Jung-Schroers, V., Álvarez-Pellitero, P., Steinhagen, D., & Sitjà-Bobadilla, A. (2013). Effects of *Enteromyxum leei* (*Myxozoa*) infection on gilthead sea bream (*Sparus aurata*) (Teleostei) intestinal mucus: Glycoprotein profile and bacterial adhesion. Parasitology Research, *112*(2), 567–576.

Estensoro I., Redondo, J., Alvarez-Pellitero, P., & Sitjà-Bobadilla, A. (2010). Novel horizontal transmission route for *Enteromyxum leei* (Myxozoa) by anal intubation of gilthead sea bream *Sparus aurata*. Diseases of Aquatic Organisms, 92(1), 51–58.

Estensoro I., (2013) *Enteromyxum leei* (MYXOZOA. MYXOSPOREA) Infection in Gilthead Sea

Bream (*Sparus aurata*): Immune-related Studies [Doctoral dissertation, University of Valencia, Faculty of Biological Science] p.195

FAO. (2020). The state of World Fisheries and Aquaculture 2020. Sustainability in action.

FAO (2022). *Sparus aurata Linnaeus*,1758. Fisheries and Aquaculture Division [online]. Rome.

Feist, S. & Longshaw, M. (2008). Histopathology of fish parasite infections - Importance for populations. Journal of Fish Biology,73(9), 2143-60.

Fekete, S. G., & Kellems, R. O. (2015). Interrelationship of feeding with immunity and parasitic infection : a review. Veterinarni Medicina, 52, 131-143.

Fioravanti, M. L., Caffara, M., Florio, D., Gustinelli, A., & Marcer, F. (2006). A Parasitological Survey of European Sea Bass *(Dicentrarchus Labrax)* and Gilthead Sea Bream *(Sparus aurata)* Cultured in Italy. Veterinary Ressearch Communication 30, 249–252.

Fleurance, R., Sauvegrain, C., Marques, A., Le Breton, A., Guereaud, C., Cherel, Y., & Wyers, M. (2008). Histopathological changes caused by *Enteromyxum leei* infection in farmed sea bream *Sparus aurata*. Diseases of Aquatic Organisms, 79(3), 219–228.

Glencross, B. (2016). Understanding the nutritional and biological constraints ingredients to optimize the application in aquaculture feeds. In *Aquafeed Formulation* (pp. 33-73) Elsevier inc.

Golomazou, E. (2006). Efficacy and Toxicity of Orally Administrated Anti-Coccidial Drug Treatment on Entero myxum leei Infections in Sharpsnout Seabream (*Diplodus puntazzo C)*. The Israeli Journal of Aquaculture,58(3), 157-169.

Golomazou, E., Athanassopoulou, F., Karagouni, E., Tsagozis, P., Tsantilas, H., & Vagianou, S. (2006). Experimental transmission of *Enteromyxum leei* Diamant, Lom and Dykova, 1994 in sharpsnout sea bream, *Diplodus puntazzo C.* and the effect on some innate immune parameters. Aquaculture, 260(1–4), 44–53.

Golomazou, E., Exadactylos, A., Malandrakis, E. E., Karatzinos, T., Gkafas, G., Dadali, O., & Panagiotaki, P. (2014). Early diagnosis of *enteromyxosis* in intensively reared sharpsnout seabream, *Diplodus puntazzo*. Aquatic Living Resources, 27(2), 99–106.

Gómez, D., Bartholomew, J., & Sunyer, J. O. (2014). Biology and mucosal immunity to myxozoans. Developmental and Comparative Immunology, 43(2), 243–256.

Henry, M. A., Kokou, F., Palenzuela, O., Pyrenis, G., & Rigos, G. (2020). Experimental infection model with the bivalvulid *Enteromyxum leei* (Myxidiidae) in the sharpsnout seabream , *Diplodus puntazzo* (Sparidae), and evaluation of the antiparasitic efficacy of a functional diet. *Folia Parasitologica*, *67*.

IUCN. (2007). Guide for the Sustainable Development of Mediterranean Aquaculture. Interaction between Aquaculture and the Environment. 107p.

Massa, F., Onofri, L., & Fezzardi, D. (2017). Aquaculture in the Mediterranean and the Black Sea : a Blue Growth perspective. Handbook on the Economics and Management Of Sustainable Oceans (4), (pp. 93-123), Edward Elgar Publishing.

