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"Use of probiotics in sole (*Solea senegalensis*) diets: Effects on growth performance, host defense, morphology and ecology of the digestive tract."

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LEGAL DETAILS

In compliance with what is stated in Decree Law n. º 115/2013 of August 7th, it is hereby declared that the author of this thesis participated in the creation and execution of the experimental work leading to the results shown, as well as in their interpretation and the writing of respective manuscripts. Includes four scientific papers published in international journals originating from part of the results obtained in the experimental work referenced to as:

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Abstract

Senegalese sole (*Solea senegalensis*) is a promising flatfish species for the diversification of the European aquaculture, due to its high commercial value and nutritional properties. Nevertheless, growth performance and survival of sole from juvenile to market-size is not fully controlled and the intensification of sole production has been severely halted due to various biological limitations and infectious diseases. The presence of antibiotic residues in products that lead to increased bacterial resistance, forced to limit their use in animal production. In this context, probiotics represent an emerging tool increasingly used in aquaculture systems, both in water and feed as prophylactic biological control agents.

Recent advances on nutritional requirements are contributing to overcome the constraints for the establishment of sole aquaculture in a large commercial scale. In fact, the ability of sole to efficiently use plant-based diets is an important finding towards the intensification and commercialization of sole. Nevertheless, there are indications suggesting some adverse impacts on gut morphology and physiology when sole fed high plant-based content diets. Given the importance of nutrition to fish health, there is an on-growing trend in exploring the functional attributes of dietary components of a non-nutritional nature to improve fish welfare and growth performance. The use of probiotics and/or immunostimulants may difficult the intestinal colonization of bacterial pathogens by modulating the microbiota. The aim of the current PhD thesis is to evaluate the effects of dietary probiotics and immunostimulant supplementation in Senegalese sole, considering growth performance, gut morphology, health status and disease resistance in order to reduce economic and environmental losses in sole production.

The **chapters** comprising in this thesis were designed to address the following questions:

- Are probiotics able to affect Senegalese sole, bringing benefits concerning growth performance, innate immune response and gut morphology? (**Chapter 2**)

- Are probiotics or other immunostimulant raw materials, able to protect Senegalese sole from the possible negative effects caused by the use of plant ingredients as fishmeal replacement? (**Chapter 3** and **Chapter 4**)

- Are autochthonous bacteria from sole intestine able to have a protective effect against bacterial pathogens? (**Chapter 5**)

In **chapter 2**, juveniles were fed for one month, with a diet supplemented with two different commercial probiotics (multispecies and monospecies) at two different concentrations. Growth performance as well as innate immune parameters analyzed were not affected by the dietary treatments. The study indicates that the use of the multispecies probiotic (*Bacilli* class) at low concentration may enhance protection against pathogen outbreaks by affecting the muscular duodenal layer thickness, whereas at the highest concentration could reduce fish size dispersion among tanks.

Chapter 3 focuses on the ability of sole to grows well when fed diets containing plant ingredients and the possible effect of probiotics and immunostimulants, preventing some negative effects caused by high plant ingredient supplementation. The probiotic used was the same multispecies tested in **chapter 2**, and an autolyzed yeast was used as immunostimulant. Juvenile Senegalese sole were fed diets formulated with low (35%) or high (72%) content of plant protein (PP) ingredients, with or without probiotic or yeast supplementation during a 73-days trial. Overall, fish fed diets with 72% of plant ingredients showed lower transcript levels of key immune- and stress-related genes in distal intestine, rectum and head-kidney than the fish fed the 35% diets. Inclusion of PP was associated with differences in gene expression and a more diverse microbiota profile but without a significant effect on growth performance. Moreover, probiotic supplementation resulted in up-regulation of some genes transcript levels (*hsp90b*, *gpx*, *cat* and *apoa1*) in distal intestine concomitantly with a growth rate reduction compared to non-supplemented fish.

In **chapter 3** it was determined that a continuous stimulation of innate immune system during 73-day administration period is possibly not the best option to detect the potential activation of innate immune response. So, **chapter 4** evaluated the effect of both factors (PP content and supplementation) in the humoral innate immune response and in the intestine histology and microbiota, during short- and log-term administration periods (2, 17, 38 and 73 days). Curiously, PP content had a stronger effect on the innate immune response than the dietary probiotic or yeast supplementation. The results presented at **chapter 4** suggested that short-term

feeding high dietary PP level may enhance the immune system (17 and 38 days of feeding) and increase intestinal surface area for absorption (2 days of feeding). However, this effect was reversed with long-term feeding (73 days), possibly by a habituation to dietary treatments and/or immunosuppression, with a reduction in the number of the goblet cells. The predominant bacteria found in sole intestine were *Vibrio* sp., whereas dietary probiotic supplementation caused a reduction in *Vibrio* content, regardless of the dietary PP level.

Finally, in **chapter 5,** a growth trial and a bacterial infection trial using *Photobacterium damselae* subsp. p*iscicida* as pathogenic agent, were carried out using independent systems supplied with filtered open flow seawater. **Chapter 5**, tested the use of two autochthonous bacteria (*Enterococcus raffinosus* and *Pseudomonas protegens*) isolated from sole intestine as a dietary probiotic treatment. No significant differences were observed in growth performance and innate immune parameters of fish, after the 36-days feeding. However, during the growth trial (36 days) fish fed *E. raffinosus* had higher muscular layer thickness and number of goblet cells counts, indicating an enhancement in the protection against pathogen outbreak. In fact, fed *E. raffinosus* had lower cumulative mortality after 17 days post infection, indicating a possible protective effect of *E. raffinosus* against photobacteriosis. In addition, there was a very clear indication that the two autochthonous bacteria, were able to modulate sole intestine microbiota, having different profiles from fish fed control diet. Fish subjected to the *P. damselae* subsp. *piscicida* infection, presented high similarity of intestinal microbiota, especially in the proximal intestine (>60% of similarity), maybe showing the dominance of *P. damselae* subsp. p*iscicida* during disease. In addition, a decrease in the peroxidase activity was observed in infected fish, revealing lowest antioxidant capacity.

Lastly, in **chapter 6 (general discussion)**, the findings from **chapters 2** to **5** are reviewed. The effects of the dietary treatments on growth performance, immune response and intestinal morphology and microbiota are summarized and discussed for advancing the research presented in this thesis. Overall, the present thesis shows that probiotics and immunostimulant effects may be controversial. They may be useful reducing size dispersion among tanks despite not bringing a clear effect on growth performance. They seem to be able to stimulate the innate immune system in some cases, but such effect is lost in long-term administration periods. Conversely, dietary PP supplementation showed to be more effective than

probiotics or immunostimulants to potentiate the immune response. Concerning the intestinal microbiota, the predominant bacteria found in sole intestine were *Vibrio* sp. and dietary multispecies probiotic used in our experiments seems to cause a reduction in *Vibrio* content.

Resumo

O linguado senegalês (*Solea senegalensis*) é um peixe plano bastante promissor para a diversificação da aquacultura Europeia, devido às suas propriedades nutricionais e ao seu elevado valor comercial. No entanto, o crescimento e a sobrevivência do linguado da idade juvenil até ao seu tamanho comercial, não se encontram totalmente controlados, sendo a intensificação da produção severamente afetada por várias limitações biológicas, tais como as doenças infeciosas. Além disso, o uso de antibióticos na produção animal tem vindo a ser limitado, devido ao aumento das resistências bacterianas. Neste contexto, os probióticos, administrados na água ou através do alimento, surgiram como uma ferramenta que pode ser utilizada nos sistemas de aquacultura como medida profilática de controlo biológico.

Recentes avanços sobre os requerimentos nutricionais do linguado senegalês, têm contribuído para ultrapassar algumas limitações que impediam a sua produção aquícola em grande escala comercial. A capacidade do linguado em usar eficientemente dietas de origem vegetal, é uma descoberta importante para a intensificação da sua produção e comercialização. Porém, há indicações de que as dietas vegetais podem causar alguns efeitos adversos tanto na morfologia como na fisiologia intestinal. Devido à importância da alimentação na saúde, tem surgido uma tendência em explorar os atributos funcionais de determinados componentes alimentares, sem relevante valor nutritivo, para melhorar o bem-estar e o crescimento dos peixes. O uso de probióticos e/ou imunoestimulantes podem modular a microbiota intestinal e assim impedir a colonização por parte de bactérias patogénicas. O objetivo da presente tese de doutoramento é avaliar os efeitos da suplementação com probióticos e imunoestimulantes na dieta do linguado senegalês, atendendo ao seu crescimento, morfologia intestinal, estado de saúde e resistência à doença, de forma a reduzir as perdas económicas e ambientais no processo da sua produção.

Os **capítulos** apresentados nesta tese foram desenhados de forma a responder às seguintes questões:

- Serão os probióticos capazes de afetar o linguado senegalês, trazendo benefícios no crescimento, resposta imune inata e morfologia intestinal? (**Capítulo 2)**

- Serão os probióticos ou outras matérias-primas imunoestimulantes, capazes de proteger o linguado senegalês de possíveis efeitos negativos causados pelo uso de ingredientes vegetais como substitutos da farinha de peixe? (**Capítulos 3** e **4**)

- Será que bactérias autóctones isoladas do intestino de linguado, terão algum efeito protetor contra invasão de bactérias patogénicas? (**Capítulo 5**)

No **capítulo 2**, juvenis de linguado foram alimentados durante um mês com dietas suplementadas com 2 probióticos comerciais distintos (multiespécie e monoespécie) e cada um deles a duas concentrações diferentes. Tanto o crescimento, como os parâmetros imunológicos inatos analisados não foram afetados pelas dietas experimentais. O estudo indica que o uso do probiótico multiespécie (da Classe *Bacilli*) na sua concentração mais baixa pode melhorar a proteção intestinal, contra a entrada de agentes patogénicos, devido ao seu efeito na espessura da parede muscular duodenal. Enquanto que a concentração mais alta pode ajudar a reduzir a dispersão no tamanho dos peixes entre os tanques.
O **capítulo 3** centra-se na capacidade do linguado em crescer eficientemente quando alimentado com dietas vegetais e no uso de probióticos ou imunoestimulantes na prevenção de possíveis efeitos negativos causados por essas mesmas dietas. O probiótico utilizado na experiência descrita no **capítulo 3**, foi o mesmo multiespécie testado no **capitulo 2**, assim como também foi testado um imunoestimulante (levedura inativa autolisada). Os juvenis de linguado foram alimentados durante um ensaio de 73 dias, com duas dietas de formulação distinta quanto ao seu teor em ingredientes vegetais, uma de baixo teor (35%) e outra de alto teor (72%), suplementadas com ou sem probiótico ou levedura. No geral, os peixes alimentados com dietas com 72% de ingredientes vegetais mostraram níveis mais baixos de expressão dos genes relacionados com o sistema imunitário e resposta ao stress, que os peixes alimentados com as dietas com 35% de teor, quando analisados ao nível do intestino distal, reto e rim anterior. A inclusão dos

ingredientes vegetais foi associada com diferenças na expressão dos genes assim como com uma maior diversidade da microbiota intestinal, apesar de não ter tido qualquer efeito significativo no crescimento dos peixes. Além disso, a suplementação com o probiótico resultou num aumento da expressão de alguns genes (*hsp90b*, *gpx*, *cat* and *apoa1*) no intestino distal em conjunto com uma redução da taxa de crescimento comparativamente com os peixes não suplementados.

No **capítulo 3**, conclui-se que a estimulação contínua do sistema imune inato, durante 73 dias de administração das dietas experiemtais, talvez não seja a melhor opção para detetar uma possível ativação dessa mesma resposta imune. Assim sendo, o **capítulo 4** avalia o efeito de ambos os fatores (dieta vegetal e suplementação com probiótico ou levedura) na resposta imune inata humoral e na morfologia e microbiota intestinais, considerando também períodos de curta administração (2, 17, 48 e 73 dias). Curiosamente, o teor de ingredientes vegetais na dieta, teve um efeito mais pronunciado na resposta inata que propriamente o uso do probiótico ou da levedura. Os resultados apresentados no **capítulo 4** sugerem que a administração da dieta vegetal 72% num curto período de tempo (17 e 38 dias de alimentação) pode melhorar a resposta do sistema imunitário e levar a um aumento da área de absorção intestinal (2 dias de alimentação). No entanto, este efeito foi revertido com a continuidade da sua administração (73 dias), possivelmente devido a uma habituação a essa dieta e/ou imunossupressão evidenciada pela redução no número de células caliciformes nos peixes que ingeriram essa dieta. Detetou-se que as bactérias predominantes no intestino de linguado pertencem à espécie *Vibrio*, e que o uso do probiótico na dieta levou a uma redução dessa mesma presença de *Vibrio* sp na microbiota, independentemente da dieta vegetal testada (35 ou 72%).

