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GROWTH PERFORMANCE, ANTIOXIDANT AND INNATE IMMUNE RESPONSES IN EUROPEAN SEABASS FED PROBIOTIC SUPPLEMENTED DIET AT THREE REARING TEMPERATURES.

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Growth performance, antioxidant and innate immune responses in European seabass fed probiotic supplemented diet at three rearing temperatures.

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“We must plant the sea and herd its animals using the sea as farmers instead of hunters. That is what civilization is all about – farming replacing hunting.”

Jacques Cousteau

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Por fim o mais importante, obrigado mãe, obrigado pai.

Abstract

Aquaculture industry growth often faces disease outbreaks wherein antibiotics are then used as control strategies. An alternative to these restricted chemicals could be the use of probiotics. Probiotics are defined as "live microorganisms, which when consumed in adequate amounts, confer a health effect on the host". The benefits of probiotic treatments are: improvements in host nutritional retention, antagonistic properties to bacterial proliferation, modulation of immune responses and anti-oxidants defenses, among others.

Temperature plays a major role in fish, since they are poikilothermic animals, i.e. internal temperature varies according to the surrounding environment. The intestinal microbiota composition in fish is, therefore, influenced by water temperature. Thus, the effectiveness of probiotic supplementation will vary with rearing temperatures. In addition, stress events may trigger a cascade of physiological changes, detectable by quantification of several analytical methods.

This study evaluated the effects of dietary probiotic supplementation on growth performance, immune and oxidative stress responses, and digestive enzyme activity in European seabass (*Dicentrarchus labrax*), reared at three different temperatures (17, 20 and 23 °C) for 100 days. A pair-feeding method was adopted, having the daily feed being fixed to the voluntary intake of fish reared at 17 °C, group with the lowest intake, in order to obtain similar probiotic intake among the temperature groups. A challenge stress trial (crowding combined with chasing with a pole for 15 minutes) was conducted at the end of the feeding trial.

Growth performance was not affected by the dietary treatment. Non-specific immune response (ACH50 activity) were temperature-sensitive ($P < 0.05$), with no dietary influence. Glutathione, an important antioxidant marker, was significantly affected by temperature $\times$ diet interaction. Digestive enzymes activities were significantly affected by the interaction between diet and temperature. The activity ratio of amylase/trypsin increased with temperature and dietary probiotic supplementation, an indication of metabolic flexibility of carbohydrate-protein utilization. Post-stress cumulative mortality were significantly higher in the 17 °C control group. The current study showed that dietary probiotic supplementation conferred some protection to seabass, with temperature playing a modulatory effect.

Keywords: Probiotics; European seabass (*Dicentrarchus labrax*); Innate immune system; Oxidative stress; Digestive enzymes.

Resumo

O aquacultura frequentemente lida com surtos infecciosos, sendo os antibióticos frequentemente utilizados como estratégias de controlo. Uma alternativa a estes produtos químicos, fortemente restringidos poderá ser o uso de probióticos. Os probióticos são definidos como "microorganismos vivos que, quando consumidos em quantidades adequadas, conferem um efeito benéfico à saúde do hospedeiro". Os benefícios do uso de probióticos são: melhorias da retenção nutricional, propriedades antagonistas para com a proliferação bacteriana, a modulação da resposta imune e defesas antioxidantes, entre outros. A temperatura desempenha um papel importante nos peixes, uma vez que se tratam de organismos poiquilotérmicos, i.e. a sua temperatura corporal varia consoante a temperatura do seu ambiente circundante. A composição da microbiota intestinal do peixe é portanto, influenciada pela temperatura da água. Por isso, a eficácia da suplementação probiótica irá variar de acordo as as temperaturas de cultivo. Adicionalmente, eventos de stress podem desencadear uma cascata de alterações fisiológicas, detetáveis pela quantificação de diversos métodos analíticos.

Este estudo avaliou os efeitos da suplementação probiótica na dieta sobre o crescimento, as respostas do sistema imunológico e antioxidante e também na actividade de enzimas digestivas em robalo (*Dicentrarchus labrax*), mantidos em três regimes de temperaturas diferentes (17, 20 e 23 °C) durante 100 dias. O método *pair-feeding* fora adoptado, tendo a taxa diária sido fixada no consumo voluntário dos peixes cultivados a 17 °C, a fim de igualar a taxa de ingestão de probióticos entre os vários grupos. Um evento de stress (aglomeração combinada com uma perseguição de 15 minutos com um bastão) fora realizado antes da conclusão experimental.

O crescimento não fora afectado pelo probiotico. A resposta imunológica inata (capacidade hemolítica) demonstrou-se sensível à temperatura ($P < 0,05$), mas sem a influência da dieta. A glutathione, um marcador importante na defesa antioxidante, foi significativamente afectada pela interacção temperatura × dieta. Aa actividades das enzimas digestivas foram também afectadas pela interacção entre temperatura e dieta. A proporção da actividade amílase / tripsina aumentou com a temperatura e suplementação probiótica, um indicador de maior flexibilidade metabólica na utilização de hidratos de carbono e proteína. A mortalidade pós-stress foi significativamente maior no grupo controlo a 17 °C. Este estudo demonstrou que a suplementação probiotica conferiu alguma protecção ao robalo, tendo a temperatura sido um forte modulador.

Palavras-chave: Probioticos; Robalo (*Dicentrarchus labrax*); Sistema imune inato; Stress oxidativo; Enzimas digestivas.

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Abbreviations

ROS – Reactive oxygen species.	GST - Glutathione s-transferase.
CAT – Catalase.	TG - Total glutathione levels.
GPX - Glutathione peroxidase.	SOD – Superoxide dismutase.
GSH – Reduced glutathione.	A / T – Amylase to trypsin ratio
GSSG – Oxidized glutathione.	T / C – Trypsin to chymotrypsin ratio.
GR – Glutathione reductase.	ACH50 – Alternative complement pathway.

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1 Introduction

1.1 Aquaculture development

The beginning of aquaculture has multiple theories, from catch-and-hold to trap-and-crop theory, both presuming a likely rudimentary scenario, without place or time description. Such details came first by S. Y. Lin, a noted Chinese aquaculturist, setting the primordium of aquaculture in China's cultivation of common carp (*Cyprinus carpio*) around 2000-1000 B.C., dating aquaculture as far as 4000 years ago (Rabanal, 1988).

The current definition of aquaculture, according to FAO, is "the farming of aquatic organisms, including fish, mollusks, crustaceans and aquatic plants", encompassing the production of over 600 species. Finfish alone attain 66% of the production volume, of which over 80% come from inland facilities (SOFIA, 2014).

World aquaculture production continues to grow, although at a slowing rate, reports claim an all-time high in 2012 (US\$144.4 billion), with China alone producing nearly half, becoming the main reason for the reduced productivity in some developed countries, e.g. the United States of America. Nevertheless, aquaculture represents the fastest growing industry in food sector, driving employment in this area to grown faster than the world's population. As cause and result, world per capita apparent fish consumption increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012. This impressive development has been driven by a combination of population growth, rising incomes and urbanization, and facilitated by the strong expansion of fish production and more efficient distribution channels. Within the world's major producers, e.g., Russia, Iceland and Norway, global importance has only managed to stabilized at 4.32 %, after a simultaneous decrease since the ninety's.

1.2 Aquaculture in Portugal

Aquaculture in Portugal is a relatively old activity, although mostly on a familiar scale. Nevertheless, for decades that it encircles several mollusks bottom cultures (*Ruditapes decussatus*, *Crassostrea angulata* and *Crassostrea gigas*) and finfish inland cultures (*Dicentrarchus labrax*, *Sparus aurata* and *Solea vulgaris*). In the late 1960's, the first large scale production was built, located in Paredes de Coura for the production of rainbow trout (*Oncorhynchus mykiss*), the Castro & Cabero enterprise operates with intensive systems and still functional in these days.

In 2012, Portuguese aquaculture attained over 10.000 tons, a total value of 54 million euros. With brackish and marine fish representing 57.3% of total production and mollusks about 38%. Within finfish, Turbot (*Psetta maxima*) represents over 90% of fish production, followed by seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*).

Today there are approximately 1500 licensed aquaculture companies in Portugal, although reducing in number, these are increasing in production volume. Within these, less than 10 companies focus on breeding, most are growing companies, especially in semi-extensive and extensive conditions (INE, 2013).

1.3 European Seabass

(*Dicentrarchus labrax*, Linnaeus, 1758)

The European seabass (**Figure 1**) belongs to the Actinopterygii class (ray-finned fishes), subclass Neopterygii (new fins), Perciformes order (Perch-likes), and within this, the Moronidae family (Temperate basses), wherein we can find the *Dicentrarchus labrax* (Resolution of ray-finnedfish phylogeny and timing of diversification).



Fig 1: European seabass (*Dicentrarchus labrax*)
Source:(Crocetta, 2014)

With an elongated body, males can reach 1 meter long, weight up to 12 Kg and live to 3 decades, with a bright silver tone trough the body. Juveniles tend to be slightly darker with black spots over the back. As fins, these have 2 sets of dorsal fins, with a total of dorsal spines of 8 - 10 and dorsal soft rays of 12 – 13. Anal fins have 3 spines and 10 – 12 anal soft rays. Posterior Operculum edge is finely serrated, with the lower edge

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possessing strong forward- directed denticles. Mouth is moderately protractile and has 2 flat opercular spines and vomerine teeth are present anteriorly in a crescent band (Fishbase, 2014).

As an euryhaline (3 ‰ to full strength sea water) and eurythermic (5-28 °C) marine teleost species , seabass can be found from Eastern Atlantic to Morocco, Canary Islands, Senegal, Mediterranean and Black sea, inhabiting very different habitats, adapting to different prey, water and bottom compositions (FAOa, 2014).

Seabass are carnivores, feeding on crustaceans, mollusks and fish. Adults tend to wander often alone, predating fish, while juveniles form school groups in search for small crustaceans and mollusks. With a protractile mouth, predatory habits include suction of small preys.

Spawning occurs in batches, as a gonochoristic species, males and females gather in large groups in open sea. There is only one breeding season per year, which takes place in winter in the Mediterranean population (December to March), and up to June in Atlantic populations. Young larvae are then passively transported to nursery areas, as shallow water lagoons and estuaries. These remain in creeks, estuaries, backwaters, and shallow bays through their first and second years, after which they migrate to over wintering areas in deeper water, returning to larger estuaries in summer (ICES, 2013).

In seabass culture, studies have revealed temperature tolerance ranging from 2 to 32 °C (Vázquez & Muñoz-Cueto, 2014). With growth rate and feed utilization having a its best performance within the range of 22 °C to 25 °C (Person-Le Ruyet *et al.*, 2004). Temperature has shown capable to modulate immune system protein expressions (Sarropoulou *et al.*, 2010) and antibody response (Cecchini & Saroglia, 2002). In oxidative stress parameters, temperatures beyond optimal range for seabass induced lipid peroxidation and catalase activity, revealing that temperature deviations may cause responses similar to environmental contaminants (Vinagre *et al.*, 2012).

Although microflora bacteria and supplementation success thermal modulation have been described (Ibrahim *et al.*, 2004, Hagi *et al.*, 2004), more work is required to better understand how this probiotic-host relationship is affected.

European seabass production is historically known for being cultured in coastal lagoons and tidal reservoirs before the development of juveniles mass-production in the late 1960s. These were associated with the reuse of salt production evaporation ponds, where their activities took place along the year, the salt would be harvested during the high evaporation season of summer and autumn, and fish cultured during winter and

spring. But in the late 1970's France and Italy had finally attained a good management of this larvae production, making the European seabass the first non-salmonid species to be commercially cultured in Europe. Representing a very economical important fish, seabass cultures activity, quickly spread along Mediterranean countries (FAOb, 2014).

Recent reports claim a global production peak of 153 000 tonnes in 2012 (**Figure 2**). Main producers are: Greece, Turkey, Italy, Spain, Croatia and Egypt.

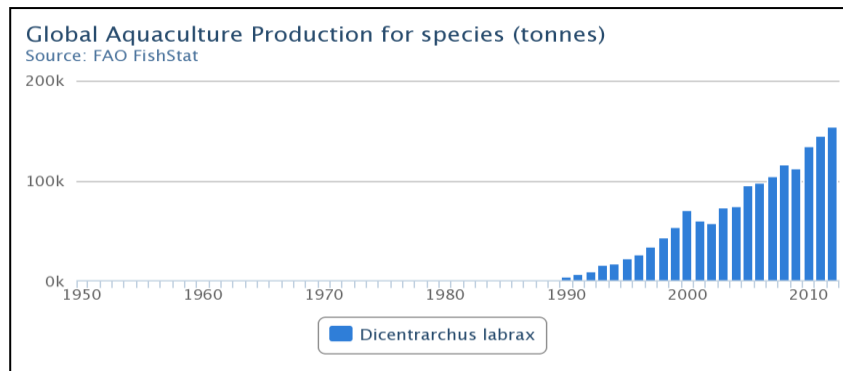


Fig 2: Global seabass aquaculture production (tonnes). Source:(FAOc, 2012).

Portugal annual seabass production peaks at 500 tonnes, a small production when facing the global 132 thousand tons produced annually, of which, Greece alone is responsible for 42 000 tonnes (FAOc, 2012).

With seabass production, scientific research around its needs increased dramatically. A natural priority was its diet. As nutritional needs, the required protein level was settled at 50% since 1988 (Hidalgo & Alliot, 1988). As protein source, seabass revealed quite dynamic as long as the absent essential amino acids were supplemented (Tibaldi & Lanari, 1991). Lipid levels revealed ideal at 12 % (Alliot *et al.*, 1974).

Starch inclusion in diet has become popular due to its use by fish metabolism as energy source, therefore sparing protein or lipids. Starch ideal levels appear to be close to 10% (Enes *et al.*, 2006a), with temperature influencing its digestibility (Enes *et al.*, 2006b).

Essential amino acids for seabass are the same as for salmonids (Kaushik, 1998), being the fish meal the best choice for protein source, with the only requirement for methionine – cysteine and lysine supplementation (Hidalgo *et al.*, 1987) (Tibaldi & Lanari, 1991). Mineral and vitamin supplementations appear to be reduced to vitamin C requirement, important for collagen synthesis. Since the majority of fish are not able produce ascorbic acid, this is required at a minimum of 5mg.Kg⁻¹ of feed (Fournier *et al.*, 2000).