Moretti, A., Pedini Fernandez-Criado, M., Cittolin, G., & Guidastri, R. (1998). Manual on

hatchery production of seabass and gilthead seabream. Volume 1. FAO. 194p.

Muniesa, A., Basurco, B., Aguilera, C., Furones, D., Reverté, C., Mona, A. S., Jansen, D., Brun, E., & Tavornpanich, S. (2020). Mapping the knowledge of the main diseases affecting sea bass and sea bream in Mediterranean., Transboundary Emerging Diseases, 67(3), 1089-1100.

Palenzuela, O., Del Pozo, R., Piazzon, M. C., Isern-Subich, M. M., Ceulemans, S., Coutteau, P., & Sitjà-Bobadilla, A. (2020). Effect of a functional feed additive on mitigation of experimentally induced gilthead sea bream *Sparus aurata* enteromyxosis. Diseases of Aquatic Organisms, 138, 111–120.

Pavlidis, M., & Mylonas, C. (2011). Sparidae: Biology and Aquaculture of Gilthead Sea Bream and other species. (4) (pp. 51-73).

Piante, C. & Ody, D. (2015). Blue Growth in the Mediterranean Sea: the Challenge of Good Environmental Status. MedTrends Project. WWF, 192 p..

Piazzon, M. C., Calduch-giner, J. A., Fouz, B., Estensoro, I., Simó-mirabet, P., Puyalto, M., Karalazos, V., Palenzuela, O., Sitjà-bobadilla, A., & Pérez-Sánchez, J. (2017). Under control : how a dietary additive can restore the gut microbiome and proteomic profile , and improve disease resilience in a marine teleostean fish fed vegetable diets. Microbiome 5(1), 164.

Plan Bleu. (2014). Economic and social analysis of the uses of the costal and marine waters in the Mediterranean,characterization and impacts of the Fisheries,Aquaculture, Tourism and recreational activities.Marine transport and Offshore extraction of oil and gas sector.Technical Report.

Rigos, G., Christophilogiannis, P., & Yiagnisi, M. (1999). *Myxosporean* infections in Greek mariculture. Aquaculture International 7, 361–364.

Rigos, G., & Katharios, Æ. P. (2010). Pathological obstacles of newly-introduced fish species in Mediterranean mariculture : a review. R*eviews in Fish Biology and Fisheries*, 20 (1), 47–70.

Ringø, E., Myklebust, R., Mayhew, T. M., & Erik, R. (2007). Bacterial translocation and pathogenesis in the digestive tract of larvae and fr*y*.Aquaculture 268, 251-264.

Ronza, P., Estensoro, I., Bermúdez, R., Losada, A. P., Pérez-Cordón, G., Pardo, B. G., Sitjà-Bobadilla, A., & Quiroga, M. I. (2020). Effects of *Enteromyxum spp*. (Myxozoa) infection in the regulation of intestinal E-cadherin: Turbot against gilthead sea bream. Journal of Fish Diseases, 43(3), 337–346.

Sánchez-García, N., Raga, J. A., & Montero, F. E. (2014). Risk assessment for parasites in cultures of *Diplodus puntazzo* (Sparidae) in the Western Mediterranean: Prospects of cross infection with *Sparus aurata*. Veterinary Parasitology, 204(3–4), 120–133.

Sitjà-Bobadilla, A., Diamant, A., Palenzuela, O., & Alvarez-Pellitero, P. (2007). Effect of host factors and experimental conditions on the horizontal transmission of *Enteromyxum leei* (Myxozoa) to gilthead sea bream, *Sparus aurata L*., and European sea bass, *Dicentrarchus labrax* (L.). Journal of Fish Diseases, 30(4), 243–250.

Sitjà-Bobadilla, A., Calduch-Giner, J., Saera-Vila, A., Palenzuela, O., Álvarez-Pellitero, P., & Pérez-Sánchez, J. (2008). Chronic exposure to the parasite *Enteromyxum leei* (Myxozoa: Myxosporea) modulates the immune response and the expression of growth, redox and immune relevant genes in gilthead sea bream, *Sparus aurata* L. Fish and Shellfish Immunology, 24(5), 610–619.