Finalmente, no **capítulo 5** foi efetuado um ensaio de crescimento (36 dias) prosseguido por um ensaio de infeção bacteriana, usando como agente patogénico o *Photobacterium damselae* subsp. *piscicida*. Estes dois ensaios, foram realizados em sistemas independentes, abastecidos com água do mar filtrada e em fluxo aberto. No **capítulo 5** testou-se o uso de duas bactérias autóctones (*Enterococcus raffinosus* e *Pseudomonas protegens*) previamente isoladas do intestino de linguado senegalês e que foram identificadas *in vitro* como potenciais suplementos probióticos. Após os 36 dias de alimentação, não se observaram diferenças

significativas na avaliação do crescimento nem na resposta imune inata dos peixes. No entanto, os peixes alimentados com a bactéria *E. raffinosus* apresentaram uma parede muscular duodenal mais espessa, assim como um maior número de células caliciformes, indicando que estes animais possam ter ganho uma melhoria na proteção contra a entrada de agentes patogénicos. De facto, após 17 dias da infeção, os peixes alimentados com *E. raffinosus* manifestaram uma mortalidade cumulativa mais baixa, revelando um possível efeito protetor da *E. raffinosus* contra a photobacteriose. Além disso, verificou-se claramente que as duas bactérias autóctones foram capazes de modular a microbiota intestinal, evidenciando perfis bacterianos distintos da microbiota dos peixes alimentados com a dieta controlo. Os peixes sujeitos à infeção com o *P. damselae* subsp. *piscicida*, mostraram uma grande similaridade na microbiota intestinal, especialmente no intestino proximal (>60% de similaridade), mostrando um possível domínio do *P. damselae* subsp. p*iscicida.* Além disso, os peixes infectados apresentaram um decréscimo da atividade da peroxidase, revelando uma menor capacidade de resposta antioxidativa desses peixes.

Por último, no **capítulo 6 (discussão geral)**, efetuou-se uma revisão tendo por base os resultados e conclusões dos **capítulos** anteriores. Os efeitos das dietas experimentais na avaliação do crescimento, resposta imunitária e morfologia e microbiota intestinais do linguado, são sumarizadas e discutidas. No geral, a presente tese mostra que os efeitos do uso de probióticos podem ser controversos. Apesar de não terem evidenciado um efeito claro no crescimento dos animais, podem ser úteis reduzindo a dispersão do tamanho dos peixes entre tanques. Eles parecem, em alguns casos, serem capazes de estimular a resposta imune inata, mas esse efeito perde-se com um período mais longo de administração. Por outro lado, o uso de ingredientes vegetais na dieta revelou-se ser mais eficiente em estimular a resposta imunitária do que propriamente o uso do probiótico ou até mesmo do imunoestimulante. No que diz respeito à microbiota intestinal, detetou-se uma predominância de *Vibrio* sp, tendo o probiótico multiespécie testado no nosso trabalho reduzido a presença de *Vibrio* sp. na microbiota.

CHAPTER 1

General introduction

1.1. General aspects of Senegalese sole (*Solea senegalensis*) biology and production

The Senegalese sole (*Solea senegalensis* Kaup, 1858) (order Pleuronectiformes and family Soleidae) is a benthonic marine flatfish species found from the Gulf of Biscay to the coasts of Senegal in sandy or muddy bottoms off the continental shelf, up to 100 m depth (Imsland et al., 2003). In its natural environment, this species feeds essentially on invertebrates living in the sediment, such as polychaetes, bivalves, molluscs and small crustaceans (Cabral, 2000). Sexual maturity is reached at age 3+ or when total length is around 32 cm. Sole spawning season occurs mostly between the months of March and June, with each female ovulating and releasing batches of eggs every few days over a period of several weeks (Imsland et al., 2003). Its life cycle can be divided between the juvenile phase, which is predominantly estuarine, and the adult phase, which is mainly marine (Cabral, 2003). Similarly, to other flatfish, this fish undergo a dramatic metamorphic process, which starts around 8-12 days post hatching (dph) and involves a 90º rotation of the body position and the migration of the left eye to join the other one on an ocular upper side (Fernández-Díaz et al., 2001). During metamorphosis, there is a rearrangement of the internal organs and digestive tract, with migration of the anus towards the pelvic fin. Only around 30 dph the digestive system completes its maturation (Ribeiro et al., 1999).

Solea senegalensis is a sole specie, found naturally in Atlantic and Mediterranean waters, and is considered potentially important for marine aquaculture owing to their high market value and consumer demand (Colen et al., 2014). Sole production increased from 110 tonnes to 500 tonnes from 2008 to 2011, especially in Portugal and Spain (Borges, 2014). The increase in Senegalese sole production has been constantly halted due to disease outbreaks, causing high mortality, growth depression and poor juvenile quality (Morais et al., 2014). Growth and survival from juvenile to market-size is not fully controlled and one of the most serious problems concerning sole production is the existence of bacterial infectious diseases (Arijo et al., 2005a; Romalde, 2002; Zorrilla et al., 1999).

Sole has a nocturnal activity pattern, peaking during the first part of the dark period (Bayarri et al., 2004) and higher metabolic rate during the dark phase (Castanheira et al., 2011). However, aquaculture facilities for indoor on-growing use mostly a 12hL:12hD photoperiod and some shading in the tanks to keep light at the surface

between 80 and 350 lux (Navarro et al., 2009; Salas-Leiton et al., 2008). Typical rearing of Senegalese sole is done either following natural thermoperiod or maintaining constant temperature around 20°C (Morais et al., 2014). Although higher growth occurs at temperatures ranging from 20 to 25°C, temperatures above 22°C entail higher risk of pathological outbreaks (Cañavate, 2005). However, sole can be exposed to high temperature fluctuations throughout its life time, which in the wild can range between 12 ºC and 28 °C (Cabral and Costa, 1999; Vinagre et al., 2006). Sole juveniles can tolerate salinities from 5 to 55 ppm (Arjona et al., 2007). However, growth was shown to be depressed at a low salinity concentration, with a clear impact on feed intake, energy metabolism and cortisol response when fish is reared at salinities between 25 and 39 ppm (Arjona et al., 2009). Densities of up to 30 kg m^{-2} have been tested with no effects on growth (Salas-Leiton et al., 2008) although a relationship has been found between high stocking densities and stress (Costas et al., 2008; Salas-Leiton et al., 2010), but it is unclear whether this is due to density per se, or rather to deteriorating water quality.

Understanding the underlying mechanisms of growth in fish has been a major focus for an effective and successful aquaculture production. Research on fish muscle growth is also important for the rapidly developing global aquaculture industry, particularly with respect to quality.That it is a very complex process involving hyperplasia (increase in number of fibers) and hypertrophy (increase in fiber size), which is controlled by an extensive network of genes (Johnston et al., 2011). Adult muscle is a heterogeneous tissue composed of several cell types that interact to affect growth patterns. Temperature is perhaps the most important single abiotic factor known to have a marked effect on myogenesis in several fish species of commercial importance, including Senegalese sole (Campos et al., 2014). Other important factor is the composition of the diet. Recently, it has been shown that an increase in the dietary lipid content or a decrease in the protein/fat ratio was shown to have a negative effect on growth or feed efficiency of Senegalese sole juveniles (Borges et al., 2009). Moreover, Campos et al. (2010) observed a decrease in the expression of myogenic regulatory factors and myosins in the muscle of Senegalese sole fed increasing dietary lipid levels, supporting the hypothesis that high lipid levels somehow depress growth by reducing protein accretion.

1.2. Nutrient requirements and plant ingredients in sole aquafeeds

Feeding strategies as well as specific dietary formulation is required to enhance production and minimize costs. Furthermore, in the last decade the increasing demand, price and world supply fluctuations of fish meal (FM) have emphasized the need to look for alternative protein sources.

Sole has a high dietary protein requirement (53% dry matter, DM) to maintain good overall growth performance (Rema et al., 2008). This represents an extremely high cost in aquafeeds, since fish meal is the main protein source, which can account for 20 to 60% of the diet, and the most costly ingredient.

In most marine fish, a significant protein sparing can be achieved by increasing digestible energy levels through an increase in fats and/or carbohydrates (Helland and Grisdale-Helland, 1998; Kaushik, 1998). However, contrary to most marine fish species, the ability of Senegalese sole juveniles to efficiently use high dietary lipid levels seems limited, in both juvenile (Borges et al., 2009; Dias et al., 2004; Guerreiro et al., 2012) and market-sized fish (Valente et al., 2011). Borges et al. (2009) clearly demonstrated a low lipid tolerance in this species and recommended a dietary lipid inclusion of up to 8% (dry matter basis) for optimal growth and feed utilization efficiency.

High-quality FM is still the major protein source currently used in sole diets. However, supplies of FM and fish oil are limited, and their replacement in aquafeed formulations with ingredients from more available plant sources is needed (Tacon and Metian, 2008). The replacement of marine-derived protein sources by plant protein (PP) ingredients in Senegalese sole feed is feasible in both juvenile (Cabral et al., 2011) and large-sized fish (Cabral et al., 2013; Valente et al., 2011). It was further evidenced that sole juveniles can grow equally well with diets completely devoid of fish meal, providing these diets of a well-balanced dietary aminoacid profile (Silva et al., 2009).

Considering the effect of plant-based diets on the sensorial characteristics of Senegalese sole flesh, the replacement of fish meal by a blend of plant ingredients did not have a significant impact on the majority of volatile compounds (Moreira et al., 2014; Silva et al., 2012) or in the sensory descriptors (Cabral et al., 2013).

Nevertheless, these plant-based diets contain some antinutritional factors (saponins, phytoestrogens, trypsin inhibitors, phytic acid, and allergens) which may hamper growth and nutrient utilization of fish (Francis et al., 2001). Thus, the impact

of long-term feeding high plant-based diets on gut integrity, liver function and immune status should be addressed.

1.3. Disease in sole aquaculture

Infectious diseases are one of the most significant threats to successful aquaculture. The high-density living conditions in aquaculture facilities and the increased animal stress due to overcrowding lead to outbreaks of diseases that normally occur at low levels in natural populations. In the aquaculture systems, fish are in permanent contact with microbial communities and fish metabolites, a feature that can affect their health and growth. The oscillation of environmental conditions (e.g. temperatures, salinity, water quality, UV light), management factors (e.g. high density and poor feeding) and host-related factors (stress, skin surface condition) play a significant role on disease outbreaks.

One of the main factors hampered Senegalese sole farming has been the high incidence and intensity of diseases (Padrós et al., 2003; Toranzo et al., 2003). Currently, the main pathological problems are bacterial diseases, mainly tenacibaculosis (or flexibacteriosis), photobacteriosis (or pasteurellosis) and vibriosis.

Tenacibaculosis, which is mainly caused by *Tenacibaculum maritimum* (or *Flexibacter maritimum*), can cause significant morbidity and mortality, limiting the culture of economically important marine fish species (Santos et al., 1999). Cepeda and Santos (2002) isolated for the first time *T. maritimum* from Senegalese sole in south-west Spain, where it caused almost 100% mortality of the affected stocks. Recently, Vilar et al. (2012) described particularly severe ulcerative disease outbreaks in cultured Senegalese sole associated with *T. maritimum*. Affected sole usually display several external signs including eroded mouth, rotten fins and skin lesions with total loss of epidermis and dermis and extensive necrosis of the muscular layers.

Photobacteriosis, caused by *Photobacterium damsela* ssp. *piscicida*, is responsible for high losses in the aquaculture industry leading to massive mortalities in several marine fish species such as gilthead sea bream (Toranzo et al., 1991), sea bass (Balebona et al., 1992), and in the flatfish Japanese flounder (Fukuda et al., 1996), among others. As it was first recorded in farmed Senegalese sole in southwest of Spain (Zorrilla et al., 1999), several sole farms, mainly in the south of Spain, have

suffered mortalities caused by this disease (Magariños et al., 2003). In most cases, peracute mortalities without apparent lesions are the most typical manifestation found mainly in juveniles. However, in subacute and chronic cases, external lesions of infected fish included only unspecific symptoms such as dark skin coloration and swelling of the abdominal cavity. This disease particularly affects Senegalese sole at temperatures above 18°C and usually triggers severe acute cases in which mortality can be extremely high (Padrós et al., 2003).