1.4 Digestive system in teleost

A better understanding of a species digestive system represents a major contribution for its rearing success. An adequate diet is closely linked to digestion and absorption; this depends on both the physical-chemical nature of the feed and from the type of enzyme activities present along the alimentary tract. Digestive enzymes are responsible for disassembling the major food components, as protein, carbohydrate and fat to their absorbable subunits. These, can then undergo transportation in the circulatory system, and be reabsorbed by the organism cells, leading to its nutrition, and therefore to growth and development. Digestion is then a progressive process, enduring from the stomach to the rectum, being expelled as feces (Smith, 1989).

Fish digestion starts in the stomach, where pepsinogen (pepsin primordium) and hydrochloric acid are produced. Pepsinogen activated by the acid, turns into pepsin and starts proteolysis, degrading peptides bonds adjacent to aromatic amino acids. Further into the digestive tract, the intestinal mucosa produces enterokinase, this, acts on the secreting pancreatic juice (trypsinogen, pancreatic amylase and lipase, and others). With the activation of trypsinogen into trypsin, a cascade of activations of other zymogens in pancreatic secretions starts, including chymotrypsinogen, proelastase, procarboxypeptidases, and some prolipases. Proteases as trypsins, chymotrypsins and caboxypeptidases A and B, will then further break down peptide chains into amino acids so these can be absorbed through the intestine brush border (Ogiwara & Takahashi, 2007, Grosell *et al.*, 2010).

Digestive enzymes activity can however be modulated by several factors, such as: diet composition (Cahu & Infante, 1994), temperature (Zhao *et al.*, 2009) and age (Kuz'mina, 1996). Therefore, evaluating digestive enzymes activities become a usefull tool in predicting the ability of a species to use different nutrient sources and/or digestibility conditions.

1.4.1 A-Amylase

The main role carbohydrates have in metabolism is of energy source upon their oxidation, acting as fuel for other metabolic functions and therefore sparing protein or lipids use as energy. Carbohydrates are used for this purpose in the form of monosaccharides, mainly glucose. Alpha-amylase is a key enzyme for carbohydrate digestion. It is secreted by the pancreas into the intestine and the pyloric caeca, where it may be adsorbed. It acts on complex polysaccharides, such as starch and glycogen, hydrolysing them up into glicose, maltose, maltotriose and a combination of branched

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(1:6) oligosaccharides and glucose (Papoutsoglou & Lyndon, 2003) (**Figure 3**). Thence, studies quantify α -amylase activity for evaluation of a species capability to digest the supplemented carbohydrates. Starch supplementation represents the most common source. This can be added raw or gelatinized, studies show that partial gelatinized starch substitution over raw increases digestibility, allowing a greater reduction in protein content without mitigating growth (Peres & Oliva-Teles, 2002).

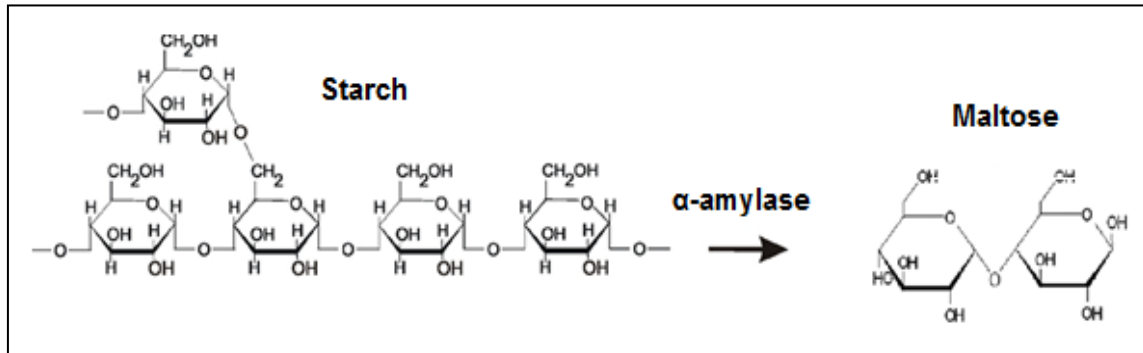


Fig 3: Starch to maltose conversion through α -amylase action. Source: Modified from (Paul, 2012).

1.4.2 Trypsin

Trypsin [EC 3.4.21.4] appears to be the predominant protease in the midgut. Its presence has been found in pancreatic tissues from both vertebrates and invertebrates. As a serine amino acid based protease, its target for hydrolysis are peptide bonds involving the carboxyl groups of lysine and arginine residues (Smith, 1989)(**Figure 4**). Its activation comes from the action of enteropeptidase over trypsinogen. Studies claim trypsin as responsible for digesting up to 50 % of the ingested protein in carnivorous fishes (Eshel *et al.*, 1993).

Trypsin activity is usually measured using synthetic substrates. These include amide and ester derivatives of the amino acids lysine or arginine that possess only one bond that can be easily broken. Trypsin is the only pancreatic protease which can activate its own preform as well as the preforms of other proteases secreted from the pancreas, having a key position in controlling the activity of the pancreatic proteases (Hjelmeland *et al.*, 1984).

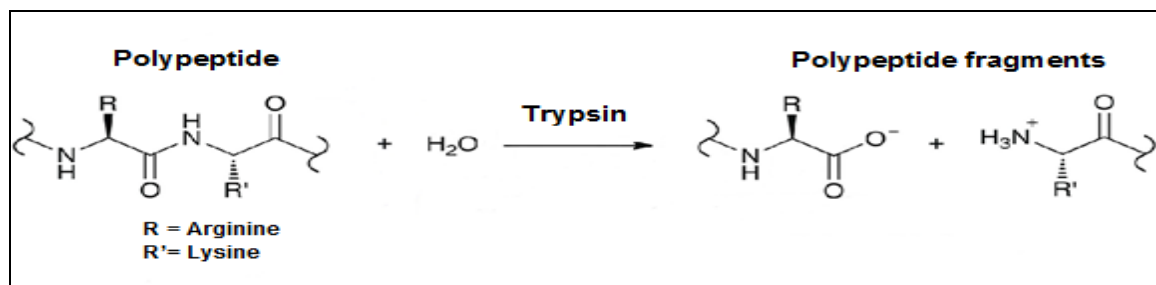


Fig 4: Polypeptides fragmentation by trypsin activity. Source: Modified from (Worthington.corp, 2014).

1.4.3 Chymotrypsin

Like trypsin, chymotrypsin is a serine type, alkaline protease produced in the pancreas and secreted in an inactive form into the lumen of the intestine. Chymotrypsin is activated upon trypsin action on Chymotrypsinogen (**Figure 5**). This enzyme then selectively hydrolyses peptide bonds on the carboxyl side of the aromatic chains of tyrosine, tryptophan, and phenylalanine and large hydrophobic residues such as methionine. Like other digestive enzymes, chymotrypsin is sensitive to temperature and pH changes (Applebaum *et al.*, 2001).

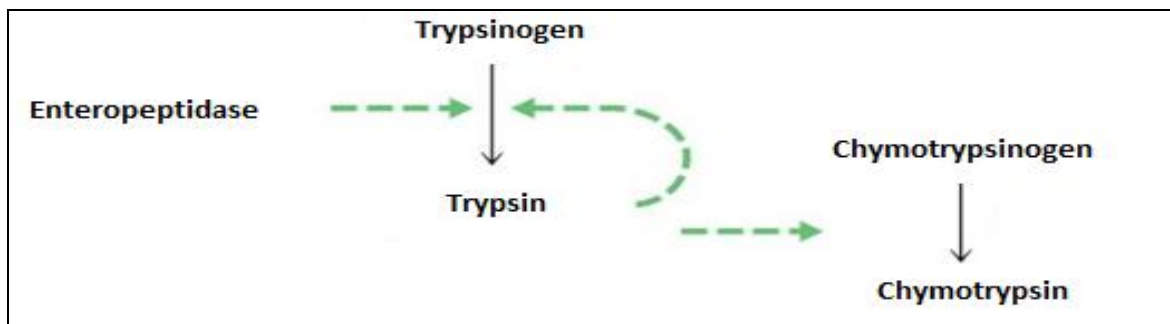


Fig 5: Trypsin and chymotrypsin activation path. Source: Modified from (Maine.University, 2014)

1.4.4 Trypsin to chymotrypsin ratio

The secretion rate of trypsin and chymotrypsin is related to feed intake and satiation levels (Rungruangsak-Torrissen *et al.*, 2006) and their activity ratio (trypsin to chymotrypsin) has been suggested as an indicator of the nutritional status of the fish, by promoting a higher absorption/transport rate of EAA for protein synthesis and plasma insulin level, hence stimulating protein synthesis (Rungruangsak Torrissen & Male, 2000).

1.4.5 Amylase to trypsin ratio

Ratio between α -amylase and trypsin is seen as an indicator of energy metabolism flexibility, showing the fish capability to use carbohydrates in substitution of proteins or lipids for energetic purposes. Often used as a sign for carnivorous feeding habits (Aragón-Axomulco *et al.*, 2012). Recent studies however tend to see this ratio from the protein saving point of view. Nonetheless, growth performance and A/T ratio, still miss correlation with better growth parameters or more efficient protein use (Thongprajukaew *et al.*, 2011).

1.4.6 Lipase

Lipids are required by four main needs in the body: energy, fatty acids, structural components and regulatory functions. If lipid recourses surpass current demand, these are stored as triglycerides within the adipose tissue beneath skin, in muscle fibers, and in the abdominal chamber. It is well known that lipids digestion differs from protein and carbohydrates digestion, since lipids are not water-soluble. To solubilize, lipids must be break down by the bile secreted from the gall-bladder. By emulsifying, lipids become vulnerable to pancreatic lipase [E.C. 3.1.1.3] hydrolization , turning triacylglycerols into diacylglycerides, then monoacylglycerides and finally free fatty acids and glycerol (**Figure 6**). These are then absorbed under the form of micelles, a mix of fatty acids, glycerol and bile salts(Webster & Lim, 2002).

Marine fishes require polyunsaturated n-3 fatty acids, such as eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) as essential fatty acids for normal growth (Cowey & Cho, 1993). The control of pancreatic secretions in fish is probably the same as in mammals, but there is no information on teleost yet. Nevertheless, studies suggest that pancreatic lipase activity results serve as a good indicator of lipase digestibility (Infante & Cahu, 1999). Studies have also suggested lipase activity modulation by dietary yeast supplementation (Tovar *et al.*, 2002a).

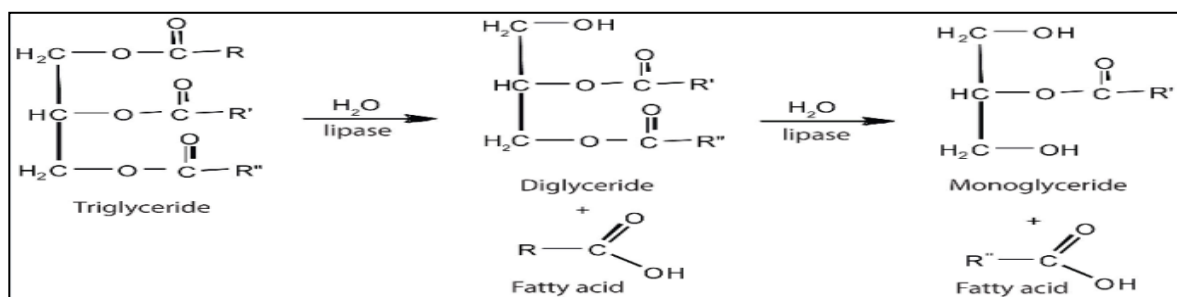


Fig 6: Triacylglycerol hydrolization to glicerol and fatty acids by lipase reaction. Source:(Namrata, 2013).

1.5 Immune system in teleost

Aquaculture rapid development has increased disease outbreaks frequency. It is well documented that the occurrence of a fish disease depends on the balance between host, pathogen and the environment, three factors with continuous interaction (Roberts, 2012).

Immune system in fish has attained interest in phylogenetic point of view, since fish were the first group of animals showing the basic aspects of the immune system of higher vertebrates such as true lymphocytes, lymphoid tissues, antibody production, T cell

cytotoxicity and long term memory (Koumans-van Diepen, 1993). As in other vertebrates, the innate immune system of fish provides the first line of immune defense. Teleost lymphoid system contains thymus, head kidney, spleen and mucosal associated to lymphoid tissue and cellular components that display humoral and cellular immune responses. Within these, non-specific cell-mediated cytotoxic, microbial killing by neutrophils and macrophages, B-cell and T-cell activities (Scapigliati *et al.*, 2002).

1.5.1 Complement system

The complement system is composed of more than 35 soluble plasma proteins and represents a very important defense system in fish, these are expressed in the liver and released into plasma. Consisting in a complex enzyme cascade and composed of several inactive glycoproteins, these can be activated by one of the three known activation routes: classical, alternative, and lectin-mediated. The functions of the complement system include lytic activity (for viruses, bacteria, and parasites) and neutralization pathogenic exotoxins (**Figure 7**). The activated proteins can also provide an alert of potential pathogens present in the host, hence contributing to the degradation of pathogens through the recruitment of immune cells and through opsonization (Boshra *et al.*, 2006).

The fish complement system have been showed to be affected by light exposure (Angeles Esteban *et al.*, 2006), environment temperature (Tort *et al.*, 1998), diet (Geay *et al.*, 2011) and more directly correlated, infection (Henry *et al.*, 2009).

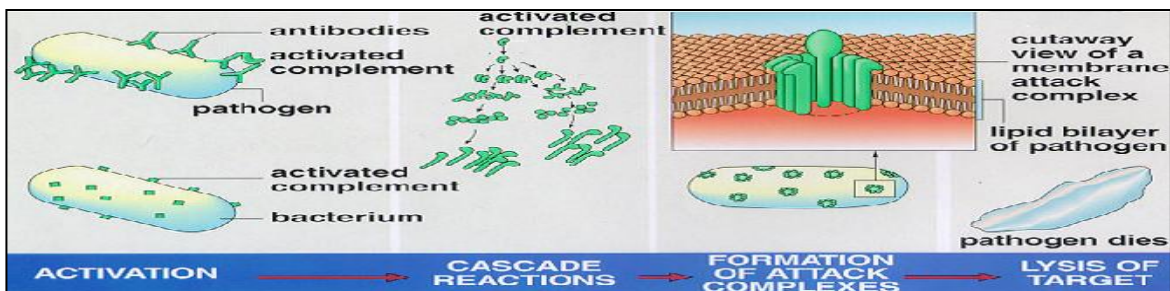


Fig 7: Complement system cascade reaction upon activation illustration. Source: (UIC, 2014)

1.5.2 Peroxidase

Both neutrophils and macrophages are important innate system components that protect the organism against microbial infection. The functions of both cells include phagocytosis, chemotaxis and bactericidal activity (Katzenback *et al.*, 2012).