Sitjà-Bobadilla, A. & Palenzuela, O. (2012). Enteromyxum Species *in Fish parasite: pathobiology and protection,* eds. Woo, P. T. K., & Buchmann. , (pp. 163-176).

Sitjà-Bobadilla, A., Estensoro, I., & Pérez-Sánchez, J. (2016). Immunity to gastrointestinal microparasites of fish. Developmental and Comparative Immunology, *64*, 187–201.

Thomson, K. J., & Venzi, L. (2006). The Economics of Aquaculture with respect to Fisheries. 95th EAAE Semiar, Civitavecchia (Rome), 9-11 December 2005.

Yokoyama, H., & Shirakashi, S. (2007). Evaluation of hyposalinity treatment on infection with *Enteromyxum leei* (Myxozoa) using anemonefish *Amphiprion spp.* as experimental host. Bullettin of European Association of Fish Pathologist 27(2), 74–78.

8.Appendix

Table 5: Data of experimental group (A,B,C) of Gilthead seabream "Sparus aurata" from histology and qPCR in the sampling time (T0,T1,T2,T3,T4).

		Group A control		***Group B 0.5% inclusion***		***Group C 0.7% inclusion***	
Time	**Fish N°**	**qPCR (mean ct)**	**Histology**	**qPCR (mean ct)**	**Histology**	**qPCR (mean ct)**	**Histology**
T0	1	31,84	-	21,8	+	35,89	-
T0	2	29,27	+	33,36	-	29	-
T0	3	31,4	+	36,07	-	25,17	+
T0	4	22,65	+	27,97	+	22,24	+
T0	5	24,9	+	22,53	+	23,93	+
T0	6	22,7	+	21,21	+	19,33	+
T0	7	32,78	-	33,35	-	31,54	+
T0	8	31,71	-	37,19	-	31,83	-
T0	9	28,2	-	35,96	-	25,39	+
T0	10	31,84	-	34,7	-	31,8	-
T1	1	34,54	-	22,19	+	20,8	+
T1	2	34	-	19,22	+	24,57	+
T1	3	33,86	-	33,46	-	32,84	-
T1	4	35,36	-	19,54	+	32,55	-
T1	5	34,42	-	17,54	+	31,19	-
T1	6	32,6	-	18,02	+	22,12	+
T1	7	17,64	+	32,42	-	20,53	+
T1	8	32,69	-	17,37	+	30,43	-
T1	9	19,35	-	22,65	+	22,98	+
T1	10	22,53	+	15,95	+	36,27	-
T2	1	undetermined	-	36,07	-	undetermined	-
T2	2	undetermined	-	19,29	+	28,79	+

T2	3	32,67	-	35,24	-	34,79	-
T2	4	37,48	-	34,33	-	undetermined	-
T2	5	undetermined	-	32,57	-	35,47	-
T2	6	undetermined	-	32,75	-	undetermined	-
T2	7	undetermined	-	17,57	+	undetermined	-
T2	8	36,27	-	29,8	-	27,18	+
T2	9	19,36	+	17,1	+	37,38	-
T2	10	35,63	-	31,56	-	36,18	-
T3	1	36,23	-	33,33	-	31,59	-
T3	2	19,46	+	undetermined	-	31,74	-
T3	3	33,03	-	undetermined	-	35,95	-
T3	4	35,74	-	17,82	+	29,07	+
T3	5	36,79	-	32,06	-	33,34	-
T3	6	35,87	-	15,85	+	27,02	+
T3	7	34,52	-	29,49	-	32,42	-
T3	8	34,27	-	34,71	-	30,51	-
T3	9	33,11	-	35,77	-	28,95	+
T3	10	33,49	-	31,43	-	36,25	-
T4	1	32,73	-	25,28	+	25,33	+
T4	2	34,93	-	undetermined	-	32,87	-
T4	3	34,43	-	21,19	+	36,79	-
T4	4	35,73	-	36,82	-	33,5	-
T4	5	35,06	-	undetermined	-	20,45	+
T4	6	36,65	-	37,58	-	33,44	-
T4	7	undetermined	-	19,06	+	36,52	-
T4	8	37,01	-	21,52	-	28,83	-
T4	9	35,26	-	25,86	+	19,62	+

T4	10	35,9	-	34,88	-	20,97	+