Vibriosis affecting Senegalese sole are usually detected as secondary infections associated with other disease, but often they can also be primary infections and its pathogenesis is still unclear (Padrós et al., 2003). *Vibrio harveyi* (Rico et al., 2008; Zorrilla et al., 2003), *V. parahaemolyticus* (Zorrilla et al., 2003) and *Vibrio alfacsensis* (Gomez-Gil et al., 2012) are pathogenic bacteria which were described in some disease outbreaks of farmed sole in Spain. Main external signs of the disease were skin ulcers and haemorrhagic areas near the fins and mouth (Zorrilla et al., 2003).

Other bacteria have also been identified as causative of infectious disease in sole, such as the *Aeromona salmonicida* subspecies *salmonicida* (Magariños et al., 2011), and *Edwarsiella tarda* (Castro et al., 2012).

Vaccination strategies have been development against these diseases (Romalde et al., 2005), and a divalent vaccine against *P. damselae* subsp. *piscicida* and *V. harveyi* that provides short-term protection is being studied (Arijo et al., 2005b). In addition, recent studies on the use of probiotics to control *Photobacteriosis* and different *Vibrio* species have given encouraging results (García de la Banda et al., 2012; Tapia-Paniagua et al., 2012).

As viral diseases, betanodaviruses have been detected in Senegalese sole (Cutrín et al., 2007; Hodneland et al., 2011; Olveira et al., 2009; Thiéry et al., 2004) as well birnavirus and lymphocystis virus (Alonso et al., 2005; Cano et al., 2010; Rodríguez et al., 1997; Toranzo et al., 2003). Fish infected with betanodaviruses and birnavirus, show abnormal swimming behaviour and moderate to high mortalities (Hodneland et al., 2011; Rodríguez et al., 1997). Lymphocystis disease is caused by an iridovirus, characterized by papilloma-like lesions typically on the skin, fins and tail (Walker and Hill, 1980).

The main parasitic problem in cultured Senegalese sole is the systemic amoebic disease. Although the condition was not associated with high mortalities, reduced

growth and high morbidity were noted. Fish show protuberances on the skin surface in addition to unspecific signs of disease (lethargy with sporadic and erratic swimming) (Constenla and Padrós, 2010). *Endolimax piscium* (Archamoeba) is the causative agent of this amoebiasis (Constenla et al., 2014), causing a granulomatous inflammatory reaction mainly in muscular but also in different internal organs of the host. Early detection of the parasite in the farm should be considered a priority for the management of this disease in sole culture, as there is no known effective treatment against these parasites.

1.4. Probiotic definition

The word “Probiotic” is derived from Latin word “pro”-for and Greek word “biotic”-life. According to the currently adopted definition by Food and Agricultural Organization/ World Health Organization (FAO, 2001), probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host”.

Dietary probiotic supplementation may beneficially affect the host by the production of inhibitory compounds, competition for chemicals and adhesion sites, immune modulation and stimulation, and improving the microbial balance (Fuller, 1989; McCracken and Gaskins, 1999; Verschuere et al., 2000). Merrifield et al. (2010d), proposed a distinct definition of probiotics, given the nature of fish farming and the closer relationship with their water environment: “Probiotic is any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved by improving the microbial balance of the fish. In this context, the direct benefits to the host are immune-stimulation, improvement of disease resistance, reduction of stress response, improvement of intestinal morphology. The benefits to the fish farmer or consumer are the improvement of fish appetite, growth performance and feed utilization, improvement of carcass and flesh quality and reduction of malformations. Therefore, several terms such as “friendly”, “beneficial”, or “healthy” bacteria are commonly used to describe probiotics (Wang et al., 2008a). Most probiotics are bacteria and lactic acid bacteria are especially popular.

Prebiotics, on the other hand can be defined as non-digestible food ingredients that selectively stimulate the growth and/or activity of one or limited microbes and

"symbiotic", the nutritional supplements combining probiotics and prebiotics (Andersson et al., 2001; Morelli et al., 2003).

1.5. Probiotic attributes

The probiotic concept requires that the bacterial strains must meet selected attributes: non-pathogenic (to the host species, aquatic animals and human consumers); free of plasmid-encoded antibiotic resistance genes; survive through the digestive tract (resistant to bile salts and low pH); adhere and colonise the intestinal epithelial surface; improve growth performance of host by improving feed efficiency, competing for energy sources and/ or produce relevant extracellular digestive enzymes and/or vitamins; exhibit antagonistic properties towards one or more key pathogens, among others (Gomez and Balcázar, 2008; Merrifield et al., 2010c; Sáenz de Rodrigáñez et al., 2009; Tinh et al., 2008; Verschuere et al., 2000).

Several works have studied the immunological and haematological stimulation of fish defence mechanisms by probiotic bacteria (Arijo et al., 2008; Brunt et al., 2008; Merrifield et al., 2010a; Merrifield et al., 2011; Merrifield et al., 2010d; Pieters et al., 2008). Furthermore, probiotics may confer protection against intestinal aggression (Sáenz de Rodrigáñez et al., 2009) caused by an increase in dietary antinutrients or antinutrional factors, as a consequence of replacing of fish meal by plant ingredients.

1.6. Regulation and safety assessment of the probiotics use for animal nutrition in the European Union

The use of probiotics is associated with a proven efficacy on the gut microflora and improved health status. Probiotics should have a role on the balance of gut microflora, increasing the resistance to pathogenic agents, both through a strengthening of the intestinal barrier and stimulating directly the immune system (Anadón et al., 2006). Microorganisms used in animal feed in the EU are mainly strains of Gram-positive bacteria belonging to the types *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus* and strains of yeast belonging to the *Saccharomyces cerevisiae* species and kluyveromyces (Anadón et al., 2006). While most of Lactobacilli and bifidobacteria are apparently safe but certain microorganisms may be problematic; particularly the enterococci, which are

associated with infections and harbour transmissible antibiotic resistance determinants (Wright, 2005).

Probiotics used in animal nutrition in the European Union must be registered as microbial feed additives. The manufacturers demonstrate the safety, efficacy and stability of their products by appropriate trials. Studies conducted in the laboratory and under practical conditions follow the requirements of the European Community for registration (Directive 70/524/EC and Regulation (EC) No. 1831/2003 on feed additives in animal nutrition, respectively, and the guidelines for the assessment of feed additives) (Busch et al., 2004). Registration comes into effect only after the European Food Safety Authority (EFSA) and the experts of all Member States have approved the quality and efficacy of the probiotic as well as its safety in humans, animals and the environment. Once the probiotic is authorised, the microorganism is registered as approved feed additives, with the dosage range and the approved target species (Busch et al., 2004).

Briefly, the use of a given microorganism as probiotic requires its isolation, characterization and testing to certify its probiotic efficiency. First a source of microorganisms (e.g. digestive tract of healthy animals) must be selected. Thereafter, the microorganisms are isolated and identified by means of selective culture. Then, *in vitro* evaluations (inhibition of pathogens; pathogenicity to target species; resistance conditions of host; among others) are performed only with the colonies of interest. In case of the absence of restrictions on the use of the target species, experiments with *in vivo* supplementation, are carried out to check if there are real benefits to the host (Azevedo and Braga, 2012). Comprehensive and accurate characterisation of the microorganism is necessary and microbiological tests and selection procedures are carried out to evaluate their suitability for animal nutrition. The behaviour of the microorganism in the animal is studied, i.e. whether it survives intestinal passage, how long it remains in the intestine and how it regulates the intestinal ecosystem. Then, for production purposes it is necessary the microorganism is capable of large-scale proliferation and it remains genetically stable (Busch et al., 2004). Finally, the probiotic that presented satisfactory results can be produced utilized commercially (Azevedo and Braga, 2012)

Final formulation and standardisation are usually achieved by mixing with a carrier to ensure a homogeneous distribution of the probiotic indifferent feed types (Busch et al., 2004).

Probiotics in aquaculture may act in a manner similar to that observed for terrestrial animals. However, the relationship of aquatic organisms with the farming environment is much more complex than the one involving terrestrial animals (Azevedo and Braga, 2012). Currently *Pediococcus acidilactici* (CNCM MA 18/5M) it the only strain authorized as a feed additive by the Regulation (CE) nº 911/2009 for salmonids and shrimps and by the Regulation (CE) nº 95/2013 for all fish except salmonids, being classified in the additive category “zootechnical additives”.

1.7. Probiotics in aquaculture

Fish is one of the richest sources of animal protein and is the fastest food producing sector in the world. Worldwide, 25% of animal protein come from fish and shellfish, and dependence on fish protein continues to climb (Naylor et al., 2000). The intensification of aquaculture and globalization of the seafood trade have led to remarkable developments in the aquaculture industry with the addition of commercial diets, growth promoters, antibiotics, and several other additives (Wang et al., 2008a). However, serious economic losses could occur in the modern aquaculture.

In fish farms, the control of bacterial pathogens is achieved by the administration of chemotherapeutic agents, which are extensively employed, leading to potential risk to public health and to environment by the emergence of drug-resistant microorganism and antibiotic residues (Miranda and Zemelman, 2001; Radu et al., 2003). Taking this into account, as well as the increasing demand for environment friendly aquaculture, it is necessary to provide aquaculture industry with alternative means to keep a microbiologically healthy environment and to enhance fish production and economic profits (Díaz-Rosales et al., 2009). A variety of useful feed additives, including probiotics and prebiotics were successfully used in aquaculture to combat diseases, to improve growth performance and to stimulate immunity response of fish (Irianto and Austin, 2002a). The use of probiotics has emerged as a potential tool to reduce mortalities in the rearing of aquatic organisms (Gatesoupe, 1999; Gomez-Gil et al., 2000; Ringø and Gatesoupe, 1998; Verschuere et al., 2000) by improving growth, being already available several commercial preparations that could be introduced as feed additives or incorporated in the water.

Boyle et al. (2006) reviewed the safety of probiotics and highlighted deficiencies in our understanding of their appropriate administration and their mechanisms of

action. They found that probiotics should be used with caution in some cases, because: a) of the risk of sepsis; b) of adverse metabolic effects from manipulation of the microbiota, even if such manipulation is temporary; c) of immune deviation or excessive immune stimulation; or even d) of possible transfer of antimicrobial resistance from probiotic strains to pathogenic bacteria in the intestinal microbiota.

1.8. Use of probiotics in sole farming

Commercial rearing of sole recently became of great interest due to their high value and increased market demand. Great research efforts have been devoted to the evaluation of various plant ingredients as sustainable alternatives to fish meal. Sealey et al. (2009) suggested that the amount of dietary plant ingredients can be increased by adding probiotics to the diet, but there are scattered studies on this matter. Moreover, several reports on the application of different probiotic strains on Senegalese sole have provided encouraging results concerning growth and survival against pathogens (Díaz-Rosales et al., 2009; García de La Banda et al., 2010; Makridis et al., 2008). Different approaches have been used in order to estimate the beneficial effects of different probiotic strains on feed efficiency and growth performance, body composition, intestine and liver morphology, immune responses (respiratory burst activity of phagocytes) and *in vivo* challenge with pathogens (Avella et al., 2011; Díaz-Rosales et al., 2009; García de La Banda et al., 2010). However, diets containing probiotics have generally been evaluated in Senegalese sole in terms of their effect on disease resistance and immune response, with little attention given to their effect on growth (Sáenz de Rodrigáñez et al., 2009).

Studies have been conducted to isolate and define the best bacterial species for probiotic applications for sole (Table 1). In particular, several strains isolated from a number of teleost species have been assessed in order to prevent bacterial diseases. Nevertheless, additional information concerning (a) the mechanism of action of probiotics on the digestive and absorptive processes within the gut of fish and, (b) the *in vivo* interaction between host and microbes, (c) the optimization of the dose and frequency of probiotic administration are still needed to define adequate selection criteria for new potential probiotics (Sáenz de Rodrigáñez et al., 2009).

Table 1 - Probiotic applications in Senegalese sole experimental farming.