Neutrophils upon activation release hydrogen peroxide and myeloperoxidase (EC 1.11.2.2), a heme-containing lysosomal glycoprotein, found predominantly in neutrophils azurophilic granules, and in much lower amounts in monocytes and some tissue

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macrophages. Myeloperoxidase then catalyzes chloride ions and the hydrogen peroxide to form hypochlorous acid. Hence damaging the invading microorganisms (Klebanoff, 1968). Similar to neutrophils myeloperoxidase, eosinophils produce an eosinophilic peroxidase (EC 1.11.1.7), consisting of a heavier glycosylated chain and a lighter non-glycosylated chain. This enzyme prefers bromide over chloride as a substrate, converting it to hypobromite, toxic to infecting pathogens (Bielek, 1981) (**Figure 8**).

Peroxidases, released from the cytoplasmic granules of phagocyte participate in the oxidative responses against pathogens. Serum Peroxide levels are known to increase in response to infection (Alvarez-Pellitero, 2008). Therefore, peroxidases released into the blood are then often used as an indicator of the immunologically active status of circulating leucocytes.

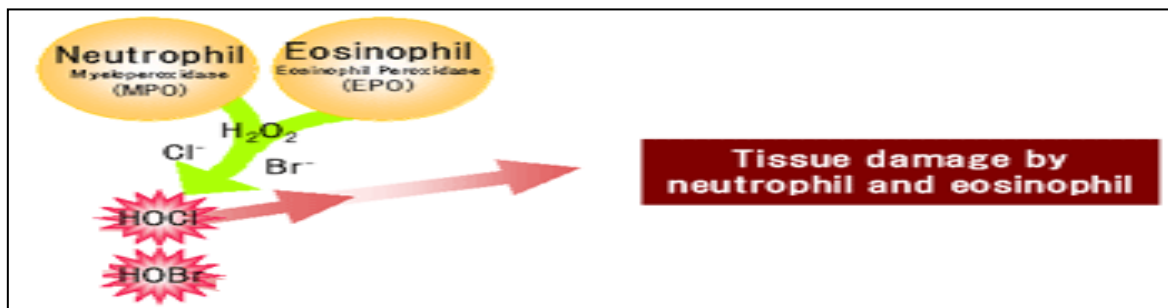


Fig 8: Myeloperoxidase and Eosinophilic peroxidase reaction. Source: Adaptation of (Jaica, 2014)

1.5.3 Lysozyme

Lysozyme was the first natural antibiotic isolated from humans in 1922 by Mr. Alexander Fleming (Nakatsuji & Gallo, 2012). As an glycoside hydrolase enzyme, lysozyme (EC 3.2.1.17) can damage bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins. Hence, lysozyme is useful tool when dealing with pathogens, especially gram positive bacteria, as these have a peptidoglycan outer layer. With of leucocyte origin, lysozyme is widely distributed in bacteriophages, microbes, invertebrates and vertebrates and its found in a large variety of animal secretions such as mucus and saliva, in many tissues including blood (Jollès & Jollès, 1984).

Studies have shown that plasmatic lysozyme activity can be modulated by several factors. Low temperatures have been shown to decrease lysozyme activity in gilthead sea bream (Tort et al., 1998), but a less linear correlation was found in Nile tilapia (*Oreochromis niloticus*), where a slight increase revealed to increase before a plateau was reached (Dominguez et al., 2005). Finally, dietary supplements provenience of probiotic

yeast (*Saccharomyces cerevisiae*) have shown capable to improve lysozyme activity as well (Torrecillas *et al.*, 2007).

1.6 Oxidative stress in teleost

A consequence of all aerobic life is the production of potentially harmful, partially reduced species of molecular oxygen radicals (ROS) which occur as a result of normal oxygen metabolism. ROS are generated by physical, chemical and metabolic processes that convert O₂ into reactive oxygen species, such as superoxide, hydroxyl radicals and non-radical hydrogen peroxide or even singlet oxygen. It has been estimated that about 1 to 3 % of O₂ consumed in animal systems is converted to ROS. Greatest incomes are the auto-oxidation of the mitochondrial electron transport chain, microsomal cytochrome P-450 and flavin-protein reductases (Livingstone, 2003). Although ROS supply the body with humoral innate protection against pathogens, as mentioned in peroxidases parameter, disturbances in the normal redox state of cells can cause toxic effect, ROS may damage all components of the cell, including proteins, lipids, and DNA. Furthermore severe oxidative stress can cause cell to trigger apoptosis, while prolonged extreme oxidative damage may cause tissue necrosis (Zong & Thompson, 2006). These effects depend upon the size of these changes. A cell can always be able to overcome small perturbations and regain its original state, without further damage. Only when the production of these aggressor outcomes the organism defense capabilities, does the animal enters the oxidative stress condition (**Figure 9**).

ROS production can be induced by external factors. Environmental pollution may well be one of the most studied sources. Transition metals (Cu, Fe) represent a major path of environmental oxidative, as they catalyze the production of hydroxyl radicals through Fenton reaction. Other chemicals like biphenyls, quinones and nitroaromatics can also induce production of superoxide by redox cycling (Sevcikova *et al.*, 2011).

Environmental influence however does not require physical or chemical hazardous conditions to induce ROS production. In aquaculture, several events common to daily maintenance may cause stress to fish, hampering fish health and growth. Studies suggest that oxidative stress in aquaculture can be correlated to several types of stressful conditions, such as: dietary influence (Olsen & Henderson, 1997), feed deprivation (Pascual *et al.*, 2003), fish crowding (Bagni *et al.*, 2007), hypoxia (Guerriero *et al.*, 2002), acute thermal conditions (Vinagre *et al.*, 2012) and infection response (Ali *et al.*, 2011).

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Within the infection response, ROS main source may well be leukocytes respiratory burst attack, as these use NADPH (nicotinamide adenine dinucleotide phosphate-oxidase) to generate ROS, and therefore performing an aggression towards the pathogen (Alvarez-Pellitero, 2008).

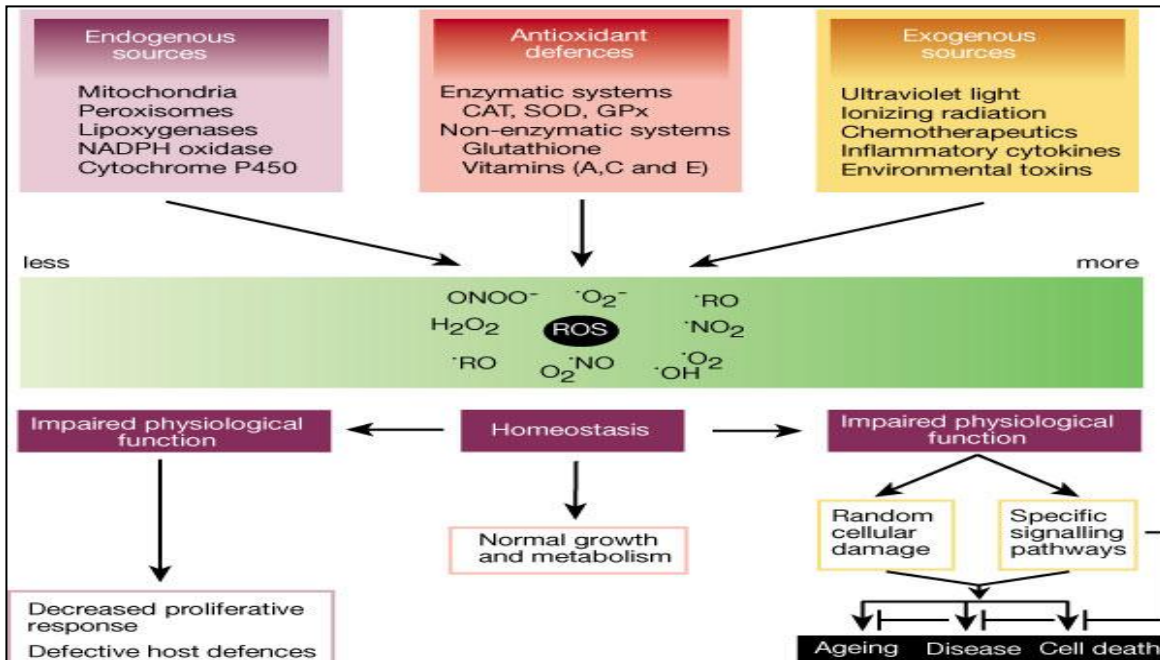


Fig 9: Reactive species of oxygen sources, defenses and consequences. Source: (Finkel & Holbrook, 2000)

Copper/zinc superoxide dismutase, catalysis the dismutation of superoxide into oxygen and hydrogen peroxide. CAT (EC 1.11.1.6.) catalysis the decomposition of hydrogen peroxides to water and oxygen, and interpreted as a protector against hydrogen peroxide. Glutathione peroxidase (GPX) (EC 1.11.1.9.), reduces lipid hydro-peroxides to their corresponding alcohols and to reduce free hydrogen peroxide to H_2O , with a co-factor, glutathione (GSH). Glutathione reductase (GR) (EC 1.8.1.7), is an enzyme responsible for recovering oxidized glutathione (GSSG) to its useful state (GSH). Glutathione s-transferase (GST) (EC 2.5.1.18), another glutathione (GSH) dependent enzyme, neutralizes certain xenobiotics, inhibiting these to cause damage over the organism cells.

Finally, total glutathione levels (TG), a sum of both GSH and GSSG, a tripeptide that when in its reduced form works as an electron donor to GPX, and as cofactor for GST, and also, as a direct thiol-based antioxidant. Studies suggest that although GSH/GSSG ratio may indicate a more prompt antioxidant state, total glutathione levels drop signals a state of great use of GSH (Eroglu et al., 2014).

These molecular parameters constitute the first line of antioxidant enzymatic defense and are used as biomarkers for oxidative stress condition in a variety of marine and freshwater organisms (Eroglu et al., 2014). ROS reaction on cells lipids is considered one of the most

prevalent mechanisms of cell (Gravato & Guilhermino, 2009). Since lipids are oxidized usually through the formation of peroxides, the process of their formation has been called “lipid peroxidation”, and is quantified to determine the degree of damage that may have been caused by ROS over the lipid layers. As this damage tends to be avoided when antioxidant enzymes surpass the oxidants, this gives a sight of the organism vulnerability (Lushchak, 2011). As site of measurement, the liver has been the focus of toxicological studies and has indeed been shown to be very sensitive organ to oxidants presence (Ben Ameer et al., 2012). Therefore, it's accepted that the monitoring of antioxidant enzymes activities in the liver may create a good evaluation of the antioxidant state.

1.7 Probiotics in teleost

The term, probiotic, means “for life”, its origin came from the Greek words “pro” and “bios”. Probiotics history starts with the fermentation of cheese, where milk sugars are turned to lactic acid. But the idea of the accommodation of these micro-organism within animals intestine only came in 1901, when a Russian scientist studied the human intestine microflora and suggested that ingestion of fermented milk could bring health benefits (Gismondo *et al.*, 1999).

In aquaculture intensive productions, aquatic species are subjected to high-stress conditions, increasing the probability of diseases incidence and causing a decrease in productivity. In order to control pathogenic infections, aquaculture uses chemical additives and veterinary medicines, especially antibiotics, which generate significant risks to public health by promoting the selection and persistence of bacterial-resistant strains (Martinez Cruz *et al.*, 2012). In the search for alternatives, other methodologies emerged: bacteriophages, anti-virulence therapy, chromosome II replication inhibition and the promising probiotic administration (Defoirdt *et al.*, 2011).

As the probiotic strain(s) proliferates in the intestine, this may then influence the host, acting as commensals, pathogens and even interacting within ecosystems micro-fauna. These may be administered intentionally, allowing a pursuit for an advantageous biodiversity of beneficial micro-flora for the host. With this feature, we approach the concept of probiotics, initially used to define substances excreted by an organism, capable of benefiting the growth of other, later in 1971, Sperti defines as tissue extracts capable of stimulate microbial growth, but only in 1974 Parker would define it as a microbial feed supplement (Vilà *et al.*, 2010). And so, reaching to the current definition of probiotics according to FAO, as “live microorganisms, which when consumed in adequate amounts, confer a health effect on the host”, in which, for use in foods, probiotic

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microorganisms should not only be capable of surviving passage through the digestive tract but also have the capability to proliferate in the gut (Pineiro & Stanton, 2007).

Regarding their source, most microbial strains were initially recovered from the intestinal tract of many aquatic animals, particularly rainbow trout, Atlantic salmon and Indian carp (*Catla catla*). Currently there are about 30 recognized strains, with considering importance, the genera *Bacillus* and *Lactobacillus*. In the European Union, there is still only one probiotic based on the lactic acid bacteria strain *Pediococcus acidilactici* that has been approved as feed additive (Bactocel®), after demonstration of beneficial effects on salmonids and shrimps (EFSA, 2012).

As administration methods, we can find: dietary supplementation, where probiotics can be directly incorporated into feed pellets at a suitable temperature; bioencapsulation, when we allow the probiotic strains to reach the intestine due to a coating, this method can use live microorganism as a delivery mechanism (e.g. artemia, rotifers); and finally, straight water enrichment, where the host get colonized through water ingestion (De et al., 2014).

The influences of probiotics already registered are diverse, encompassing improvements in nutritional retention (Mohapatra et al., 2012), antagonistic properties towards bacterial proliferation (Sun et al., 2010), nutrient competition towards possible pathogens (Fredrickson & Stephanopoulos, 1981), accommodation space struggle (Vine et al., 2004), modulation of host's immune system responses (Taoka et al., 2006). Also, recent findings indicate the properties of probiotics as promoter of better protection against oxidative stress (Chiu et al., 2007, Tovar-Ramírez et al., 2010).

These defensive properties, make the use of probiotics an promising alternative to antibiotics, improving the range of prophylactic treatments possible, and also aiding the on growing stringent regarding the use of chemotherapeutic products in animal in European countries (EC, 2003).

Although attractive, probiotic use, action and proliferation are affected by several factors that may limit their applications in fish models, such as water temperature, osmotic pressure and mechanical friction, pH and oxygen levels (Nayak, 2010b). Other interesting limitation, is the “quorum sensing” defined as a process of bacterial cell-to-cell communication. This theory defends that the homeostasis of the microflora living in their ecosystem, e.g., intestinal mucosa (Mohapatra et al., 2013).

1.7.1 Growth and Digestive enzymes

Despite the promising potential benefits demonstrated in the current literature, the probiotic mechanisms which mediate host benefits are poorly understood. Digestibility however, may well be the better explained propriety. Digestive exo-enzymes have been reported in *Bacillus* genera, in fact, the bacteria isolated from the digestive tract of aquatic animals have been shown to produce chitinases, proteases, cellulases and lipases (De et al., 2014, Mohapatra et al., 2012). A better availability of exo-enzymes produced by probiotics can lead to a more efficient nutrient digestibility (Vine et al., 2006). Consequently, it becomes important to quantify the activity of the digestive system enzymes in an assay with dietary probiotic supplementation.

1.7.2 Immune system

In the defense from pathogens, competitive exclusion, antagonistic compounds production and feed competition revealed success strategies. The simple idea of culturing beneficial bacterial, preventing other to house in the host intestine, therefore reducing the infection rate, represents a theory, mostly applied in fry, referred as “getting in there first” strategy (Irianto & Austin, 2002).

Probiotics can interact with the host's immune cells such as mononuclear phagocytic cells (monocytes, macrophages), poly-morphonuclear leucocytes (neutrophils) and natural killer cells to enhance innate immune responses. Experiments report influences in the organism phagocytic activity, respiratory burst activity, lysozyme levels, peroxidases activity and complement system activity. More detailed approaches mention cytokines modulation (Nayak, 2010a). Within probiotic bacteria, *Lactobacillus* and *Enterococcus* genera appear to be the most influent in the immune system modulations. It's most common action appears to be the improvement of complement system activity (Sun et al., 2012), peroxidase (Wang et al., 2008) and cytokine expression (Biswas et al., 2013).

Probiotics action methods still require future work in evaluating the mechanisms by which probiotics interact with the host and pathogens. Pursuing this objective, more importance has been given to a peculiar area, the axenic and gnotobiotic organisms experiments. Axenic organisms are animals raised or treated to eliminate microbes derived from parents, gametes or environment; hence considered germ free animals. Gnotobiotic organisms are animals harboring a known microbe or microbiota, that could formerly derived from a germ free animal, posteriorly colonized by a known microbe. This area has provided interesting results, such as an adaptive trace found in zebrafish (*Danio rerio*), where its immune system has revealed tolerance to probiotic strains. More

precisely, the intestinal wall cells, enterocytes, genetic expression for alkaline phosphatases, has shown a capability to reduce the immune system response aggression upon facing microbiota lipopolysaccharide (Bates *et al.*, 2007). This divulging an evolutionary trait on hosts defensive system.

1.7.3 Oxidative stress

Probiotic supplementation has been correlated with antioxidant parameters modulation, although not completely understood, possibilities encompass two major theories: improved diet utilization, hence increasing the assimilation of dietary antioxidants from feed, and also, an active role in antioxidants activity or availability.

Studies with quantified enzymatic activities and oxidation products account greater SOD and CAT in shrimp (*Litopenaeus stylirostris*) and in leopard grouper (*Mycteroperca rosacea*) fed *Pediococcus acidilactici* and *Debaryomyces hansenii* supplemented diets, respectively (Castex *et al.*, 2009, Reyes-Becerril *et al.*, 2011). Also, a damage reduction upon lipids and DNA was observed in zebra fish fed *Lactobacillus rhamnosus* (Gioacchini *et al.*, 2014). Also, an interaction between time and dosage level of supplementation in antioxidant and oxidative damage results was found in coruh trout (*Salmo coruhensis*) fed a probiotic blend (Kefir) (Can *et al.*, 2012).

Although probiotic seem to influence the antioxidant potential, more work is necessary to understanding how is this connection being established. Nonetheless, results reveal greater resilience to ROS inducing stress events like thermal shocks, pH variations and pesticides contaminations when the organisms are fed probiotic supplemented diets (Mohapatra *et al.*, 2013).

1.8 Temperature in teleost

As stated by Sengupta and Garrity in "*Sensing temperature*" (2013): "Temperature is an omnipresent physical variable reflecting the rotational, vibrational and translational motion of matter". For what matters in biology, temperature decides a great part of a species success and possible proliferation, and this is mostly due to temperature's capacity to mold matter. Temperature dominant modulations affect proteins and lipids re-conformation, as well as genetic modulation, playing as an on/off trigger in genetic transcription, translation or path activation (Sengupta & Garrity, 2013). A more immediate effect is the increase of kinetic energy in molecules, such as enzymes, temperature can then make molecule collisions more frequent, enabling the enzyme-substrate complex to occur more incessantly, hence increasing the reaction rate (Athel, 2004).

Expectedly temperature is often considered one of the most important variables in biochemical and physiological processes, and so, a highly concerning factor in the environment of a poikilothermic organism.

2 Objectives

The current study aimed to determine the effects of dietary probiotic supplementation on growth performances, immune system response, antioxidant parameters and digestive enzymes activity, in seabass reared at three temperatures. This experiment intends to search for the direct influence of probiotic supplementation as well as possible interactions of these influences with the auxiliary factor of temperature.

3 Materials and Methods

The current trial was conducted under the supervision of an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal), as according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

3.1 Fish and facilities

Fish were provided by “*L’ecloserie marine de gravelines*”, a hatchery company, situated near Calais, France. After arriving at the experimental unit, fish were kept in the quarantine system for 2 weeks at CIIMAR - UP. Then, fish were transferred to the experimental system at the aquatic production department in ICBAS – UP, and acclimatized to the rearing conditions for 15 days (**Figure 10**). During this period fish were fed a commercial diet (non-supplemented) three times a day until satiation.



Fig 10: Aquatic engineering Laboratory (ICBAS – UP).

The experimental system was composed of eighteen tanks of 80-liters connected to a single recirculation system (TMC® *System 5000P Marine*), at a $1.5 \text{ L} \cdot \text{min}^{-1}$ per tank flow, with continuous aeration. Three water temperatures were tested (17, 20 and 23 °C) in a 2 x 3 factorial design. Six 17°C tanks were acclimatized by pre-chilling the water through two aquaria chiller (Teco® – Model TR60), the 23 °C tanks each with 2 thermostats heaters (Trixie® – 200w), and the 20 °C tanks were obtained by acclimatization of room atmosphere through air conditioning set to 20 °C and also by the total water mixing (17 °C and 23 °C) in the water reservoir, allowing the 20 °C mid-term.

3.2 Experimental diets

The basal diet (**Table 1**) was prepared without probiotic supplementation (control). Half of the basal diet was used as control and the other half was supplemented with probiotic blend (*Bacillus sp.*, *Pediococcus sp.*, *Enterococcus sp.*, and *Lactobacillus sp.*) at 3g per kilogram feed (3×10^9 CFU/Kg feed).

All diets were formulated and manufactured by Sparos.Ltd, the main ingredients were grinded (below 250 micron) in a micro-pulverizer hammer mill (Hosokawa Micron, SH1, the Netherlands). Powder ingredients and oil sources were then mixed accordingly to the target formulation in a paddle mixer (Mainca RM90, Spain). After the incubation the probiotic mixtures were added to the feed mash under continuous mixing at 3g per feed kilogram ratio. All diets were manufactured by temperature controlled-extrusion (30-35 °C) with pellet size of about 1.5 to 2.0 mm by means of a low shear extruder (Italplast P55, Italy). Upon extrusion, all feed batches were left overnight to dry at 45°C. For preservation, diets were stored at 4 °C, throughout the duration of the feeding trial.

Table 1: Basal diet analysis and composition (%). Experimental diet differed only in the probiotic supplementation with a probiotic blend, composed by 4 bacteria strains (*Bacillus spp.*, *Pediococcus spp.*, *Enterococcus spp.*, e *Lactobacillus spp.*).

Control Diet composition	%
Fishmeal 70 LT (Norvik)	32.00
Fishmeal SOLOR	15.00
CPSP G	5.00
Squid meal	2.50
Wheat gluten	3.00
Corn gluten	4.00
Soybean meal 48	6.00
Rapeseed meal	5.00
Wheat meal	7.40
Peas gelatinized (Aquatex 8071)	8.00
Fish oil	9.30
Vit & Min Premix PV01	1.00
Choline chloride	0.10
Soy lecithin	0.50
Binder (guar gum)	1.00
Antioxidant liquid (Naturox)	0.20
Content	%
Crude protein	50.03
Crude fat	16.69
Fiber	1.20
Starch	9.63
Gross Energy	20.47
Lys	3.54
Met + Cys	1.95
Total P	1.22
Available P	1.05

3.3 Experimental design

The experimental trials were conducted using seabass in a fast growing stage, reared under three water temperature conditions (17, 20 and 23° C) in a 2 x 3 factorial design. Fish were fed either a control or a probiotic diet for 100 days, 3 times a day by means of automatic feeders (8:00-13:00-18:00). Diets were fed at a fixed feeding level. The trial were carried out at pair feeding, i.e. at the end of the trial, all aquaria received approximately the same amount of feed (**Table 2**), fixed according to the voluntary feed intake of the fish groups reared at the lower temperature (17 °C).

Table 2: Total feed intake (FI, (g)) and voluntary feed intake (VFI, % BW/day) of fish feed for all the treatments.

	Control			Probiotic		
Temperature	<u>17 °C</u>	<u>20 °C</u>	<u>23 °C</u>	<u>17 °C</u>	<u>20 °C</u>	<u>23 °C</u>
FI (g)	30.7±0.5	36.8±0.4	40.9±0.5	30.7±0.2	35.8±0.4	39.4±0.7
VFI (% BW/day)	1.83±0.04	1.81±0.03	1.73±0.05	1.71±0.02	1.68±0.03	1.74±0.02

3.4 Experimental procedure and sampling

After fish arrival, these were equally distributed into 18 tanks at a density of 26 fish (initial body weight: 12.8 ± 0.5 g) per tank, (4 Kg.m^{-3}). After acclimatization that lasted for 2 weeks, triplicate tanks were conditioned to one of the three testing temperatures (17, 20 or 23 °C). All tanks were kept in the same recirculation systems with continuous aeration with seawater (**Figure 11**), providing a flow of approximately 1.5 L water/ min per tank. Oxygen levels near saturation (9.7 mg.L^{-1}), pH (8 to 8.5), ammonia ($< 1 \text{ mg / L}$) and nitrite ($< 2 \text{ mg / L}$) were daily monitored. Temperature was analysed at every tank 3 times a day (**Table 3**). Photoperiod was set for 12:12 light:dark, and water sterilization was provided by 8 ultraviolet lamps of 54 watts each.

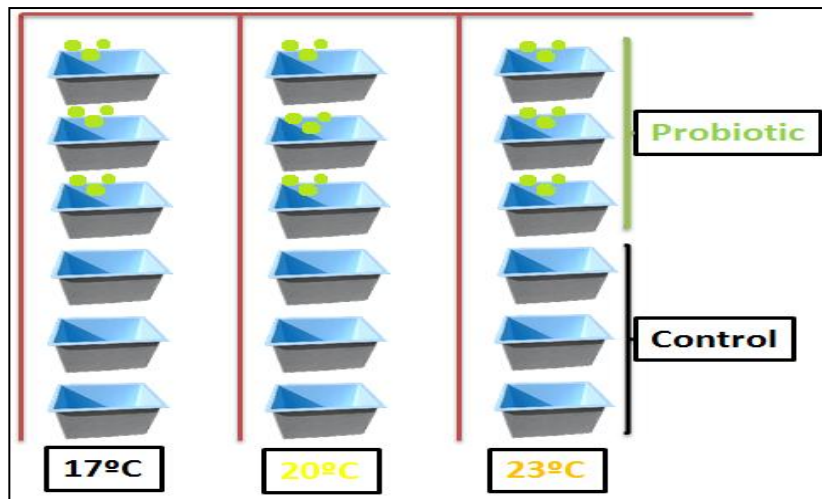


Fig 11: Experimental trial schematic design. Six tanks at each temperature (17, 20 and 23 °C). With three tanks of each temperature for each diet (Control and experimental), in a total of 18 tanks of 80 liters each.

3.4.1 Samples preparation

Two samplings were performed, at day 70 and 100. At day 70, four fish were removed from each tank and immersed in crushed ice for hypothermic anesthesia. These were then weighted and sampled for blood before cervical dislocation, followed by liver extraction. The blood was immediately centrifuged for plasma sampling, later to be used for the immunological parameters, while the liver was stored at -80 °C for posterior homogenization. This sampling would then contribute for growth performance, immune system responses and antioxidant parameters quantification.

At the hundredth day, an acute stress would be conducted to all tanks previously to sampling. After this, 4 fish were removed per tank. These were then immersed in crushed ice for hypothermic anesthesia and then executed by cervical dislocation. Intestine would then be sampled from all 4 fish.tank⁻¹, for posterior digestive enzyme activity quantification. Finally, a total of 18 fish would remain in each tank for accumulative mortality quantification.

3.4.2 Handling Stress

To simulate a handling stress, capable of replicating a common acute stress in aquaculture production, we followed a procedure carried out (Olsen et al., 2005). This was characterized by an initial crowding, increasing animal density by 6 fold (from 4.3kg m^{-3} to 25.5kg m^{-3}), followed by a 15 min chase challenge with a pole (**Figure 12**). No other alterations were made in water flow, rearing temperature or oxygenation conditions during this event. Mortalities were registered for 10 days to perform an accumulative chart.

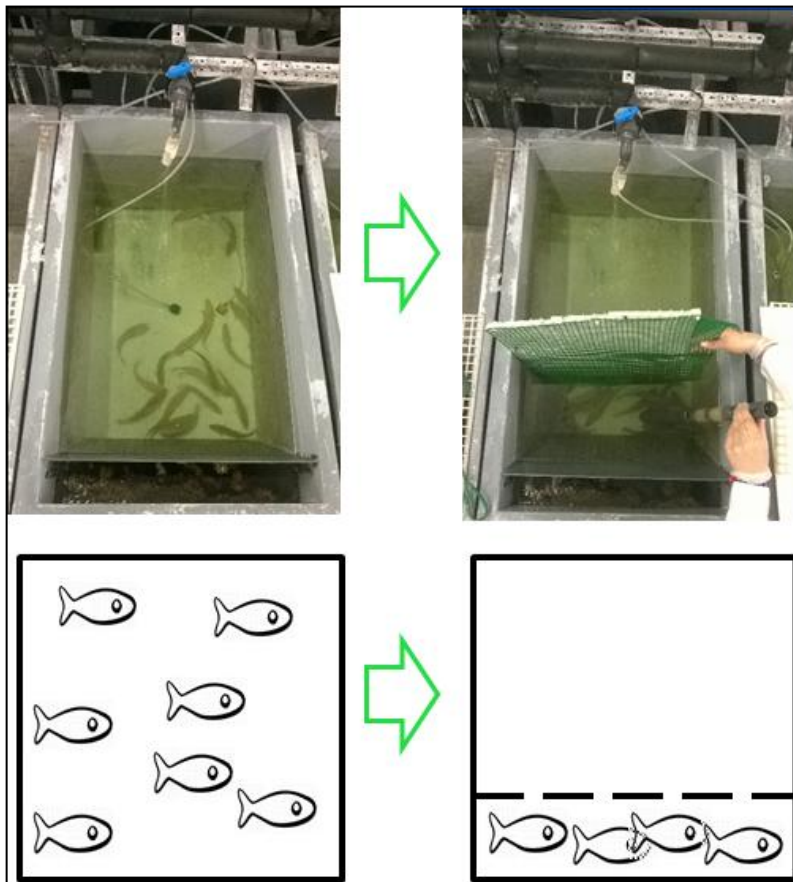


Fig 12: Representative illustration of the stress event. Composed of a crowding that increased fish density 6 times fold. Followed by a pole pursuit for 15 minutes.