Fish	Objective	Probiotic and origin	Method	Effects	Reference
Solea senegalensis	Evaluate the adhesive ability to Senegalese sole mucus and the host specificity of several microbial isolates from farmed fish.	Ten isolates. Isolated from healthy farmed Senegalese sole.	*In vitro* test. Test their adhesion to skin and intestinal sole mucus, and were also screened for their antagonistic capability against *P. damselae* subsp. *piscicida*.	Mucus adhesion of certain isolates is strain-dependent rather than host-dependent. 21% of the microorganisms isolated exhibited antibacterial activity against *P. damselae* subsp. *piscicida*.	Chabrillón et al. (2005b)
Solea senegalensis juveniles	Evaluate the adhesive competitiveness of four potential probiotic strains isolated from the microbiota of a farmed fish, with the pathogen *V. harveyi*.	Pdp5 (*Micrococcus*) Pdp9 (*Pseudomonodaceae*) Pdp11 51M6 (*Vibrionaceae*) Recovered from skin mucus of healthy farmed gilthead sea bream.	*In vivo* and *in vitro* tests. 15 days trial. Bacteria were mixed with the daily feed dose in a blender to obtain a dose of 10^8 cfu g^{-1} feed. Challenge with *V. harveyi.*	Pdp11 was selected, based on its adhesion to intestinal mucus, its antagonistic effect on *V. harveyi*, and its inhibition of the attachment of the pathogen to intestinal mucus under exclusion and displacement conditions. Pdp11 significantly reduced mortality in challenged fish.	Chabrillón et al. (2005a)
Solea senegalensis larvae	Determine the effect of the candidate probiotic strains on: (a) survival of unfed sole yolk-sac larvae (in vivo test) (b) survival of larvae and postlarvae in a feeding experiment (c) number of culturable bacteria present in the water and the fish gut.	Three candidate probiotics, which had shown antimicrobial activity in vitro against two fish pathogens. Isolated from the culturable heterotrophic gut microflora of Senegalese sole juveniles fed natural prey.	*In vivo* and *in vitro* tests. During the first phase of the rearing (0–20 days after hatching), bacterial cultures were added daily to the water in tanks. In the second phase of the rearing (20–60 days after hatching), bacteria were added via bioencapsulation in Artemia.	Addition of probiotic bacteria increased the survival of the larvae during the first phase of rearing. In the second phase of rearing, showed a low rate of colonization of the gut and no increase of survival in the sole postlarvae.	Makridis et al. (2008)

Fish	Objective	Probiotic and origin	Method	Effects	Reference
Solea senegalensis juveniles	Assess the effect of two probiotics on growth and feed efficiency, enzymatic activities of the brush-border membrane, intestine histology and microbial community.	Pdp11 (*S. putrefaciens*) Pdp13 (*S. baltica*) Isolated from the skin of gilthead seabream	*In vivo* test. 60 Days of supplementation period. Lyophilized bacterial cell suspension (10^9 cfu g^{-1} feed) sprayed into the feed under continuous agitation.	Increase growth and nutrient utilization in fish receiving probiotics. Accumulation of lipid droplets in the enterocytes of fish receiving the control diet, but not in those fed on probiotics.	Sáenz de Rodrigáñez et al. (2009)
Solea senegalensis juveniles	The effects of dietary administration of the two probiotics, on growth, respiratory burst activity of phagocytes, and survival of fish challenged with *Photobacterium damselae* subsp. p*iscicida*.	Pdp11 (*S. putrefaciens*) Pdp13 (*S. baltica*) Isolated from the skin of gilthead seabream	*In vivo* test. 60 Days of supplementation period. Lyophilized bacterial cell suspension (10^9 cfu g^{-1} feed) sprayed into the feed under continuous agitation. Challenge with *P.damselae* subsp. *Piscicida*	Increase respiratory burst activity of phagocytes from fish fed diet Pdp11. Increase growth, and survival against the pathogen. Cumulative percentage of mortality after the challenge: 100% in the control diet groups, 75–100% in the Pdp11 and 65–80%, in the Pdp13.	Díaz-Rosales et al. (2009)
Solea senegalensis juveniles	Study of intestinal microbiota (PCR + DGGE) diversity following probiotic administration.	Pdp11 (*S. putrefaciens*). Isolated from the skin of gilthead seabream.	*In vivo* test. 60 Days of supplementation period. Fresh or lyophilized bacterial cells diluted in a suspension (10^9 cfu g^{-1} feed) sprayed into the feed under continuous agitation.	Incresase in the predominant species related to *Vibrio* genus in the intestinal microbiota. Differences in the microbial composition from fishes receiving the commercial diet, compared to those fed with a diet supplemented with fresh or lyophilized probiotics.	Tapia-Paniagua et al. (2010)

Fish	Objective	Probiotic and origin	Method	Effects	Reference
Solea senegalensis juveniles	Evaluate the influence of dietary administration of two probiotic strains on growth, biochemical composition, histology and digestive microbiota.	Pdp11 (*S. putrefaciens*) Pdp13 (*S. baltica*) Isolated from the skin of gilthead seabream	*In vivo* test. Fish fed for 2 months. Pdp11 was incorporated at concentration of 10^9 cfu g^{-1} Challenge with *Photobacterium damselae* subsp. *piscicida* (intraperitoneal) was performed.	Probiotic confered protection against *P. damselae* subsp. *Piscicida*. Pdp11 diet promoted better digestive and liver condition. Fish fed the Pdp13 diet showed significant differences in growth and body composition. Intestinal microbiota was differently influenced depending on the strain assayed.	García de La Banda et al. (2010)
Solea senegalensis juveniles	Tested the health protection and nutritional effects of probiotic (fresh and lyophilized cells) on juveniles.	Pdp11 (*S. putrefaciens*). Isolated from the skin of gilthead seabream.	*In vivo* test. Fish fed for 2 months. Pdp11 was incorporated at concentration of 10^9 cfu g^{-1} Challenge with *Photobacterium damselae* subsp. *piscicida* (intraperitoneal) was performed.	Fresh Pdp11 enhanced growth performance. Both fresh and lyophilized Pdp11 cells conferred protection against P. damselaesubsp. piscicida.	García de la Banda et al. (2012)
Solea senegalensis larvae	Study the influence of probiotic supplementation on growth, body composition and gut microbiota, during larval and weaning development.	Pdp11 (*S. putrefaciens*). Isolated from the skin of gilthead seabream.	*In vivo* test. Pdp11 was incorporated using Artemia as live vector (2.5×10^7 cfu mL^{-1})	Pdp11 modulated gut microbiota and increased protein contents and DHA/EPA ratios. Pdp11 promoted higher growth and a less heterogeneous fish size in length at 90 days after hatching.	Lobo et al. (2014)

Fish	Objective	Probiotic and origin	Method	Effects	Reference
Solea senegalensis juveniles	Evaluate the effect of the dietary administration of two probiotics on the intestinal microbiota and on the fatty acid contents of their liver.	Pdp11 (*S. putrefaciens*) Pdp13 (*S. baltica*) Isolated from the skin of gilthead seabream	*In vivo* test. Fish fed for 69 days. Pdp11 was incorporated in a dose of 10^9 CFU/g feed	Modulation of intestinal microbiota by probiotic diets, increasing the presence of *Shewanella* spp and decreasing of *Vibrio* spp. Correlation between bacteria species observed in fish fed Pdp13 and liver linoleic and linolenic acid levels. Species comprising the intestinal microbiota in fish fed Pdp11 were related to lower lipid droplet presence in liver and enterocytes.	Tapia-Paniagua et al. (2014)
Solea senegalensis juveniles	Determine the effect of a dietary multispecies probiotic on growth, gut morphology and immune parameters.	Commercial probiotic (*Bacillus* sp., *Pedicoccus* sp., *Enterococcus* sp. and *Lactobacillus* sp.)	*In vivo* test. Fish fed for 72 days. A sub-lethal bath challenge with *Photobacterium damselae* subsp. *piscicida* was performed after the growth trial.	No significant differences were found in growth performance and humoural immune parameters. Gut morphology showed a significant increase in intestinal villi height of fish fed the probiotic. Probiotic supplementation increased thrombocytes levels whereas a decrease in the proportion of lymphocytes was observed. Bath challenge differentially affected leucocyte counts and increased peroxidase activity.	Barroso et al. (2014)

Fish	Objective	Probiotic and origin	Method	Effects	Reference
Solea senegalensis juveniles	Evaluate the effect of the dietary administration of oxytetracycline (OTC) in isolation or combined with probiotic on the intestinal microbiota and hepatic expression of genes related to immunity, oxidative-stress and apoptosis in the liver.	Pdp11 (*S. putrefaciens*). Isolated from the skin of gilthead seabream.	*In vivo test.* Fish fed for 10 days. Pdp11 was incorporated at concentration of 10^9 cfu g^{-1}	Richness and diversity of intestinal microbiota of fish was changed by the use of Pdp11. Fish received OTC and Pdp11 jointly showed a decreased intensity of the DGGE bands related to *Vibrio* genus and the presence of DGGE bands related to *Lactobacillus* and *Shewanella* genera. Pdp11 induced the up-regulation of genes related to antiapoptotic effects and oxidative stress regulation.	Tapia-Paniagua et al. (2015)

1.9. Factors affecting the immunomodulation capacity of probiotics

Modulation of host immunity is one of the most alleged benefits of probiotics. Selection of probiotics is very critical because inappropriate microorganisms can lead to undesirable effects in the host. An ideal probiotic, regardless of its origin, should be able to colonise, establish and multiply in the host gut (Gómez-Gil and Roque, 1998).

1.9.1. Type and form of strain

The dominant groups of probiotics used in aquaculture are Gram+, especially lactic acid bacteria (LAB) and bifid bacteria groups (Kesarcodi-Watson et al., 2008). Each strain has unique properties and they greatly differ in their mode of action, including the ability to activate immune system. The probiotic effects of a specific strain should not be extrapolated to other strains (Boyle et al., 2006; Pineiro and Stanton, 2007). Different strains of the same species may exert different effects on the host, as well as strains of the same species can exert different, and sometimes, opposite effects (Aureli et al., 2011). Recently a study in sole using *Shewanella putrefaciens* and *Shewanella baltica* as probiotic bacteria showed difference that both bacteria have different mechanisms in triggering the respiratory burst activity (Díaz-Rosales et al., 2009).

Commercially available probiotics are sometimes ineffective. They are unable to survive and/or remain viable at optimum concentration in gut, possibly due to their non-fish origin (Abraham et al., 2008). Autochthonous bacteria isolated from fish tissues and/or its natural environment or aquaculture systems are currently being studied as the best approach for increasing efficacy as fish probiotic (Verschuere et al., 2000). The strategy on isolating probiotics from the gut of mature animals and then use in immature animals of the same species has been successfully applied in fish (Gatesoupe, 1999; Gildberg et al., 1997; Gomez-Gil et al., 2000; Gram et al., 1999).

These autochthonous probiotics have a greater chance of competing with resident microbes and of becoming predominant within a short period of intake and persist in the colonic environment for some time after the withdrawal of probiotics (Carnevali et al., 2004). In this context, the identification of the strain is necessary for safety reasons and to prove their beneficial action.

It is unlikely to find a single probiotic that fulfil all the desirable characteristics of an ideal probiotic. So several studies were designed to explore the possibilities of simultaneously using probiotic blend or probiotics-prebiotics (termed symbiotic) approaches (Patterson and Burkholder, 2003). According Timmerman et al. (2004), multistrain and multispecies probiotics approach have shown to provide synergistic bacteria with complementary modes of action with enhancing protection.

A wide range of probiotics, containing either mono- or multi-species microorganisms are commercially available for aquaculture practices. The multispecies/multistrain probiotic treatment may be considered more effective and more consistent than the monospecies probiotic treatment, by promoting synergistic properties (Timmerman et al., 2004). The use of multispecies probiotics in fish, may induce immune response (Cabral and Costa, 1999; Irianto and Austin, 2002b; Salinas et al., 2006) and be more effective in triggering the local gut immunity (Salinas et al., 2008).

Bacteria belonging to both spore former and non-spore formers are used as probiotics. Several spore forming bacteria which produce a wide range of antagonistic compounds can be valuable as probiotics (Moriarty, 2003). Among spore formers, *Bacillus* spores are routinely being used as probiotics in human and animal practices due to their immunostimulatory properties (Casula and Cutting, 2002; Hong et al., 2005). Due to the physical and biological spore forming bacteria can resist adverse environmental conditions having a prolonged shelf life, they are heat-stable and can survive transit across the stomach barrier, properties that cannot be assured when using non-spore forming bacteria (Huang et al., 2008). The production cost of probiotic from spore-forming bacteria is lower with respect to production of purified components (Huang et al., 2008). However, the majority of probiotics currently available are bacteria which are non-spore formers, supplemented to fish diet in the vegetative form. Nevertheless, the combination of both spore- and non-spore forming bacteria are also found to increase immunity in fish (Salinas et al., 2008; Salinas et al., 2005; Taoka et al., 2006b).