3.5 Growth parameters

All fish were weighted at the beginning and end of trial. From these measurements the following parameters were calculated, feed conversion ratio (FCR), daily growth index (DGI) and protein efficiency rate (PER).

$$FCR = \frac{\text{Feed consumption}}{\text{Body weight gain}}$$

$$PER = \frac{\text{Wet weight gain}}{\text{protein intake}}$$

$$DGI = \frac{[100 \times (\text{fish final weight } 1/3 - \text{fish initial weight } 1/3)]}{\text{days}}$$

3.6 Humoral immune parameters

3.6.1 Alternative complement pathway (ACH50)

The ACH50 was determined according to Sunyer et al., (1995). The main principle of this procedure is to evaluate the required plasma sample to have 50% hemolysis in the added rabbit red blood cells according to the Sunyer method (Sunyer *et al.*, 1995). The alternative complement pathway (ACH50) units were defined as the concentration of serum giving 50% hemolysis of rabbit's blood cells. All analysis were conducted in triplicates.

3.6.2 Peroxidase

The total peroxidase content present in the plasma was measured according to Quade and Roth (Quade & Roth, 1997). Using TMB (3,3',5,5'-tetramethylbenzidine) as substrate, in the presence of hydrogen peroxide. Final unit is presented as enzymatic units, with one enzymatic unit (EU) being defined as the amount producing an absorbance change of 1.

3.6.3 Lysozyme

Lysozyme concentration was determined by the turbidimetric assay as described by Ellis method (Ellis, 1990). By measuring its action upon *Micrococcus lysodeikticus* bacteria. Final unit is presented as enzymatic units made by the convention of 1 EU = 0.001 absorbance units.minute⁻¹.

3.7 Oxidative Stress: Enzymatic and non-enzymatic analyses

After liver extraction, a liver homogenate was made with a K-phosphate buffer (pH 7.4, 0.1M) in a 1:15 (p / v) ratio. For the lipid peroxidation analysis, 150 µl of liver solution were add with 2.5 µL BHT (2,6-Di-tert-butyl-4-methylphenol) at 4% in methanol, for preservation, and stored at -80 °C until quantification. The remaining enzymes (CAT, GPX, GR and TG) samples were prepared by centrifuging liver homogenate at 10000 g forces for 20 min at 4 °C, here post-mitochondrial supernatant (PMS) was isolated and stored in separate microtubes at -80 °C. For CAT and GST quantification, the soluble protein content required to be close to 0.7mg.ml⁻¹ concentration. For this calibration a previous protein quantification and adequate dilution were made.

3.7.1 Protein quantification

Protein quantifications were required for protein levels calibration (CAT and GST) and final activity unit reference in all anti-oxidant enzymes. This quantification followed the Comassie binding principle of Bradford (Bradford, 1976). Final unit is protein mg. ml⁻¹.

3.7.2 Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) are formed as a byproduct of lipid peroxidation, which can be detected by the TBARS essay using thiobarbituric acid as a reagent (Ohkawa *et al.*, 1979). TBARS essay then measures malondialdehyde (MDA) present in the sample, and represents the main product of lipid peroxidation. Final unit presents as nmoles of MDA formed.mg protein⁻¹.

3.7.3 Catalase

With the soluble protein levels near 0.7 mg.ml^{-1} , activity quantification followed the principle of catalase action on peroxide hydrogen as described by Clairborne (Claiborne, 1985). Final unit is presented as hydrogen peroxide $\mu\text{mol.min}^{-1}.\text{protein mg}^{-1}$.

3.7.4 Glutathione s-transferase

With the soluble protein levels near 0.7 mg.ml^{-1} , the analysis was as described by Habig (Habig *et al.*, 1974). Where CDNB (1-chloro-2,4-dinitrobenzene) is conjugated with GSH, forming the measurable conjugate of GSH-CDNB (1-chloro-2, 4-dinitrobenzene). Final unit is expressed in $\text{pmol GSH-CDNB conjugate formed.min}^{-1}.\text{protein mg}^{-1}$.

3.7.5 Glutathione peroxidase

Glutathione peroxidase quantification followed the method of Mohandas, (Mohandas *et al.*, 1984). In this method the reaction is measured by the formation of oxidized NADPH (NADP⁺), and presented in the final unit of NADP⁺ $\text{pmol.min}^{-1}.\text{protein mg}^{-1}$.

3.7.6 Glutathione reductase

The measure of glutathione reductase in an assay based on reduction of 5,5'-dithiobis(2-nitrobenzoic acid) to reduced glutathione (GSH), generated from an excess of oxidized glutathione (GSSG) as described by Cribb (Cribb *et al.*, 1989). Again, the activity is quantified by the formation of oxidized NADPH (NADP⁺), and presented in the final unit of NADP⁺ $\text{nmol.min}^{-1}.\text{protein mg}^{-1}$.

3.7.6 Total Glutathione

Total glutathione is quantified by reaction of GSH with DTNB (5,5'-dithio-*bis*-2-nitrobenzoic acid), which produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB) quantified at 412 nm, as described by Baker (Baker *et al.*, 1990). During the reaction the GSSG is concomitantly reduced to GSH, hence enabling the measure of all glutathione. Final unit is presented as TNB $\mu\text{mol.protein mg}^{-1}$.

3.8 Digestive enzymes

3.8.1 Enzyme extraction and protein quantification

The extraction of the digestive enzymes was made through the homogenization of the intestine as described by Torrison 2007 (Rungruangsak-Torrissen, 2007). Protein quantification was conducted according to Lowry (Lowry *et al.*, 1951), following the folin phenol method. Final unit expressed as protein mg. ml⁻¹.

3.8.2 A-Amylase

Amylase activity was measured from the increase in reducing sugar (maltose) by the hydrolysis of α -D (1,4) glycosidic bond in polysaccharides, and stained with 3,5-dinitrosalicylic acid (DNS) as described by Bernfeld (Bernfeld, 1951). Final unit is expressed as enzymatic unit per protein mg.

3.8.3 Trypsin

Trypsin activity was determined by the Torrison 1984 (Torrissen, 1984), using benzoyl-L-arginine-*p*-nitroanilide as substrate. Final product (nitroaniline) was measured spectrophotometrically at 410 nm. Final unit is expressed as enzymatic milli enzymatic units per protein mg.

3.8.4 Chymotrypsin

Chymotrypsin activity was determined by incubating Torrison and Sundby 2000 method (Rungruangsak-Torrissen & Sundby, 2000), using succinyl-Ala-Ala-Ala-Pro-Phe-*p*-nitroanilide as substrate. Similar to trypsin assay, nitroaniline was measured spectrophotometrically at 410 nm. Final unit is expressed as enzymatic milli enzymatic units per protein mg.

3.8.5 Lipase

Lipase activity assay was performed using *p*-nitrophenyl substrate as described by Winkler and Stuckmann (Winkler & Stuckmann, 1979). The formation of *p*-nitrophenol is then quantified by reading at 410 nm. Final unit is expressed as the other enzymatic activities, as enzymatic milli enzymatic units per protein mg

3.9 Statistics analyses

One-way and two-way ANOVA analyses were carried out using R Studio Desktop v0.98.1028 - Windows XP/Vista/7/8 software package. Data transformation was applied when homogeneity and normality of the samples were not achieved. Tukey test was used for pairwise comparisons between treatments. Confidence level of 95% was considered in all statistical analysis.

4 Results

No mortalities were registered during the 100 days of experiment. Temperatures were measured daily to ensure the experimental design (**Table 3**).

Table 3: Temperature mean $\pm$ deviation through the 100 day trial, with measurements noted 3 times a day (Celsius).

Treatment	Experiment unit	°C
17°C Control	Tank 1	17.0 $\pm$ 0.7
	Tank 2	17.1 $\pm$ 0.8
	Tank 3	17.1 $\pm$ 0.8
17°C Probiotic	Tank 1	17.0 $\pm$ 0.8
	Tank 2	17.1 $\pm$ 0.8
	Tank 3	17.0 $\pm$ 0.9
20°C Control	Tank 1	20.2 $\pm$ 0.6
	Tank 2	20.2 $\pm$ 0.6
	Tank 3	20.3 $\pm$ 0.4
20°C Probiotic	Tank 1	20.3 $\pm$ 0.4
	Tank 2	20.1 $\pm$ 0.4
	Tank 3	20.1 $\pm$ 0.5
23°C Control	Tank 1	23.2 $\pm$ 0.3
	Tank 2	23.2 $\pm$ 0.3
	Tank 3	23.2 $\pm$ 0.4
23°C Probiotic	Tank 1	23.2 $\pm$ 0.4
	Tank 2	23.1 $\pm$ 0.4
	Tank 3	23.2 $\pm$ 0.3

4.1 Growth performance

Fish final weight

Fish grew from the initial mean of 12.8 ± 0.5 g to a final 32.1 ± 6 g in the 100 day trial (**Chart 1**). Weight gain were significantly different between fish reared at 17 °C treatments and 20°C ($P < 0.01$), between 20 °C and 23 °C ($P=0.01$) and between 17 °C to 23 °C ($P < 0.01$). No significant differences were found between probiotic supplementation treatments.

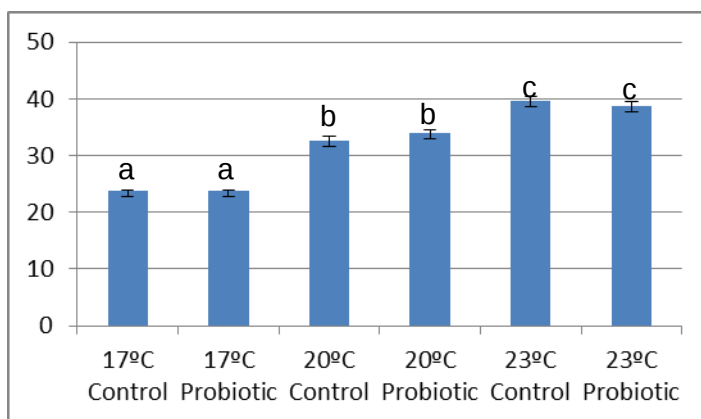


Chart 1: Fish final weight mean values from all treatments (g). (ANOVA, $P < 0.05$)

Feed conversion ratio – FCR

Feed conversion ratio (FCR; **Chart 2**) were significantly different between temperatures, having the lowest temperature (17 °C) an average FCR of 2.8, significantly greater than the 20 °C trials (P -value < 0.01) and nearly twice higher than fish reared at 23 °C (P -value < 0.01).

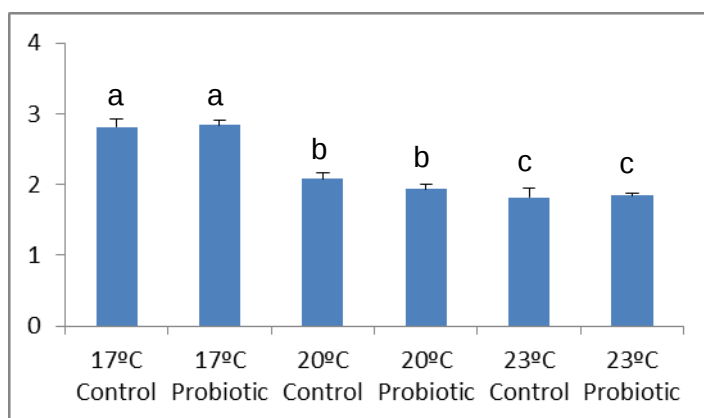


Chart 2: FCR mean values from all treatments. (ANOVA, $P < 0.05$).

Protein efficiency rate – PER

Protein efficiency rate (*PER*; **Chart 3**) followed the same pattern as *FCR* (**Chart 2**), with significant differences between all temperatures (P -value < 0.01). But again, no significant interaction was found in a Two-way ANOVA.

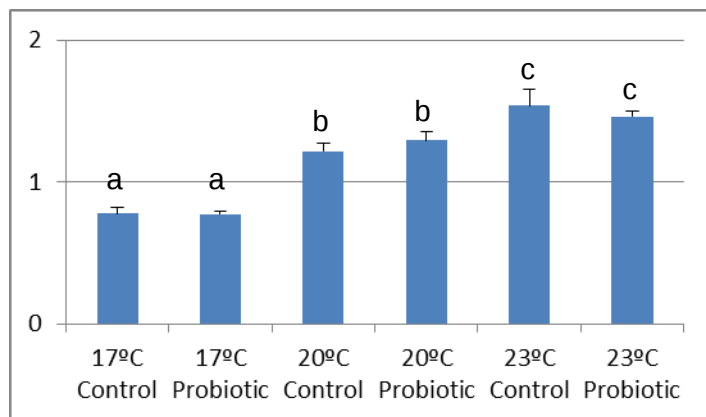


Chart 3: PER mean values from all treatments. (ANOVA, $P < 0.05$).

Daily growth index- DGI

Growth rate (*DGI*; **Chart 4**) increased with temperature upper progression, showing significant difference between all temperatures (P -value < 0.01). Two-way ANOVA showed no significant difference between treatments.

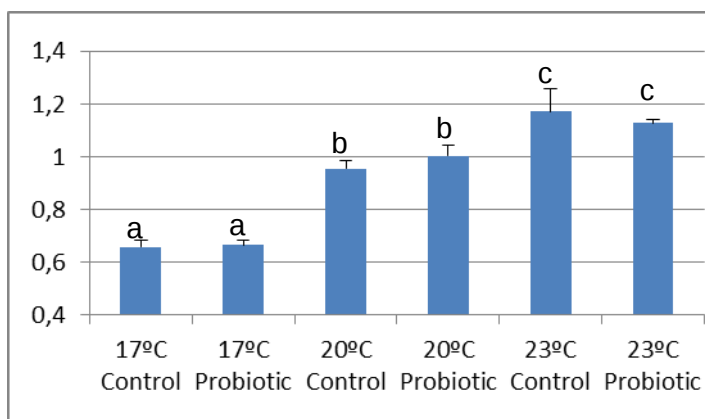
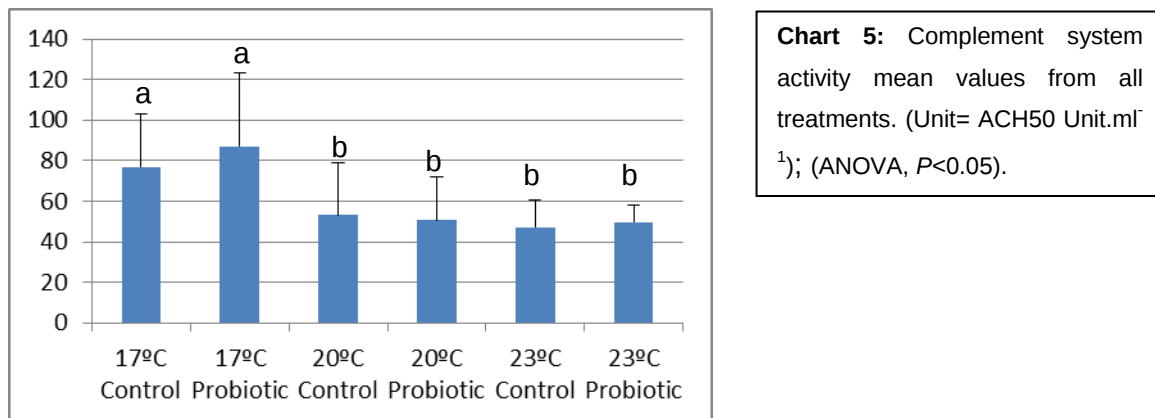


Chart 4: DGI mean values from all treatments. (Unit = % BW.day⁻¹); (ANOVA, $P < 0.05$).

4.2 Humoral immune parameters

Alternative complement pathway (ACH50)

ACH50 activity (**Chart 5**) revealed differences between temperatures, with 17 °C group having higher values than the 20 °C and 23 °C groups (P -value < 0.01).



Peroxidase and lysozyme activities

No significant differences were found in peroxidase and lysozyme activities (**Table 4**), although both activities revealed a low result in 1 treatment (23 °C probiotic and 17 °C control respectively). The characteristic high deviations in these immune system parameters quantifications may have prejudice the statistical analysis (ANOVA, P <0.05).

Table 4. Peroxidase (EU) and lysozyme (EU.min⁻¹.ml⁻¹) activities (mean ± SD, n=4 fish.treatment⁻¹).

	Control			Probiotic		
	17 °C	20 °C	23 °C	17 °C	20 °C	23 °C
Peroxidase	24.3±9.06	32.7±21.7	26.7±13.7	34.7±19.9	40.2±23.3	12.5±4.1
Lysozyme	275.9±93.6	384.3±115.1	404.6±176.2	333.3±119.2	395.4±140.4	379.6±45.2

4.3 Oxidative Stress: Enzymatic and non-enzymatic analyses

Catalase, glutathione peroxidase and glutathione s-transferase activities

All the anti-oxidant enzymatic activities (**Table 5**) measured did not show any significant differences between treatments. High deviations within fish of the same experimental conditions may have eluded possible distinctions; (mean $\pm$ SD, n=12 fish.treatment⁻¹).

Table 5. Catalase (umol.min⁻¹.mg Prot⁻¹), glutathione peroxidase (pmol.min⁻¹.mg Prot⁻¹) and glutathione s-transferase activities; (pmol.min⁻¹.mg Prot⁻¹); (mean $\pm$ SD, n=12 fish.treatment⁻¹); (ANOVA, $P < 0.05$).

	Control			Probiotic		
	17 °C	20 °C	23 °C	17 °C	20 °C	23 °C
Catalase	1.5 $\pm$ 0.7	1.1 $\pm$ 0.4	1.0 $\pm$ 0.3	1.5 $\pm$ 0.7	0.8 $\pm$ 0.4	1.5 $\pm$ 0.9
Glutathione peroxidase	2.1 $\pm$ 1.2	4.0 $\pm$ 1.4	3 $\pm$ 3	4.2 $\pm$ 3.0	2.2 $\pm$ 0.79	2.1 $\pm$ 0.54
Glutathione s-transferase	63.2 $\pm$ 52.4	65.3 $\pm$ 36.6	67.2 $\pm$ 42.0	84.1 $\pm$ 80.2	34.6 $\pm$ 21.8	109.3 $\pm$ 71.4

Lipid peroxidation

LPO (**Chart 6**), as a form of damage from ROS over the cells lipid layers, appeared to show a clear tendency for greater damage at the lowest temperatures, with significant differences between all temperatures groups (P -value < 0.01); (mean $\pm$ SD, n=12 fish.treatment⁻¹).

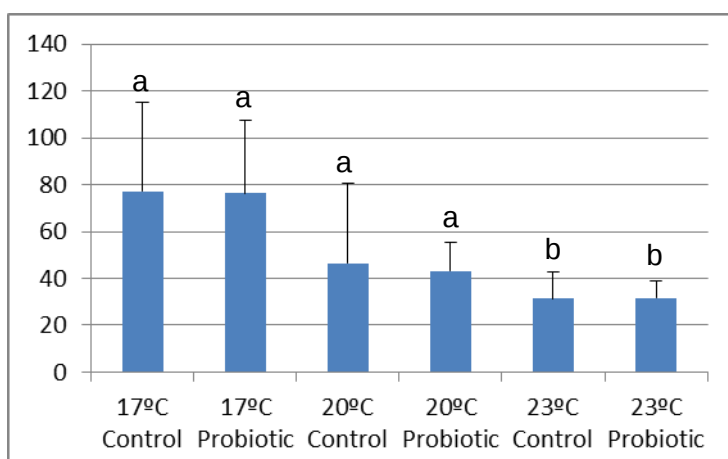


Chart 6: Lipid peroxidation mean values from all treatments. (Unit = nmol TBA.g⁻¹); (ANOVA, $P < 0.05$).

Glutathione reductase

In glutathione reductase (**Chart 7**) results, mid temperature (20 °C) treatments demonstrated significant differences within diets (P -value= 0.04); (mean $\pm$ SD, $n=12$ fish.treatment⁻¹).

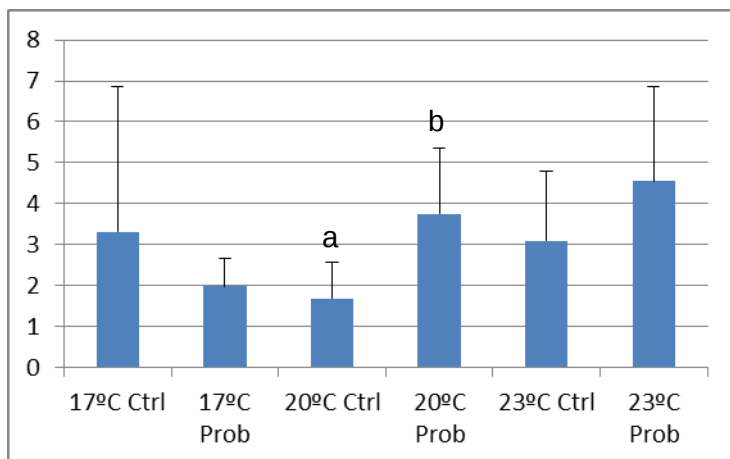


Chart 7: Glutathione reductase activity mean values from all treatments. (Unit = nmol.min⁻¹.mg Prot⁻¹); (ANOVA, $P<0.05$).

Total Glutathione

Total Glutathione concentrations (**Chart 8**) revealed significant differences between temperatures and diets. Having the 17 °C and 20 °C trials exhibiting an increasing effect in the supplemented diets treatments (P -value= 0.03). This not confirmed in the 23 °C trials. Demonstrating an interaction between our two independent factors, temperature and diet, where the 23 °C appear to shift the tendency for the probiotic to increase TG values (P -value < 0.01); (mean $\pm$ SD, $n=12$ fish.treatment⁻¹).

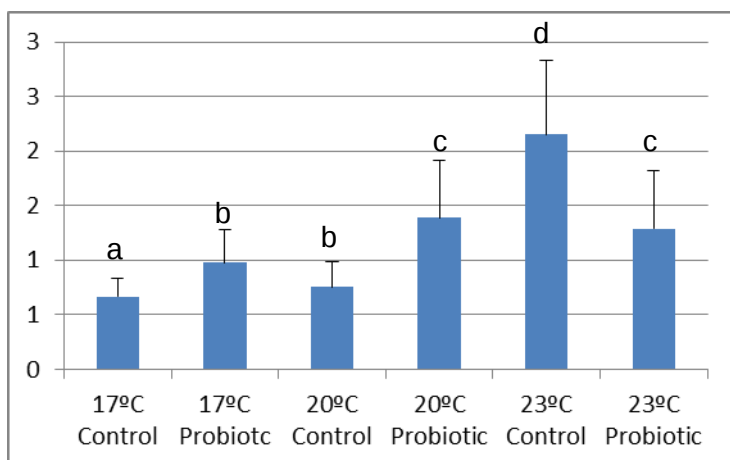


Chart 8: Total Glutathione concentration mean values from all treatments. (Unit = (μmol.g⁻¹); (ANOVA, $P<0.05$).

4.4 Digestive enzymes

A-Amylase

Alpha-amylase activities (Chart 9) showed a significant difference between the 23 °C rearing treatments and the remaining 17 °C and 20 °C treatments (P -value= 0.04 and 0.03 respectively); (mean $\pm$ SD, $n=12$ fish.treatment⁻¹).

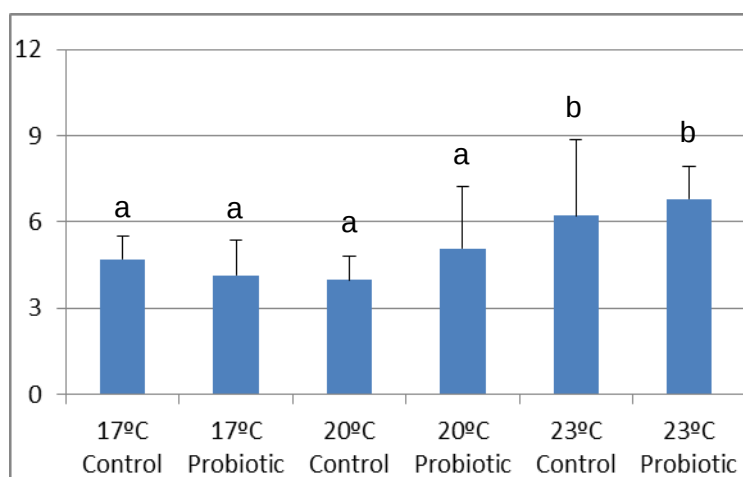


Chart 9: A-amylase enzymatic activities mean values from all treatments. (Unit = EU.mg Protein⁻¹); (ANOVA, $P<0.05$).

Trypsin

Trypsin enzymatic activities (**Chart 10**) showed a significant difference between temperatures, being the 17 °C treatments results significantly higher than the 20 °C and 23 °C groups (P -value < 0.01); (mean $\pm$ SD, $n=12$ fish.treatment⁻¹).

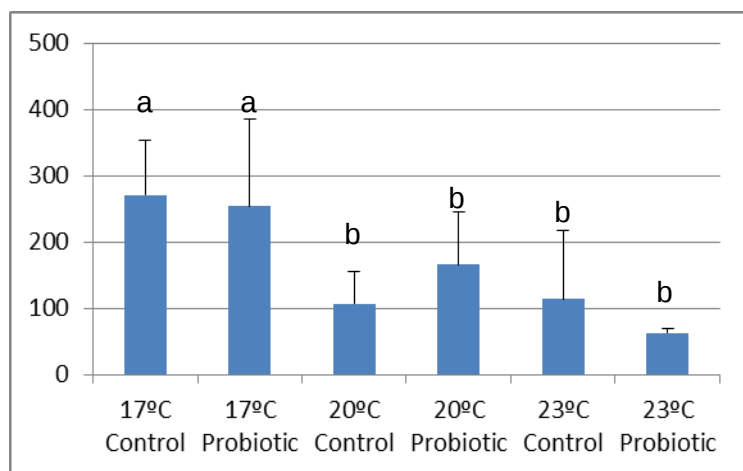


Chart 10: Trypsin enzymatic activities mean values from all treatments. (Unit = mEU.mg Protein⁻¹); (ANOVA, $P<0.05$).

Chymotrypsin

Chymotrypsin enzymatic activities (**Chart 11**) showed a significant difference between temperatures, having the 17 °C treatments values as significantly higher than the 20 °C and 23 °C groups (P -value < 0.01). A two-way ANOVA showed a temperature-diet interaction, since the increasing tendency of the probiotic diet on the chymotrypsin activity values, changes in the lowest rearing temperature, 17 °C (P -value < 0.01); (mean $\pm$ SD, $n=12$ fish.treatment⁻¹).

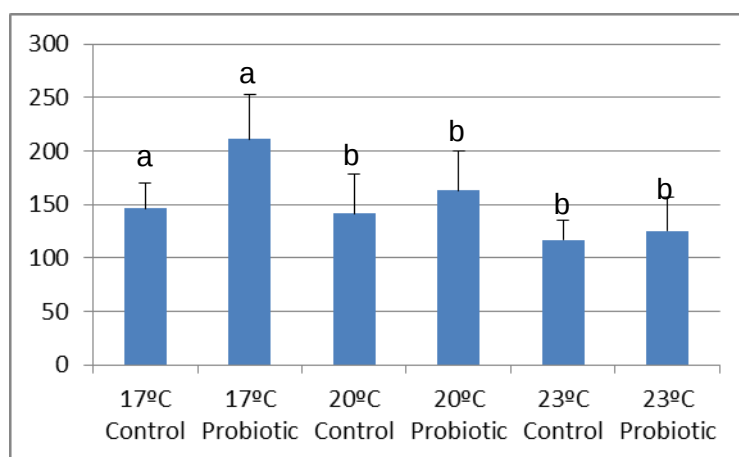


Chart 11: Chymotrypsin enzymatic activities mean values from all treatments. (Unit = mEU.mg Protein⁻¹); (ANOVA, $P<0.05$).

Amylase / Trypsin ratio

Amylase / Trypsin ratio (**Chart 12**) showed a significant difference between all temperatures, being the 23 °C treatments results significantly higher than the 20 °C and 17 °C (P -value= 0.01). No difference was found between diets; (mean $\pm$ SD, $n=12$ fish.treatment⁻¹).