Viability is an important property of any probiotics which enable them to adhere and colonize the host intestine. Although, viable bacteria are better stimulator of immune system (Taoka et al., 2006b), certain probiotic bacteria can potentially elicit similar beneficial effects in host in inactivated form. Different probiotics in inactivated form exhibited promising immunomodulatory and protection effects in various fish species, under *in vitro* (Salinas et al., 2006) and *in vivo* (Irianto and Austin, 2003;

Panigrahi et al., 2005) conditions. The immunomodulating activity of non-viable probionts could possibly be attributed to the presence of certain conserved microbial components such as capsular polysaccharides, peptidoglycans and lipoteichoic acids which are the potent stimulator of fish immune system (Secombes et al., 2001).

1.9.2. Dosage of probiotics

Determination of the adequate amount of live probiotic bacteria to be administered to fish is not an easy task (Aureli et al., 2011). The inadequate dose of probiotics treatment could limit to achieve the optimum effects. A lower dose can be insufficient to stimulate the piscine immune system, whereas too high a dose can exert deleterious effects. Nikoskelainen et al. (2001a), recorded higher percentage of mortality in *O. mykiss* fed at high dose of *L. rhamnosus* (10^{12} CFU g feed^{-1}) compared to lower dose (10^{9} CFU g feed^{-1}). The optimum concentration of probiotics is not only required for bacteria colonization and proliferation in the intestine but it is also needed to effectively exert the beneficial effects, including immunostimulatory activity, enhancing growth, protection and host protection, among others. Panigrahi et al. (2005) observed that *in vivo* immune response of fish varies with the concentration of probiotics. The dose of probiotics ingested is an important factor to obtain high concentrations in the various compartments of the gastrointestinal tract. It is often said that probiotic concentrations must be greater than or equal to 10^{6} CFU mL^{-1} in the small intestine and 10^{8} CFU g^{-1} in the colon (Sanders, 2003). In aquaculture, the dose of probiotics usually varies from $10^{6\text{-}10}$ CFU g feed^{-1}, but the optimum dose of a probiotics can vary with respect to host and also type of immune parameters (Panigrahi et al., 2004). Furthermore, stimulation of a particular immune response with respect to different tissue/organ also varies with dose. Therefore, the dose of the individual probiotics needs to be determined for a particular host.

1.9.3. Mode of supplementation

In fish, probiotics are applied by different methods such as bath immersion, suspension and dietary supplementation. Dietary supplementation is considered the best method for successful colonization and establishment in gut. Oral administration of probiotics is more effective in enhancing immunity as well as subsequent protection compared to bath immersion (Taoka et al., 2006b). However,

several probiotics are also directly used as water additives with health benefits to the fish, but also to the rearing environmental. The application of probiotic directly to the rearing water may play a significant role in the decomposition of organic matter, reduction of nitrogen and phosphorus level as well as control of ammonia, nitrite, and hydrogen sulfide (Boyd and Massaut, 1999; Zhou et al., 2010).

1.9.4. Environmental conditions

Several factors may influence the establishment of probiotics and subsequent actions, namely water quality, water hardness, dissolved oxygen, temperature, pH, osmotic pressure and mechanical friction (Das et al., 2008). Water temperature plays an important role for probiotic settlement in the intestine. Fish are poikilothermic (temperature in intestine is similar to the surround environment), thus the probiotic bacteria activity is most effective if the fish rearing temperature coincides with the optimum temperature range of the probiotic bacteria (Panigrahi et al., 2007). Stress due to high stocking density can affect the performance of the probiotics (Mehrim, 2009). Probiotics can help to overcome stress due to salinity (Taoka et al., 2006b) or due to high temperature (Asli et al., 2007).

1.9.5. Duration of treatment

Duration of the probiotic feeding is another important factor that may affect the establishment, persistence and subsequent induction of host immune responses. In fish, most of the beneficial effects have been recorded within a dietary probiotics feeding regime of 1-10 weeks (Nayak, 2010). The time course for optimum induction of immune response differs with respect to probiotic strain and also with the type of immune parameter to determine (Choi and Yoon, 2008). Similarly, difference in stimulating specific immune parameter is also dependent on feeding duration. Díaz-Rosales et al. (2009) observed significant enhancement of respiratory burst activity by feeding trout with probiotics for 60 days. Nevertheless, Díaz-Rosales et al. (2006b), did not observed an enhancement of respiratory burst activity when fed for 4 weeks the same probiotic.

Several probiotics are often found to stimulate the piscine immune system within 2 weeks of supplementation. Sharifuzzaman and Austin (2009) recorded highest cellular and humoral immunity at 2 weeks of feeding regime and further supplementation lead to lowering at weeks 3 and 4 of supplementation. However, a

short-term probiotic feeding can also cause sharp decline in immune response in fish (Panigrahi et al., 2005). Such type of decline may be due the failure of the probiotic strains to establish and multiply in the fish gut.

1.9.6. Probiotic viability and survival

High levels of viable probiotics microorganisms are recommended for efficacy (Gatesoupe, 1999). Consequently, the retention of high viability during preparation and storage presents particular challenge in commercial probiotic production. Most liquid/frozen probiotic cultures require refrigeration for storage and distribution, adding expense and inconvenience to their widespread use in aquaculture. To maintain confidence in probiotic products used in aquaculture, it is important to demonstrate good survival of the bacteria in products during their storage (Wang et al., 2008a). To be effective and confer health benefits, probiotic cultures must be able to retain their properties after processing, and in sufficient numbers of viable bacteris during shelf life/storage. However, the stability of probiotics is influenced by various factors and so special attention and techniques are needed during the process of probiotic production. Probiotic manufacturers should apply modern molecular techniques to ensure that the species of bacteria used in their products are correctly identified, for quality assurance as well as safety (Wang et al., 2008a).

1.10. Probiotics and fish immunity

Most of the earlier studies with probiotics in fish dealt with growth promoting and disease protective ability, modulating various immunohaematological parameters in teleosts (Nayak, 2010).

The main role of the innate immune system, also known as non-specific immune system, is the first line of host defense in opposing pathogenic organisms and to deal with any foreign material until the adaptive immune system is able and potent enough to take over (Sinyakov et al., 2002). This means that the cells of the innate system recognize and respond to pathogens in a generic way, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host (Alberts et al., 2002).

The adaptive immune system, also known as the specific immune system, is composed of highly specialized, systemic cells and processes that eliminate or prevent pathogenic growth (Janeway et al., 2001). The cells of the adaptive immune

system are a type of leukocyte, called lymphocyte, being B and T cells the major types (Janeway et al., 2001).

The major functions of the vertebrate innate immune system include (Alberts et al., 2002; Janeway et al., 2001): 1) Recruiting immune cells to sites of infection; 2) Activation of the complement cascade to identify bacteria, activate cells and to promote clearance of dead cells or antibody complexes; 3) The identification and removal of foreign substances present in organs, tissues, the blood and lymph, by specialized white blood cells; 4) Activation of the adaptive immune system through a process known as antigen presentation; 5) Acting as a physical and chemical barrier to infectious agents.

The innate immune system is separated into two branches, the humoral immunity, for which the protective function of immunization is observed in the humor (cell-free bodily fluid or serum) and cellular immunity, for which the protective function of immunization was associated within the cells (Janeway et al., 2001). Some parameters allow us to evaluate the immune response, like the humoral activity of some enzymes (lysozyme and peroxidase) and the system of complement activity related degradation of pathogens by lysis or the cellular production of antibacterial components by the macrophages (respiratory burst activity and the nitric oxide production) (Nayak, 2010).

Lysozyme is an important bactericidal enzyme in the innate immunity and an indispensable tool in the fight against infectious agents (Lindsay, 1986). Some studies (Balcázar et al., 2006; Kim and Austin, 2006a; Panigrahi et al., 2004) show that probiotics, individually or in combination, affect the level of lysozyme in teleost fish. On the contrary, in other studies, dietary supplementation of probiotics in *S. trutta* (Balcázar et al., 2007a) or in *O. mykiss* (Balcázar et al., 2007b; Panigrahi et al., 2005) as well as in water supplementation in *O. niloticus* (Zhou et al., 2010) failed to elevate lysozyme levels.

Peroxidase uses oxygen radicals to produce hypochlorous acid which kills the pathogens, and it is mostly released by the azurophilic granules of neutrophils, during oxidative respiratory burst (Nayak, 2010). Certain probiotics can successfully elevate this activity in fish (Brunt et al., 2007; Sharifuzzaman and Austin, 2009), but in Salinas et al. (2008), probiotic formulation failed to enhance the peroxidase activity of head kidney leucocytes of *S. aurata*.

The complement system is a biochemical cascade of more than 35 soluble and cell-bound proteins, 12 of which are directly involved in the complement pathways related to degradation and phagocytosis of pathogens by lysis (Janeway et al., 2001). Three biochemical pathways activate the complement system of teleost fish: a) the classical pathway, b) the alternative pathway; and c) the lectin pathway. All three pathways converge to the lytic pathway, leading to opsonisation or direct killing of microorganisms (Holland and Lambris, 2002). Basically, the classical complement pathway typically requires antibodies for activation and is a specific immune response, while the alternate pathway can be activated without the presence of antibodies (Janeway et al., 2001). Many studies show that probiotics, administered in the diet or the surrounding water, can improve the activity of natural complement of the fish (Panigrahi et al., 2005; Panigrahi et al., 2007; Salinas et al., 2008). It is also worth noting that non-viable probiotics can stimulate complement components in fish, as observed by Choi and Yoon (2008).

Respiratory burst activity is an important innate defense mechanism of fish. The findings of respiratory burst activity following probiotic treatment in fish are often contradictory. While some studies indicate probiotics do not have significant impact on this non-specific defense mechanism of fish (Díaz-Rosales et al., 2009; Sharifuzzaman and Austin, 2009), several other studies showed significant increase in respiratory burst activity by various probiotics in fish (Nikoskelainen et al., 2003; Salinas et al., 2005; Salinas et al., 2006; Zhou et al., 2010).

The production of nitric oxide (NO) is known to play an integral part in the regulation of the immune system. In fish, macrophage NO production by iNOS (inducible NO synthases), plays an important role in the cellular defense mechanisms against some viral, parasitic and bacterial infections, and it has been demonstrated in stimulated macrophages in several fish species (Buentello and Gatlin III, 1999; Neumann et al., 1995; Tafalla and Novoa, 2000). In teleosts fish, head-kidney is an important haematopoietic organ, serving as a secondary lymphoid organ in the induction and elaboration of immune responses. Furthermore, the head-kidney is also the major site for antibody production and melanomacrophage accumulations (Ronneseth et al., 2007).

1.11. Probiotics effect on intestinal and liver morphology in fish

Health maintenance in aquaculture is a concept in which fish should be reared under conditions that optimize the growth rate, feed conversion efficiency, and survival while minimizing problems related to infectious, nutritional, and environmental diseases (Plumb and Hanson, 2010). The overall effect of all these factors and therefore the health condition of farmed fish can be unraveled by histological analysis.

Cytoplasm alterations in fish hepatocytes are a very early and unspecific signal of disturbance of hepatocellular homeostasis being difficult to establish a threshold for what should be considered a fish farm healthy liver (Braunbeck, 1998). Hypertrophy, vacuolar degeneration and increase of lipid droplets in hepatocytes of fish are some of parameters normally analyzed to predict liver health and integrity. In farmed fish it is known that commercial feed causes lipid droplet accumulation, hepatic cell membrane degeneration, and hepatocyte vacuolization and can cause circulatory disturbances (Bilen and Bilen, 2013; Coz-Rakovac et al., 2002). However, these histological changes are not considered pathological if they did not cause extensive hepatic necrosis (Saraiva et al., 2015).

The gastrointestinal tract is seen generally as an organ of digestion/ absorption of nutrients, but nowadays great interest revolves around its role as a physical and immunological barrier and as an organ involved in the immunity, since it is accepted that digestion and immunity are complicated physiological processes that have coevolved (Van Loo, 2007).

The distal intestine is the principal site for the endocytosis of proteins (Rombout et al., 1985) and normally used to evaluate potential negative effect of diet on intestinal histology. Integrity of intestine is assumed to be a key factor for the growth and welfare of farmed fish. Some of the quantitative or semi-quantitative parameters normally used to do the intestinal morphometric study, comply the detection of changes in the epithelial integrity, presence of cell debris in the lumen, disintegrated tight junctions, villus height, presence of goblet cells, leucocytes infiltrations, microvilli disorganization/disruption, and oedema (Bakke-McKellep et al., 2007; Caballero et al., 2003; Cerezuela et al., 2013; Dimitroglou et al., 2010; Pirarat et al., 2011; Rombout et al., 2011; Sáenz de Rodrigáñez et al., 2009).

The increased use of plant feedstuffs in farmed fish diets can affect the gut integrity and increase the deleterious effect of gut pathogens (Couto et al., 2014; Mourente

et al., 2007; Oliva-Teles, 2012; Urán, 2008). Some tissue damage in fish has been related to the use of certain plant protein sources such as soybean or vegetable oils, and was reverted by probiotic administration (Caballero et al., 2003; Sáenz de Rodrigáñez et al., 2009).

1.12. Probiotics and gut microbiota in fish

Given the significant role of microbiota in the homeostasis of numerous physiologic processes, it is not surprising that an imbalance of microbiota has been implicated in many disease states. Consequently, it is necessary to develop new techniques that could reestablish a sustained balance in disrupted microbiota. For example, a new successful technique showing great promise for treating many diseases in humans, is the fecal microbiota transplantation, an innovative attempt to restore the disturbed microbiota, by infusion of fecal suspension from a healthy individual (Woo Jung et al., 2015).

The intestinal microbiota is an ecosystem formed by a variety of ecological niches, made of several bacterial species and a very large amount of strains (Aureli et al., 2011). Both microbiota and mucosa, along with mucus, form the mucosal barrier against potentially pathogenic factors present in the lumen (Aureli et al., 2011). The microbial community in the fish gut is strongly influenced by the environment and within this complex ecosystem the microbes compete for space and nutrients for their survival (Rombout et al., 2011). For a better appreciation of how the microbial ecology of the gut influences the organism health, it is important to understand how bacteria interact with each other within the gut to influence the dynamics of colonisation and their subsequent activities (Swift et al., 2000). Quorum sensing is one of the main forms of communication between bacteria, a two-component term in which quorum means “threshold” and sensing means “feel” (Moghaddam et al., 2014). The QS is used by bacteria populations to communicate and coordinate their group interactions, which is typically applied by pathogens in infection processes (Moghaddam et al., 2014). The evidence so far accumulated suggests that population cell density and cell-to-cell communication will be an important factor in the regulation of microbial activity within the high cell density bacterial population of the gut (Swift et al., 2000).

Over the years various strategies to modulate the composition of the gut microbiota for better growth, digestion, immunity, and disease resistance of the host have been

investigated. The gut is the organ where probiotics get established and also execute their functions including immunostimulatory activity (Nayak, 2010). So, there is an important cross-talk between probiotics, epithelial cells and gut immune system. Interactions between the endogenous gut microbiota and the fish host are integral in mediating the development, maintenance and effective functionality of the intestinal mucosa and gut associated lymphoid tissues (Dimitroglou et al., 2011). These microbial populations also provide a level of protection against pathogenic visitors to the gastrointestinal tract and aid host digestive function via the production of exogenous digestive enzymes and vitamins (Dimitroglou et al., 2011). Manipulation of these endogenous populations may provide an alternative method to antibiotics to control disease and promote health management (Dimitroglou et al., 2011). The innate immune system protects the host by maintaining the integrity of the intestinal barrier, using the pathogen recognition receptors (Rombout et al., 2011). The knowledge of the sole intestinal microflora is very scarce but *Vibrio* is regarded as the dominant genus (Tapia-Paniagua et al., 2010).

1.13. Probiotics and disease protection in fish

Probiotic therapy offers a possible alternative for controlling pathogens thereby overcoming the adverse consequences of antibiotics and chemotherapeutic agents. The effectiveness of probiotics in terms of protection against infectious pathogens is often attributed to elevated immunity. Protection against several diseases is successfully accomplished through probiotics feeding (Irianto and Austin, 2003; Kim and Austin, 2006b; Nikoskelainen et al., 2001a; Nikoskelainen et al., 2003; Raida et al., 2003; Sharifuzzaman and Austin, 2009; Taoka et al., 2006b)

1.14. The use of immunostimulants in fish

Immunostimulants are substances (drugs and nutrients) that stimulate the immune system by activating or increasing activity of any of its components. Immunostimulants can be grouped as chemical agents, bacterial preparations, polysaccharides, animal or plant extracts, nutritional factors and cytokines (Sakai, 1999).

Some immunostimulants are proved to facilitate the function of phagocytic cells (Jørgensen et al., 1993; Sakai et al., 1993) and increase their bactericidal activities (Grayson et al., 1987). Li and Lovell (1985) and Hardie et al. (1991) reported that

fish given large amounts of vitamin C had increased levels of complement activity. Atlantic salmon injected with yeast glucan also showed increased complement activity (Engstad et al., 1992) and lysozyme activity was also influenced by the administration of immunostimulants (Engstad et al., 1992; Jørgensen et al., 1993).
The most effective method of administration of immunostimulants to fish is by injection. However oral administration is non-stressful and allows mass administration regardless of fish size but its efficacy decreases with long-term administration (Sakai, 1999). Anderson (1992) proposed that immunostimulants should be applied before the outbreak of disease to reduce disease-related losses. The effects of immunostimulants are not directly dose-dependent, and high doses may not enhance and may inhibit the immune responses. For the effective use of immunostimulants, the timing, dosages, method of administration and the physiological condition of fish need to be taken into consideration (Sakai, 1999).

1.15. Objectives

The aim of this current PhD study was to evaluate the effects of dietary probiotic supplementation in juvenile sole (*Solea senegalensis*) with emphasis on growth performance, host defence and intestinal morphology and microbiota.
The chapters proposed in this thesis were designed to evaluate:

- The beneficial effects of probiotics on growth performance, innate immune response and gut morphology in Senegalese sole (**Chapter 2**);
- The protective effects of probiotics and other immunostimulant raw materials on high dietary plant ingredients supplementation (**Chapter 3** and **Chapter 4**);
- The use of autochthonous bacteria from sole intestine with probiotic effects against bacterial pathogens (**Chapter 5**).

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CHAPTER 2

Immune responses and gut morphology of Senegalese sole (*Solea senegalensis*, Kaup 1858) fed monospecies and multispecies probiotics

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Immune responses and gut morphology of Senegalese sole (*Solea senegalensis*, Kaup 1858) fed monospecies and multispecies probiotics

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Abstract

The current study aimed to determine the effects of dietary probiotic supplementation on growth, gut morphology and non-specific immune parameters in Senegalese sole (*Solea senegalensis*) juveniles during a 1-month trial. Fish were fed for 1 month two diets with 1.0 or 4.6 × 10^9 CFU kg^{-1}) of probiotic A (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp. and *Lactobacillus* sp.) and two diets with 3.5 or 8.6 × 10^8 CFU kg^{-1} of probiotic B (*Pediococcus acidilactici*), and tested against an unsupplemented diet (control). Growth performance, as well as respiratory burst activity, nitric oxide (NO), alternative complement pathway (ACH50), lysozyme and peroxidase activities were not affected by the dietary treatments. Probiotic supplementation tended to increased growth homogeneity between tanks having diet A_1 the best possible alternative to decrease costs associated to size grading. Villus length and number of goblet cells of the anterior intestine did not vary among treatments. Muscular duodenal layer was significantly thicker in fish fed probiotic A compared to probiotic B, when included at the lowest level (A_2 vs B_2). The current study indicates that the use of the multispecie probiotic at 1.0 × 10^9 CFU kg^{-1} might enhance protection against pathogen outbreak and increase nutrient absorption, whereas at the highest concentration could reduced size dispersion among tanks.

2.1. Introduction

Senegalese sole (*Solea senegalensis*) is a high-value flatfish that presents a great potential for future farming at commercial scale in Mediterranean countries. The major concern to the aquaculture industry is the eradication of disease outbreaks, although, growth performance from juvenile to market-size is still not fully optimized (Arijo et al., 2005a). In fish farms, bacterial diseases outbreak is a constant problem in aquaculture and the control of bacterial diseases is achieved by the administration of chemotherapeutic agents. However, the increasing prevalence of drug-resistant bacteria poses a significant threat to aquaculture sector, public health and to environment. The use of probiotics as prophylaxis may emerge as an alternative to antibiotic treatment, but is controversial how effective is dietary probiotic supplementation to enhance fish protection.

The functional integrity of the epithelial cells of gut mucosa depends on a coordinated action involving mucus layers, intestinal epithelial cells, microbiota, and host immune system (Merrifield et al., 2010c). Some studies demonstrated the importance of the complex microbe-host interactions on gut well-being. Rawls et al. (2007) observed in zebrafish (*Danio rerio*) a strong influence of endogenous microbiota on the gut integrity and development. Moreover, they were successful to correlate microbiota as a key element in the regulation of mucosal tolerance, development and differentiation.

According to FAO (2001), probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". The application of probiotic bacteria in the prevention of fish diseases has received considerable attention in recent years. Díaz-Rosales et al. (2009) observed that dietary supplementation of *Shewanella putrefaciens* (Pdp11) and *Shewanella baltica* (PdP 13) were effective to improve growth and the survival of sole (*Solea senegalensis*) against pseudotuberculosis caused by *Photobacterium damselae* subsp. *piscicida*, in comparison with those fish receiving the control diet. These two bacteria belonging to *Shewanella* genus, were isolated from gilthead seabream (*Sparus aurata*) skin (Salinas et al., 2006).

The suitability of bacteria isolates for probiotic use is evaluated according to some functional attributes (Bairagi et al., 2002; Gomez and Balcázar, 2008; Gram et al., 1999; Kim and Austin, 2006a; Merrifield et al., 2010c; Sáenz de Rodrigáñez et al., 2009; Sugita et al., 1996; Vine et al., 2004). The candidate bacteria should be non-

pathogenic and free of antibiotic resistance genes. They should survive through the digestive tract, competing for adhesion sites, growing and colonising the intestinal surface. Ideally, they should be indigenous to the host or the rearing system and exhibit antagonistic properties towards one or more key pathogens. From the industry viewpoint, probiotics should be viable during storage conditions and industrial processes. Recent studies showed activation of the immune response and haematological traits of rainbow trout, *Oncorhynchus mykiss* (Kim and Austin, 2006a; Merrifield et al., 2010d; Nikoskelainen et al., 2003), Senegalese sole, *Solea senegalensis* (Díaz-Rosales et al., 2009; Díaz-Rosales et al., 2006a; Sáenz de Rodrigáñez et al., 2009) and seabream, *Sparus aurata* (Salinas et al., 2006) fed with dietary probiotic supplementation.

The *Pediococcus acidilactici* is currently the only bacteria authorized in aquaculture by the European Commission Regulation (EC) nº 911/2009 as a feed additive for salmonids and shrimps. Nevertheless, multispecies probiotics may work synergistically for greater benefits for fish health (Timmerman et al., 2004).

The aim of this study was to evaluate the effects of dietary supplementation of commercially available multispecies (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.) and monospecies (*Pediococcus acidilactici*) probiotics on the innate immune response and gut morphology of Senegalese sole.

2.2. Materials and methods

2.2.1. Fish

One hundred and sixty-five juvenile soles (mean initial bodyweight: 82.70 ± 3.25 g) were obtained from a commercial fish farm (Aquacria S.A., Portugal). Fish were transported to the rearing facilities of University of Trás-os-Montes e Alto Douro (UTAD - Vila Real, Portugal) and maintained for a 5-week period in quarantine/acclimation. The five dietary treatments were randomly assigned to triplicate 50 L fibreglass tanks (11 fish per tank), supplied with filtered and heated closed recirculation seawater (2 L min^{-1}) system. Temperature (17.2 ± 0.5 ^{o}C), dissolved oxygen (9 ± 0.4 mg L^{-1}), salinity (23.8 ± 0.5 ppm), pH (7.6 ± 0.3), NH_4^+ (0.10 ± 0.07 mg L^{-1}), NO_2^- (0.63 ± 0.25 mg L^{-1}) and photoperiod (12 h light : 12 h darkness) were periodically monitored during the entire trial. Fish were hand-fed *ad libitum* for 1 month, three times per day. The current study was conducted under the

supervision of an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations), according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

2.2.2. Diet formulation and composition

The experimental diets were formulated to be nutritionally identical (540 g kg^{-1} crude protein, 178 g kg^{-1} crude lipid, 22 kJ g^{-1} gross energy, dry-matter basis, Table 1).