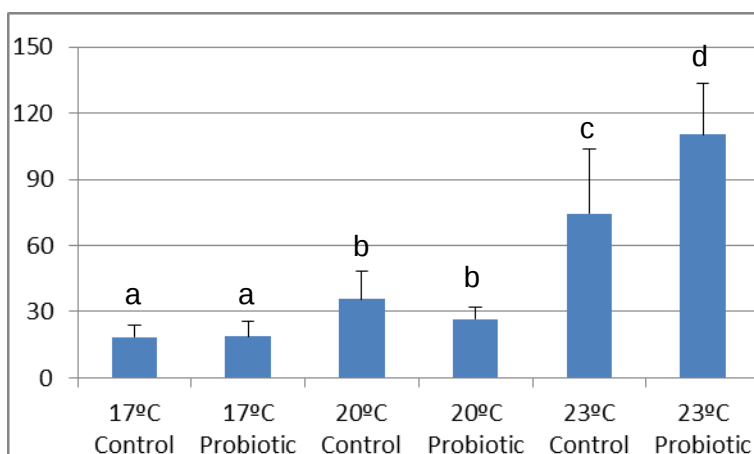


Chart 12: Amylase/Trypsin ratio mean values from all treatments. (ANOVA, $P<0.05$).

Trypsin / Chymotrypsin ratio

Trypsin / Chymotrypsin ratio (**Chart 13**) showed a significant difference between temperatures, being the 17 °C treatments results significantly higher than the 23 °C and 17 °C, revealing a tendency in the lowest temperatures to act differently on the same protein sources (P -value < 0.01) ; (mean $\pm$ SD, $n=12$ fish.treatment⁻¹).

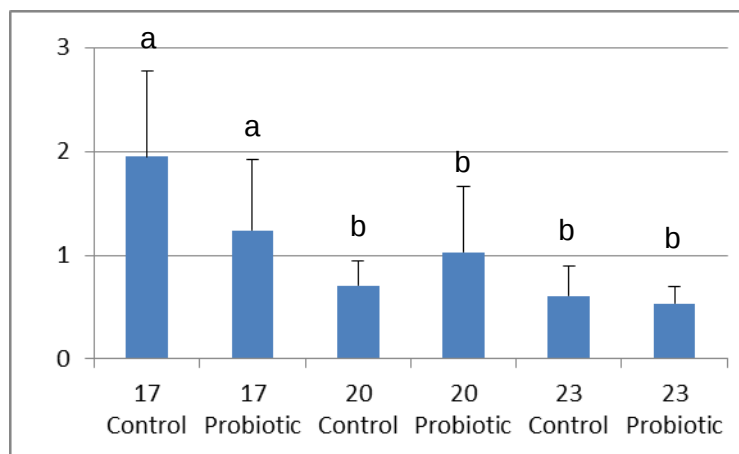


Chart 13: Trypsin enzymatic activities mean values from all treatments. (ANOVA, $P<0.05$).

Lipase

Lipase enzymatic activities (**Chart 14**) showed significantly higher results in the 23°C treatment when compared with the other temperatures groups (P -value < 0.01). No difference was found between diets; (mean $\pm$ SD, $n=12$ fish.treatment⁻¹).

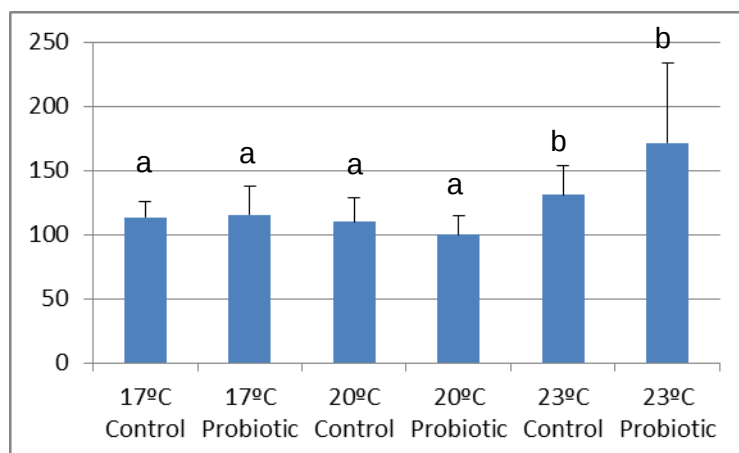


Chart 14: Lipase enzymatic activities mean values from all treatments. (Unit = mEU.mg Protein⁻¹); (ANOVA, $P<0.05$).

4.5 Cumulative mortality

Cumulative mortalities (**Chart 15**) were noted for 10 days, starting with the stress event day ($n=3$ tanks.treatment⁻¹). Casualties differ between temperatures, having the lowest temperature (17 °C) a greater damage on fish stock, specially the control treatments. Remaining temperatures did not seem to diverge considerably.

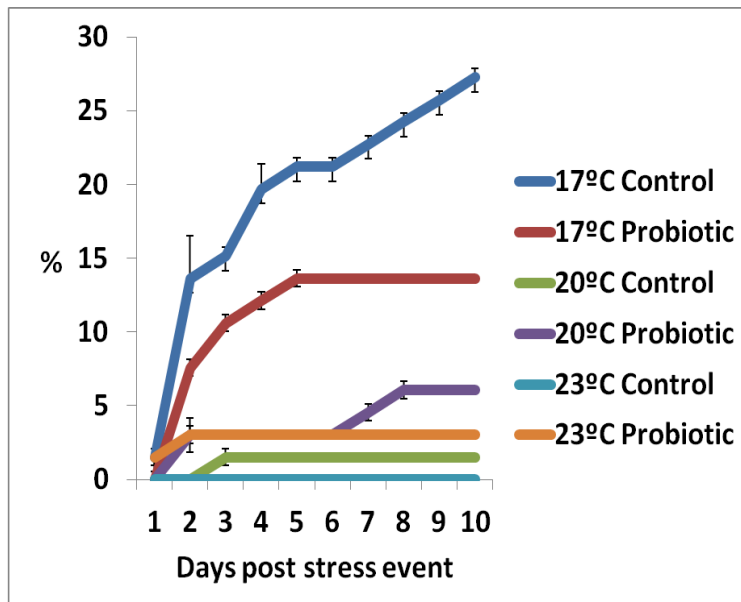


Chart 15: Cumulative mortalities results, referring to the ten days following the stress event.

5 Discussion

Growth performance

Temperature and dietary probiotic supplementation can play an important role in fish growth performance, since both have a modulatory effect on feeding, metabolism and feed digestibility. Within the tested probiotic blend, *Bacillus* and *Lactobacillus* genera seem to be the most correlated with growth improvement, either by influencing appetite, conversion ratio or reducing myostatin transcription (Lamari *et al.*, 2013, Carnevali *et al.*, 2006, Ringø & Gatesoupe, 1998, Ai *et al.*, 2011) a protein responsible for mitigating muscle growth and development. In the current study, growth was not significantly improved by dietary probiotic supplementation (**Chart 1, 2 and 3**). It is plausible to infer that the use of pair-feeding method instead of the *ad libitum* feeding technique (Riddell *et al.*, 2010) may have restricted growth by the stimulation of appetite of probiotic supplementation. The temperature effect alone in growth results was expectable, as it fits the described optimum temperatures for seabass rearing (Person-Le Ruyet *et al.*, 2004).

Humoral immune parameters

Probiotic supplementation and rearing temperature have shown to increase innate immune responses, often as a response to infection challenges (Touraki *et al.*, 2012, Aly *et al.*, 2008).

Lysozyme activity (**Table 4**) did not show any significant differences. Nevertheless, response to rearing temperature has been shown to be seasonal, but only when temperature decrease below 15 °C, therefore our temperature range (17 – 23 °C) might have evaded this decrease (Pascoli *et al.*, 2011, Valero *et al.*, 2014). Peroxidase activity lacked significant differences as well, but this has not shown any constant temperature influence in previous studies (Valero *et al.*, 2014). Regarding probiotic influence, the characteristic reactive role in the immune system when facing a pathogen threat may have eluded significant differences.

Complement system alternative pathway results (**Table 4**) showed differences between temperatures, with the lower temperatures presenting higher activities, similar to what was observed in European seabass monitored during seasonal variation (Valero *et al.*, 2014). Valero *et al.* (2014) observed that colder months tended to induce higher hemolytic activity in seabass. This response although similar to the cyprinidae family results (Collazos *et al.*, 1994), remains contradictory to what was found in seabream (Tort

et al., 1998). In our study, the lack of pathogen challenge trial may have reduced possible immune response differences between dietary groups.

Oxidative Stress: Enzymatic and non-enzymatic analyses

The effects of probiotic supplementation on antioxidant capacity has been described with and without pathogen challenge (Reyes-Becerril et al., 2011, Tovar *et al.*, 2002b). External factors alone such as rearing temperature are long-known for influencing oxidative stress indicators (Vinagre et al., 2012).

In our study, lipid peroxidation (LPO) (**Chart 6**), glutathione reductase (GR) (**Chart 7**) and total glutathione (TG) (**Chart 8**) showed significant differences between treatments. LPO results are in agreement with other studies (Vinagre et al., 2012), as it increases when temperature deviates from optimal range for the species. Damage of lipid layers increases with the decrease in rearing temperature, as indicated by the malondialdehyde results. As a consequence, fish is less fit to cope to oxidative stress radicals. However no tendency for probiotic influence was found here.

TG showed to increase significantly by the probiotic supplementation, except at 23 °C, in which TG values decreased with probiotic supplementation. Thus, the probiotic effect is temperature sensitive for TG content, indicating an inverse relationship at the highest temperature. When comparing the TG with the LPO results, it seems plausible to infer that the greater TG levels may have caused a decrease in the lipid damage. Here the protective role of glutathione appears more understandable. The fact that the 23 °C probiotic treatment response shows a mitigated response in TG when compared to 23 °C controls, which reveals a temperature differential effect on the TG levels. This may be due temperature modulation over the probiotic strains metabolic paths.

The effects of dietary probiotic supplementation on the antioxidant responses in aquatic organisms are poorly understood. Reyes et al. (2008b) observed an increase in superoxide dismutase activity in gilthead seabream and leopard grouper (*Mycteroperca rosacea*) fed *Debaryomyces hansenii*, but contrasted with Tovar-Ramírez et al. (2010) (Tovar-Ramírez et al., 2010) work with European seabass larvae. In this seabass work, yeast supplement did not affect catalase activity (CAT) but reduced glutathione peroxidase activity (GPX). In shrimp (*Litopenaeus vannamei*), GPX activity increased with dietary supplementation *Bacillus subtilis* (Shen et al., 2010).

In fish reared at 20°C, in contrast to fish reared at 17 °C and 23 °C, probiotic supplementation increased the glutathione reductase (GR) activity. As a consequence, more oxidized glutathione (GSSG) are converted to reduced glutathione (GSH), a substrate necessary for the detoxification processes.

Digestive enzymes

Amylolytic (**Chart 9**) and lipolytic enzyme activities (**Chart 14**) increased with temperature, with no detectable diet effect. The effect of temperature on alpha-amylase was observed in Rohu (*Labeo rohita*) (Alexander *et al.*, 2011), where higher temperatures tended to increase carbohydrates digestibility.

The optimal temperature for lipids digestion varies according to the species optimal range, but in general, lipolytic enzyme shows higher activities at higher temperatures (*Oreochromis niloticus*, *Oncorhynchus mykiss*, *Gadus morhua*, (Kurtovic *et al.*, 2009). In the current study, probiotic treatment did not influence the amylase and lipase activities, contrasting to other studies. Askarian *et al.* (2011) observed an improvement of gut amylase and lipase activities in Persian sturgeon (*Acipenser persicus*) and beluga (*Huso huso*) fed lactic acid bacteria (*Bacillus* sp. and *Lactobacillus* sp.).

Trypsin activity (**Chart 10**) decreased with temperature, with no dietary influence. Temperature influence in trypsin activity has been shown to directly correlated until the 25 °C in walking catfish (Ahmad *et al.*, 2014) and in yellowtail kingfish (*Seriola lalandi*) until the 22 °C (Bowyer *et al.*, 2012). Some studies have also suggested trypsin as an indicator for digestibility and growth performance (Sunde *et al.*, 2004, Thongprajukaew *et al.*, 2011). However this information is contradictory to our results. A possible theory for our proteases activities relays in the need for energy. As suboptimal temperatures turning more to protein deamination as energy source instead of carbohydrates, hence improving proteolytic activities, but misleading its enterpretation as a growth parameter. This metabolic tendency is known to occur when fish are reared outside of their optimal temperature range (FAO, 1978). Another explanation may come from the compensatory theory. A study in spotted wolffish (*Anarhichas minor*), reported lower trypsin activity at higher temperatures, suggesting a possible compensatory effect from the trypsin release in the intestine reared at optimal temperatures. Where proteolytic enzymes are over-expressed not because protein in being well converted, but as an attempt to surpass the mitigated growth (Savoie *et al.*, 2008). Another data that aids this energetic use for protein theory comes from our own results, in the protein efficiency ratio, where the lowest temperature treatments although with greater proteases activities, converted their consumed feed protein into growth less efficiently.

Chymotrypsin activity (**Chart 11**) has also been positively correlated with temperature. In catfish (*Clarias batrachus*), chymotrypsin activity increased at 25 °C and then again at 35 °C (Ahmad *et al.*, 2014) but this tendency has been contradicted in Atlantic salmon, where chymotrypsin activity was greater at 6 °C. Moreover, chymotrypsinogen is activated by trypsin, creating a limiting factor for chymotrypsin

activity. Chymotrypsin was significantly increased with probiotic treatment, whereas it decreased with increase in temperature, showing an interaction between temperature and diet. Our results are in agreement with (Zacarias-Soto *et al.*, 2011), which observed an increase in chymotrypsin activity in California Halibut, (*Paralichthys californicus*) fed *Pediococcus acidilactici* supplemented diet. However, chymotrypsin seems to have not yet been correlated with temperature and probiotic supplementation before.

Ratio between α -amylase and trypsin (A/T) (**Chart 12**), is often used as indicator of energy metabolism flexibility, showing the fish capability to use carbohydrates and sparing protein as energy source. In our study, fish reared at 23 °C outpaced fish reared at 17 °C and 20 °C, with a greater amylolytic capability, especially when fed supplemented probiotics. Highlighting a possible exocytosis amylase production from the probiotic strains as previously described (C De *et al.*, 2014). The lower carbohydrate digestion in the lower temperatures may have influenced as well the amino acid deamination for energy use.

The activity ratio of trypsin to chymotrypsin (T/C) (**Chart 13**), has been used as an indicator of growth performance, where lower T/C tend to indicate lack of growth stimulation (Rungruangsak-Torrissen, 2007). Such a decrease can be caused by a reduction of trypsin or an increase of chymotrypsin. In our study, the T/C decreased due to a decrease in trypsin, which may be correlated to trypsin expressions factors caused by feed limitation. Generally, well-fed fish have high trypsin activity, whereas starvation acts as a mitigating factor for this trypsin activity (Rungruangsak-Torrissen *et al.*, 2006). Fish fed below satiation in the 23 °C groups, caused by the pair-feeding method may have their trypsin activity also influenced by the lack of satiation effect, hence decreasing their T/C ratio.

Cumulative mortality

Finally, post-stress cumulative mortality (**Chart 15**) were significantly higher in the 17°C control group, showing an effect of temperature and dietary supplementation. Studies have shown strains like *Bacillus* spp. and *Lactobacillus* spp. to be capable of reducing mortalities of host when dealing with stress factors or direct pathogens challenges (Rollo *et al.*, 2006, Cerezuela *et al.*, 2012), and *Pediococcus* spp. and *Enterococcus* spp. by inhibiting pathogens infection (Rodriguez-Estrada *et al.*, 2013, Villamil *et al.*, 2010).

Interestingly, at 17 °C the alternative pathway complement system activity showed a more active pathogen attack complex, which were not translated into less mortality after

Discussion

the *stress* challenge. This raises the question if a stimulated immune system can always be considered a good condition.

Future perspectives

As future perspective, considerations could be made regarding the probiotic installation success, as this may have been a major cause for the probiotic influence. For this, techniques as denaturing gradient gel electrophoresis (DGGE), or pyrosequencing could be applied. Also, the probiotic potential to improve carbohydrate and protein digestion could be better analysed, by quantifying the digestive enzymes activity in experimental diets with different levels of protein and carbohydrates. And finally, feed limitation over the 20°C and 23°C trials could be removed, by feeding with *ad libitum* method, thence possible influence of probiotic in daily feed intake could be analysed.

6 Conclusion

The use of dietary supplementation of probiotic blend was effective in modulating host anti-oxidant and digestive parameters. Nevertheless, no influences in the innate immune responses were observed. Probiotic supplementation was able to reduce fish mortality after a handling and crowding *stress* challenge in fish reared at 17 °C. An interaction between probiotic supplementation and rearing temperature was observed in the anti-oxidant responses and the digestive enzyme activity.

7 References

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8 Attatchments

8.1 Laboratory analysis procedures

Complement system – (Sunyer et al., 1995)

The procedure goes as follows: firstly the sample of rabbit red blood cells must be cleaned with physiological serum (NaCl 0.9%) in a 1:4 ratio (blood:serum), and a quick 5 minutes centrifugation at 2000 g forces. From this we should collect a clear pink solution with about $2,8 \times 10^8$ cell/ml (RaRBC), using a 1:200 dilution for counting cells under a microscope at a total amplification of 100 times. From here, we should start to measure the correct dilution required for the 50% hemolysis, by adding 10ul of our plasma sample, and a variable amount of rabbit's blood solution ate the previously mentioned concentration to the 96-well microplate. The testes dilutions would be of 1:16, 1:32, :48, 1:64, 1:96. This incubation would go for 100 minutes and stopped with a solution composed of: 0.1% gelatin, 5mM sodium barbiturate, 0.13mM sodium chloride and 20mM EDTA, with a final pH calibration for 7.3.

Finally the microplates would be centrifuged at 122 g forces and from this, 150ul would be pipetted to a new microplate and the lysed cells measured at the absorbance of 414nm wavelength. The alternative complement pathway (ACH50) units were defined as the concentration of serum giving 50% haemolysis of RaRBC. All analysis were conducted by triplicates.

Peroxidase - (Quade & Roth, 1997)

To estimate the plasmatic peroxidase content, initially 135 ul of a stabilizing environment of Hank's Balanced Salt Solution, (HBSS Ca²⁺ free) solution was added to a flat bottom 96-well microplate, then 15ul of plasma sample, 50ul of tetremethylbenzidine hydrochloride (TMB) and 50ul of hydrogen peroxide (H₂O₂), this would allow the reaction to occur. Later, 50ul of a 2mM of sulfuric acid (H₂SO₄) would stop the reaction. The actual measurement would be made by spectrophotometry at 450 nm wavelength. The principle behind the reaction is, TMB can act as a hydrogen donor for the reduction of hydrogen peroxide to water by peroxidase enzymes, such as the exocytosis myeloperoxidase and eosinophilic peroxidase. The reaction when halted by addition of sulfuric acid turns TMB yellow. Finally, the colour may be read at 450 nm. Final unit is presented as enzymatic units, with one enzymatic being defined as the amount producing an absorbance change of 1.

Lysozyme - (Ellis, 1990)

This procedure starts with the preparation of a 0.5 mg/ml of *Micrococcus lysodeikticus* in a 0.05M sodium phosphate dibasic (Na_2HPO_4) solution. This bacteria solution would be used as target subtract for the lysozyme samples. Actual measurement would come from comparison with 15 known concentrations of Hen egg-white lysozyme (HEWL), from 1mg/ml to 0,0049ug/ml. A reaction mix of 15ul plasma sample and 250ul of bacterial suspension would then be read at 450nm wavelength in 2 timings, 30 seconds and 270 seconds. From these absorbances values the conversion to enzymatic units (EU) would be made by the convention of 1 EU = 0.001 absorbance units / minute.

Protein quantification - (Bradford, 1976)

Initially a standard curve of 250 μl BioRad® with 10ul of a known concentration of bovine gamma globulin (0; 0.2; 0.5 and 1 mg.ml^{-1}) was created. Followed by a 15 minutes incubation reaction in the dark to avoid light damage, and final reading at 600 nm, we were then able to calculate the concentration of our samples placed under the same conditions.

Lipid peroxidation - (Ohkawa et al., 1979)

The reaction complex is composed of: 150 μl of liver homogenate, 500 μl of TBA (Thiobarbituric acid), 500 μl of TCA (Trichloroacetic acid), 400 μl of Tris-HCl (Trisaminometane) at 60mM with 0.1 mM DTPA (diethylene triamine pentaacetic acid). After an incubation period of 60 minutes at 100 °C, a centrifugation of 11500 g forces at 25 °C for 5 minutes would be made. Only then the samples would be read at 535nm. The principle of this analysis is: the MDA-TBA adducts formed by the reaction of MDA and TBA under high temperature (100°C) and acidic conditions (TCA) can be measured colorimetrically at 535 nm. With the absorbance of this measurement and the extinction coefficient of the MDA-TBA complex of $1.56 \times 10^5 \text{ M}^{-1}.\text{cm}^{-1}$, the actual concentration of the lipid peroxidation product (MDA), can then be quantified. Finally, units are presented as nmoles of MDA formed.mg protein⁻¹.

Catalase - (Claiborne, 1985)

The reaction complex was based on 15 μl of the PMS, 135 μl of K-phosphate buffer at 0.05 M (pH 7) and 150 μl of peroxide hydrogen at 0.03 M. The peroxide hydrogen concentration would then be pursuit by spectofotometry at 240 nm for the first minute. This absorbance together with the peroxide hydrogen extinction coefficient (40 M^{-1}

¹. cm⁻¹) allowed the calculation of the catalase activity slope. Final unit is presented as hydrogen peroxide $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{protein mg}^{-1}$.

Glutathione s-transferase - (Habig et al., 1974)

For this reaction, 100 μL of PMS was added to 4.95 ml of phosphate buffer (0.1M pH 6.5), 900 μL of reduced glutathione (GSH) at 10 mM and 150 μL of CDNB (1-chloro-2,4-dinitrobenzene) at 10 mM. Spectofotometer readings were made at 340 nm wavelength every 20 seconds for 5 minutes after all reagents were combined. The enzyme glutathione-S-transferase (GST) conjugates CDNB (substrate) with glutathione (GSH) originating a complex. The formation of this compound ($\epsilon = 9.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) can be monitored at 340 nm, registering an increase in the absorbance over time. This is finally expressed in $\mu\text{mol GSH-CDNB conjugate formed} \cdot \text{min}^{-1} \cdot \text{protein mg}^{-1}$.

Glutathione peroxidase - (Mohandas et al., 1984)

This requires the peptide GSH and peroxide hydrogen to recreated reaction mix composed of: 840 μL of K-Phosphate 0.05M (pH 7.0) with EDTA 1 mM and Sodium azide (1 mM) and GR at 1U/ml; 50 μL GSH 4 mM, 50 μL NADPH (Nicotinamide adenine dinucleotide phosphate) 0.8 mM, 10 μL H₂O₂ 0.5 mM and finally 50 μL of PMS. Measures were made at 340 nm for 30 seconds. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to NADP⁺ is indicative of GPX activity, since GPX is the rate limiting factor of the coupled reactions.

Glutathione reductase - (Cribb et al., 1989)

In this method a 950 μL solution with 0.0172g of NADPH, 0.0654g of GSSG and 0.0196g of DTPA is mixed. Readings were then made at 340 nm wave length during 1 minute. The rate of increase in absorbance is directly proportional to the amount of glutathione reductase in the sample. Then measured at 340 nm during the oxidation of NADPH to NADP⁺ is indicative of GR activity.

Total Glutathione - (Baker et al., 1990)

Total glutathione (TG) required a previous treatment, where 10 μL of ultra pure water were added to 200 μL PMS and left to incubate for 1 hour at 25 °C. After this, 200 μL of 12% pure TCA (Trichloroacetic acid) were added and another incubation of 1 hour at 4°C, followed by a centrifugation at 10000 g forces for 5 minutes. The reaction per se, included 150 μL of treated PMS, 2.1 mL Na-K phosphate (0.1M pH 8.0), 75 μL of NADPH, 100 μL of DTNB and 25 μL of GR. Reading was made at 412nm wave length for 1 minute.

Calculations of the formulation of TNB (5-Mercapto-2-nitrobenzoic acid); ($\epsilon = 14.1 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Enzyme extraction and protein quantification - (Rungruangsak-Torrissen, 2007, Lowry et al., 1951)

Intestine was homogenized in 50 mM Tris-HCl buffer pH 8 containing 200 mM NaCl (1:1 w/v) using a homogenizer/blender, followed by centrifugation at 15000 g forces for 30 minutes at 4 °C, and finally prepared the required samples for protein and digestive enzymes activity quantification. The quantification was made following the folin phenol method. A volume of 25 μl enzyme extract was combined with 750 of 0.05% Na_2CO_3 , 2 mM NaOH, 0.5 % CuSO_4 and 1 % sodium or potassium tartrate with 50% folin solution. Left incubate for 30 minutes in dark and finally measured at 700 nm wave length. Comparison of values was made with a grade of known values of bovine serum albumin concentrations.

A-Amylase - (Bernfeld, 1951)

A 5% Starch solution was used as substrate, added to 37.5 μl of NaCl (20 mM) and 125 μl of enzyme extract, finally left to incubate at 37 °C for 15 minutes. Reaction was stopped with the addition of 2.5 ml water and maltose stained with 250 μl DNS, followed by 5 minutes at 100 °C. The produced maltose was determined by measuring the changes in absorbance at 540 nm, as DNS-Maltose complex turned from yellow to red-brown color. Calculations were made in comparison to a grade of 11 known maltose concentrations (0 to 50 $\mu\text{mol} \cdot \text{ml}^{-1}$).

Trypsin - (Torrissen, 1984)

Trypsin activity was determined by incubating 100 μl of enzyme extract with 700 μl of trypsin substrate [1.25 mM benzoyl-L-arginine-*p*-nitroanilide (dissolved with dimethylformamide (5% final concentration) before made up to final volume with 0.2 M Tris-HCl buffer pH 8.4)] at 25 °C for 10 min. The reaction was stopped by the addition of 800 μl 30% acetic acid. The production of nitroaniline was measured spectrophotometrically at 410 nm, and compared with a nitroaniline standard curve.

Chymotrypsin - (Rungruangsak-Torrissen & Sundby, 2000)

Chymotrypsin activity was determined by incubating 100 μl enzyme extract with 700 μl of chymotrypsin substrate [0.1 mM succinyl-Ala-Ala-Ala-Pro-Phe-*p*-nitroanilide (dissolved with dimethylformamide (5% final concentration) before made up to final

volume with 0.2 M Tris-HCl buffer pH 8.4]] at 25 °C for 10 min. The reaction was stopped and measured at 410 nm in the same way as trypsin.

Lipase - (Winkler & Stuckmann, 1979)

Lipase activity assay was performed using *p*-nitrophenyl substrate as described by Winkler and Stuckmann (Winkler & Stuckmann, 1979). The reaction mixture consisted of 0.01 M *p*-Nitrophenyl palmitate, 800 µl of 0.2 M buffer (0.1M Sodium carbonate pH 9) and 10 µl of enzyme extract. This was left to incubate for 15 minutes at 30 °C. The reaction was stopped with 250 µl 1 M Na₂CO₃. Followed by a 10000 g forces centrifugation for 15 minutes and reading at 410 nm. Calculations were made according to a *p*-nitrophenol standard curve.

8.2 Presentations

IJUP – Meeting of young researchers at University of Porto

Februart 12th – Oral Presentation



Certifica-se que Luis Ferreira Pereira esteve presente no IJUP'14 – 7º Encontro de Jovens Investigadores da Universidade do Porto, que decorreu nos dias 12, 13 e 14 de fevereiro de 2014, na Reitoria da Universidade do Porto, tendo apresentado a comunicação oral “Growth performance, antioxidant and immune responses in European seabass fed dietary probiotic supplementation under different rearing temperatures.”.

Pela Comissão Organizadora

(O Vice-Reitor, Prof. Doutor Jorge Gonçalves)

IMMR'14 – International Meeting on Marine Research 2014

July 11th – Oral Presentation

Published in *Frontiers in Marine Science Journal*

DOI: 10.3389/conf.FMARS.2014.02.00016 - (Pereira *et al.*, 2014)



Oral Communication

This is to certify that,

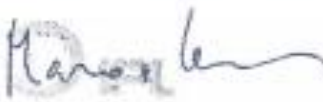
Luís F. Pereira

attended to the International Meeting on Marine Research 2014 [IMMR'14] held at Peniche (Portugal), on the 10th and 11th of July, and presented an oral communication during the event.

Dietary probiotic supplementation improved gut amylase to trypsin ratio in European seabass reared at different rearing temperatures and survival after handling stress
PEREIRA LF, GONÇALVES J, DOMINGUES AF, PEIXOTO MD, BRAGA SF, SANSUWAN K, SANTOS G, OZARIO R

Peniche, the 11th of July 2014

On the behalf of the organisation,


Maria Helena
coordenadora académica de turismo