Table 1 - Feed ingredients and proximate composition of the experimental basal diet

Feed ingredients	**Basal diet (g kg^{-1})**
Fishmeal (700*g kg^{-1}* crude protein)	185
Fishmeal (600 *g kg^{-1}* crude protein)	250
CPSP[1]	25
Squid meal	25
Soybean meal (micronized)	50
Soycomil PC	62
Pea (Lysamine GP)	100
Wheat meal	73
Corn gluten	50
Fish oil	93
Aquatex	50
Di-calcium phosphate	15
Binder (kilseghur)	10
Vit[2] and Min Premix[3]	10
Proximate composition	
Crude protein (*g kg^{-1}* DM)	548.1
Crude fat (*g kg^{-1}* DM)	178.0
Gross Energy (kJ g^{-1} DM)	21.81

DM, dry matter; [1] Soluble fish protein hydrolysate (75 g kg^{-1} crude protein, Sopropêche, France); [2] Vitamins (mg or IU kg^{-1}diet): Vitamin A (retinyl acetate), 20000 IU; vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1 (thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B12 (cyanocobalamin), 0.1 mg; vitamin B5 (pantothenic acid), 100 mg; vitamin B3 (nicotinic acid), 200 mg; vitamin B9 (folic acid), 15 mg; vitamin H (biotin), 3mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1000 mg; vitamin C (stay C), 1000 mg; vitamin E, 100 mg; [3] Minerals (g or mg kg^{-1}diet): Mn (manganese oxyde), 9.6 mg; I (potassium iodide), 0.5 mg; Cu (cupric sulfate), 9 mg; Co (cobalt sulfate), 0.65 mg; Zn (zinc oxide), 7.5 mg; Se (sodium selenite), 0.01 mg; Fe (iron sulfate), 6 mg; Cl (sodium chloride), 2.41g; Ca (calcium carbonate), 18,6g; NaCl (sodium), 4g.

The dietary ingredients were mixed without fish oil and extruded (3 mm granules) with a pilot-scale twin-screw extruder (Clextral BC45, St. Etienne, Firminy, France). Thereafter, probiotics were blended in the fish oil and vacuum coated (Dinnisen Pegasus vacuum mixer, PG-10VCLAB, Horsterweg, Sevenum, the Netherlands) to the extruded diets. The basal diet (control) was supplemented with commercial probiotic A [A_1, 4.6×10^9 CFU kg^{-1}; A_2, 1.0×10^9 CFU kg^{-1} diet, colony-forming unit

(CFU)] or with commercial probiotic B (B_1, 8.6×10^8 CFU kg^{-1}; B_2, 3.5×10^8 CFU kg^{-1} diet). Probiotic A is a blend of probiotic bacteria (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp. and *Lactobacillus* sp.) and probiotic B is a live concentrate of lactic acid bacteria, *Pediococcus acidilactici*. Diets were prepared according to the ISO 6887-1:1999, ISO-7218:2007 and ISO-6498:2012 normatives, for sample preparation in microbiology of food and animal feeding stuffs. Thereafter, the isolation and enumeration of bacteria in the diets followed by the BS EN 15788:2009 (*Enterococcus* spp.), BS EN 15787:2009 (*Lactobacillus* spp.), BS EN 15786:2009 (*Pediococcus* spp.) and BS EN 15784:2009 (*Bacillus* spp.).

2.2.3. Sampling Procedures

During sampling, all fish were quickly netted from each tank and anaesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222, 200 mg L^{-1}, Sigma, Portugal). At the beginning and end of the trial all fish were individually weighed and measured. Two fishes from each tank were collected and stored at −20 °C for subsequent whole-body composition analysis. Liver and viscera were weighted for the calculation of the hepatosomatic (HSI) and viscerosomatic (VSI) indexes. For histological evaluation, the anterior intestinal tract of six fish from each treatment was collected and fixed in phosphate buffered formalin 4 (v v^{-1}), pH 7 (VWR, Carnaxide, Portugal) for 24 hours. The samples were subsequently transversally sectioned, dehydrated and embedded in paraffin according to standard histological procedures. Thereafter, three micra sections were made and stained with haematoxylin and eosin (H&E, Merck, Algés, Portugal) and Periodic acid-Schiff (PAS, Merck, Algés, Portugal) and examined under a light microscope (cell^B software, Olympus BX51, GmbH, Hamburg, Germany). The length of ten selected *villi* was measured in three intestinal sections of each animal, from the submucosa to the top of the enterocytes, according description of Pirarat et al. (2011). Three different gut sections of each animal were used to count the goblets cells (mucus-producing cells) positive to PAS. The results were expressed in average number of goblet cells per section according description of Pirarat et al. (2011). The muscular layer thickness, of intestinal wall, measured from serosa to submucosa was determined from three sections of each animal (Fig. 1). For the evaluation of the innate immune response, plasma and head-kidney were used for the measurements of the humoral and cellular parameters. Blood was withdrawn with heparinized

syringes from the caudal vein (six fish per treatment), centrifuged (5.000 g for 10 min at 4ºC), and the resulting plasma was stored at -80ºC for further analysis. Head-kidney was aseptically dissected under ice-cold condition from the same animals.

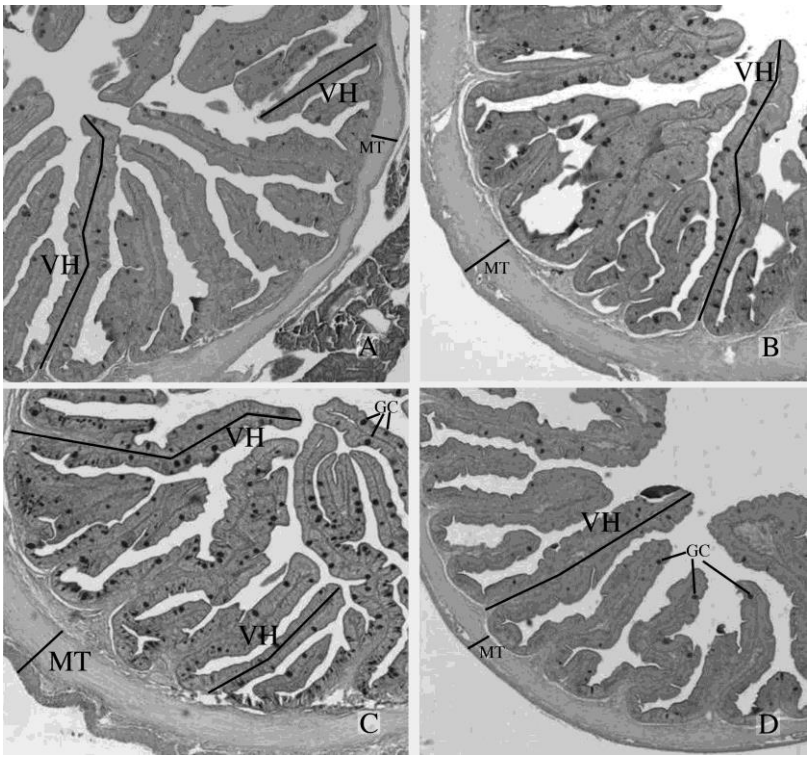

Figure 1 - Histological section (A-D: PAS stained, obj 4×) of anterior intestine of Senegalese sole and the different parameters measured. Villus height (VH), Goblet cells (GC) and Muscular thickness (MT). Notes - A: control diet; B: diet A_1; C: diet A_2; D: diet B_1. Note the difference observed in muscular layer between diet A_2 and diet B_1 and control. The number of GC is notably different between the figure C and the others.

2.2.4. Chemical analyses of diets and body composition

All chemical analyses were carried in duplicate according to AOAC (2006). Fish sampled from each tank were pooled and minced using a meat mincer, and moisture content was determined before freeze-drying. Diets and freeze dried fish samples were analysed for dry matter (105 °C for 24 h), ash (Nabertherm L9/11/B170, Bremen, Germany, 550ºC for 6 h), crude protein (N × 6.25, Leco N analyser, Model FP-528, Leco Corporation, St. Joseph, MI, USA), crude lipid (petroleum ether extraction, 40 - 60ºC, Soxtherm, Gerhardt, Germany), total P (molybdate-blue/ascorbic acid method at 820 nm after mineralization and acid digestion)

according to AFNOR (1992) and gross energy (adiabatic bomb calorimeter, Werke C2000, IKA, Staufen, Germany).

2.2.5. Innate Immune parameters

All analyses were conducted in triplicates on a Power-Wave^{TM} microplate spectrophotometer (BioTek Synergy HT, Vermont, USA).

2.2.5.1. Cellular parameters

Leucocytes from head-kidney were isolated and maintained as described by Secombes (1990) and followed by Costas et al. (2011). Respiratory burst activity (ROS) was based on the reduction of ferricytochrome C (Cyt C) method for the detection of extracelular superoxide anion (O_2^-) production. Nitric oxide (NO) production was based on the Griess reaction (Green et al., 1982) that quantifies the nitrite content of the leucocytes supernatant (Neumann et al., 1995), modified by (Tafalla and Novoa, 2000). The molar concentration of nitrite was quantified using a standard curve produced with known concentrations of sodium nitrite (1.562 µM - 100 µM).

2.2.5.2. Humoral parameters

Lysozyme activity was determined using a turbidimetric assay based on the method described by (Ellis, 1990) with minor modifications (Costas et al., 2011; Wu et al., 2007). Total peroxidase activity was measured following the procedure described by Quade and Roth (1997) and Costas et al. (2011). The peroxidase activity (units mL^{-1} plasma) was determined by defining that one unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway (ACH50) was measured using washed rabbit red blood cells (2.8×10^8 cells mL-1, Probiológica, Belas, Portugal) as target cells in the presence of ethylene glycol tetraacetic acid (EGTA, Sigma, Portugal) and Mg^{2+} ($MgCl_2.6H_2O$, VWR, Portugal) as described by Sunyer and Tort (1995).

2.2.6. Growth Performance and Nutrient Retention

Feed conversion ratio (FCR) was calculated as dry feed intake (g) × wet weight $gain^{-1}$ (g) and the daily growth index (DGI; g kg^{-1} BW day^{-1}) as DGI = 100 × $[(W1)^{1/3} - (W0)^{1/3}]$/ trial duration in days, where W_0 and W_1 are the initial and the final fish

mean weights in grams. Voluntary feed intake (VFI; g kg^{-1} BW day^{-1}) was calculated as VFI = (dry feed intake /ABW/ trial duration), where average body weight (ABW) was calculated as $(W_1+W_0)/2$. The protein efficiency ratio (PER) was calculated as PER = weight gain (g) /protein ingested (g). Daily nutrient intake (g kg^{-1} ABW day^{-1}) was calculated as nutrient intake/ABW/trial duration. Daily nutrient gain, (g kg^{-1} ABW day^{-1}) was calculated as (final body nutrient content − initial body nutrient content)/ ABW/ trial duration. Nutrient retention (g kg^{-1} intake) was calculated as (nutrient W_1 – nutrient W_0)/ nutrient intake. The HSI and VSI indexes (g kg^{-1}, wet weight basis) were calculated as HSI = liver weight/ whole body weight, and VSI = 100 × viscera weight / whole body weight.

2.2.7. Statistical analysis

Statistical analyses were carried out following the methods outlined by Zar (1999). Results are expressed as mean ± standard deviation and with a $P \leq 0.05$ as level of significance. Data were analysed for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) and were log-transformed whenever necessary. Data were analysed by a one-way ANOVA (IBM SPSS STATISTICS, 17.0 package, IBM Corporation, New York, USA) with probiotic treatment and respective concentration level as a dependent variable. When significant differences were observed, Tukey's *post hoc* tests were carried out to identify significantly different groups fed the experimental diets. When data did not meet the assumptions of ANOVA, the non-parametric ANOVA equivalent (Kruskal–Wallis test) was performed.

2.3. Results

2.3.1. Growth and body composition

No measurable effects were observed on growth performance among treatments (Table 2). Overall, size variation was lower in the dietary probiotic groups. The coefficient of variation as a function of daily growth index (DGI) and weight gain varied between 0.06 – 0.07 in A_1, 0.13 – 0.15 in A_2 and 0.11 – 0.13 in B_1 groups and 0.24 in fish fed unsupplemented diet had, suggesting higher growth homogeneity in fish fed probiotic diets (data not shown).

Hepatosomatic index varied from 12.1 ± 1.0 to 13.1 ± 4.4 g kg^{-1} and viscerosomatic index varied from 15.4 ± 1.7 to 19.1 ± 0.6 g kg^{-1} (Table 3). Both indexes were not

affected by dietary treatments. Whole body composition, dietary dry matter and protein retentions (Table 3) were not significantly affected (P>0.05) by probiotic supplementation, except ash, which was significantly lower in fish fed B_1 than in control fish (P<0.05).

2.3.2. Gut histology

Histological measurements of the intestinal mucosa of fish are present in Table 4. No significant differences were observed among treatments (P < 0.05) for villus height (µm). Thickness of muscular layer (µm) showed significantly differences (P < 0.05) between fish fed A_2 (163 ± 32) and B_2 (115 ± 14) probiotic diets. Goblet cells counting did not vary among dietary treatments.

2.3.3. Innate immune parameters

Plasma lysozyme and peroxidase activities, expressed as Enzyme Unit (EU) mL^{-1} plasma, were not affected (P < 0.05) by the probiotic supplementation. Lysozyme ranged from 160 (Control) to 500 EU mL^{-1} plasma (B_2) and peroxidase from 88 (B_2) to 150 EU mL^{-1} plasma (A_2). Alternative complement pathway activity (ACH50) varied between 35 (Control) and 63 (B_2), and was not significantly different (P < 0.05) between treatments. ROS and NO production showed no significant differences among treatments (P < 0.05, Table 5). ROS (nmoles de O_2^-) range from 4.9 (A_2) to 7.4 (B_1) and NO (concentration of nitrites, µM) range from 6.41 (A_1) to 6.46 (B_2).

Table 2 - Growth performance of Senegalese sole after 1 month of feeding the dietary treatments.

	Dietary treatments					
	Control	**A_1**	**A_2**	**B_1**	**B_2**
Growth					
IBW (g)	80.9 ± 4.53	80.8 ± 0.25	84.8 ± 1.29	83.5 ± 3.55	82.0 ± 5.03
FBW (g)	100 ± 5.47	104 ± 1.95	99.1 ± 3.49	99.8 ± 5.04	97.2 ± 9.05
DGI (g kg^{-1} BW day^{-1})	11.0 ± 2.7	13.2 ± 0.8	8.1 ± 1.1	9.2 ± 1.0	8.2 ± 1.9
FCR (g g^{-1})	1.11 ± 0.28	0.87 ± 0.02	1.37 ± 0.22	1.23 ± 0.26	1.45 ± 0.38
VFI (g kg^{-1} BW day^{-1})	7.8 ± 0.8	7.5 ± 0.3	7.3 ± 0.1	7.4 ± 0.8	7.7 ± 0.5
PER (g g^{-1})	1.69 ± 0.44	2.10 ± 0.05	1.35 ± 0.19	1.53 ± 0.30	1.33 ± 0.35
Intake (g kg^{-1} ABW day^{-1})					
Dry matter	7.85 ± 0.78	7.54 ± 0.28	7.25 ± 0.12	7.40 ± 0.80	7.67 ± 0.45
Protein	4.40 ± 0.46	4.15 ± 0.21	3.93 ± 0.06	4.07 ± 0.45	4.10 ± 0.28

Values represent mean ± standard deviation. IBW, initial body weight; FBW, final body weight; DGI, daily growth index; FCR, feed conversion ratio; VFI, voluntary feed intake; PER, protein efficiency ratio; ABW, average body weight.

Table 3 - Whole body composition and nutrient utilization of Senegalese sole after 1 month of feeding the dietary treatments.

	Dietary treatments														
	Control			**A_1**			**A_2**			**B_1**			**B_2**		
Somatic indexes (g Kg^{-1})															
HSI	13.0	±	2.1	13.1	±	4.4	13.3	±	1.2	12.1	±	1.0	12.5	±	1.3
VSI	19.1	±	0.6	17.5	±	1.8	18.6	±	1.3	18.9	±	2.7	15.4	±	1.7
Whole body composition															
Dry matter *(g Kg^{-1})*	266.5	±	9.1	262.6	±	6.6	261.9	±	14.8	265.6	±	6.0	277.3	±	12.3
Ash *(g Kg^{-1})*	25.9	±	1.4^{a}	23.8	±	1.5^{ab}	22.9	±	2.3^{ab}	20.8	±	0.8^{b}	23.9	±	0.6^{ab}
Protein *(g Kg^{-1})*	181.4	±	8.8	184.0	±	4.4	180.0	±	4.4	180.5	±	3.4	184.2	±	3.7
Lipid *(g Kg^{-1})*	57.6	±	4.8	54.70	±	3.4	60.4	±	14.5	70.4	±	2.9	73.9	±	9.5
Energy (kJ)	62.1	±	2.6	62.16	±	3.5	61.9	±	5.0	63.7	±	2.4	66.8	±	4.5
Gain (g kg^{-1} ABW day^{-1})															
Dry matter	1.31	±	0.80	1.53	±	0.1	0.57	±	0.45	0.92	±	0.32	1.17	±	0.77
Protein	0.92	±	0.65	1.27	±	0.1	0.48	±	0.09	0.64	±	0.16	0.66	±	0.35
Retention (g Kg^{-1} intake)															
Dry matter	169.9	±	105.8	203.0	±	22.2	78.5	±	61.9	127.9	±	57.9	155.9	±	109.8
Protein	213.8	±	152.3	306.6	±	28.3	122.3	±	23.0	163.3	±	58.7	162.0	±	94.8

Values represent mean ± standard deviation. In each line, different superscript letters indicate significant differences between treatments ($P< 0.05$). ABW, average body weight; HIS, hepatosomatic index; VSI, viscerosomatic index.

Table 4 - Intestinal morphology and goblet cells counting of Senegalese sole after 1 month of feeding the dietary treatments.

	Dietary treatments					
	Control	A_1	A_2	B_1	B_2
Villus height (µm)	729 ± 102	741 ± 53	712 ± 93	724 ± 91	652 ± 154
Muscular layer thickness (µm)	144 ± 12^{ab}	146 ± 22^{ab}	163 ± 32^{a}	141 ± 21^{ab}	115 ± 14^{b}
Goblet cells (number per section)	287 ± 173	298 ± 191	288 ± 169	235 ± 122	394 ± 155

Values represent mean ± standard deviation. In each line, different superscript letters indicate significant differences between treatments ($P < 0.05$).

Table 5 - Effects on humoral and celular non-specific immune parameters of Senegalese sole after 1 month of feeding the dietary treatments.

	Dietary treatments				
	Control	A_1	A_2	B_1	B_2
Humoral parameters					
Lysozyme (EU mL^{-1})	159.7 ± 109.5	298.6 ± 375.5	261.1 ± 250.7	302.8 ± 94.5	500.0 ± 270.9
Peroxidase (EU mL^{-1})	137.29 ± 30.3	124.0 ± 59.0	149.8 ± 27.0	102.8 ± 26.7	87.8 ± 12.7
ACH50 (units mL^{-1})	35.1 ± 14.6	40.6 ± 10.4	44.0 ± 4.6	49.5 ± 23.9	63.2 ± 55.7
Celular parameters					
Respiratory burst activity (nmoles O_2^{-})	6.63 ± 3.31	6.42 ± 2.82	4.85 ± 3.59	7.35 ± 2.37	4.62 ± 3.96
Nitric oxide production (nitrite µM)	6.44 ± 0.02	6.41 ± 0.10	6.45 ± 0.02	6.45 ± 0.02	6.46 ± 0.03

Values are means ± standard deviation.

2.4. Discussion

The probiotics choice was based on their effects on fish health and growth in aquaculture (Table 6). Some of these bacteria are known to present ability of spore-forming, allowing greater viability after pelleting and high resistance to gastric conditions (Hong et al., 2005).

Table 6 - Different applications of *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp. and *Pediococcus acidilactici* in aquaculture.

Aplication	Probiotic bacteria	Aquatic specie	Reference
Growth promoter	*Bacillus* sp	Catfish	Queiroz and Boyd (1998)
	Bacillus coagulans	*Cyprinus carpio koi*	Lin et al. (2012)
	Lactobacillus helveticus	*Scophthalmus maximus*	Gatesoupe (1999)
	Lactobacillus lactis AR21	*Brachionus plicatilis*	Shiri Harzevili et al. (1998)
	L. casei	*Poeciliopsis gracilis*	Hernandez et al. (2010)
	Pediococcus acidilactici	*Pollachius pollachius*	Gatesoupe (2002)
	Pediococcus acidilactici	*Oncorhynchus mykiss*	Merrifield et al. (2011)
Pathogen inhibition	*Bacillus* sp	*Penaeids*	Moriarty (1998)
	Bacillus spp, *Enterococcus* sp	*Farfantepenaeus brasiliensis*	Souza et al. (2012)
	Enterococcus faecium SF 68	*Anguilla anguilla*	Chang and Liu (2002)
	L. rhamnosus ATCC53103	*Oncorhynchus mykiss*	Nikoskelainen et al. (2001a)
	Lactobacillus acidophilus	*Clarias gariepinus*	Al-Dohail et al. (2011)
	Lactococcus lactis	*Epinephelus coioides*	Sun et al. (2012)
Adhesion to intestinal mucosa	*E. faecium*, *Bacillus* spp, *P. acidilactici*	*Oncorhynchus mykiss*	Merrifield et al. (2010b)
	Pediococcus acidilactici	*Oncorhynchus mykiss*	Merrifield et al. (2011)
Nutrient digestibility	*L. helveticus*	*Scophthalmus maximus*	Gatesoupe (1999)
	Lactobacillus acidophilus	*Clarias gariepinus*	Al-Dohail et al. (2009)
Stress tolerance and Health	*Lactobacillus delbrueckii*	*Dicentrarchus labrax*	Carnevali et al. (2006)
	B. subtilis, L. acidophilus, S. cerevisiae	*Paralichthys olivaceus*	Taoka et al. (2006a)
	L. casei	*Poecilopsis gracilis*	Hernandez et al. (2010)
	Pediococcus acidilactici	*Litopenaeus stylirostris*	Castex et al. (2009)
	Pediococcus acidilactici	*Oncorhynchus mykiss*	Merrifield et al. (2011)

There is little information about the most effective dose and supplementation duration time for the probiotics tested in this study. However, previous findings showed that a short-term supplementation (3-6 weeks) was sufficient for their successful colonization in the gut, stimulation of immune system and protection against disease (Brunt et al., 2007; Newaj-Fyzul et al., 2007).

The beneficial effect of probiotics on the growth performance and dietary nutrient utilization has been commonly reported for difference fish species (Carnevali et al.,

2006; El-Haroun et al., 2006; Taoka et al., 2006a; Wang et al., 2008b). Díaz-Rosales et al. (2009), Sáenz de Rodrigáñez et al. (2009) and García de la Banda et al. (2012) reported an improvement in growth performance in Senegalese sole fed dietary probiotic supplementation. In the current study, fish fed the multispecies (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.) and monospecies (*P. acidilactici*) probiotics at different concentrations did not have any effect on growth performance. Nevertheless, fish fed probiotic diet A_1 presented the lowest growth variation in traits such as growth rate and weight gain, suggesting higher growth homogeneity in fish fed probiotic diets. This finding was also observed in previous studies (García de la Banda et al., 2012; Sáenz de Rodrigáñez et al., 2009; Varela et al., 2010; Wang et al., 2008b). Probiotics are seen to be effective in modulating gut microbiota and reducing health problems (Djouvinov et al., 2005). As they modulate gut microbiota and morphology, nutrient assimilation may improve and so the growth performance, which might explain a reduction in variability within groups. Merrifield et al. (2010d) showed that *P. acidilactici* significantly improve microvilli length in proximal intestine of the rainbow trout compared to the control group, but did not affect microvilli density. In our study, no significant differences were observed on villus length, but villi length increased in fish fed A_1. The increase in villi length involves an increase of epithelial surface area, which may improve absorption of available nutrients (Caspary, 1992) and ultimately improve growth performance. The dietary probiotic supplementation may interfere with gut health, by altering the height, width, and surface area of the villi and muscular layer thickness, improving the tract absorption capacity of the intestine and enhancing the animal protection against pathogen outbreak (Liu et al., 2007; Peinado et al., 2012). Tsirtsikos et al. (2012) provides evidence in broilers that mucus layer thickness increased with probiotic inclusion level. Although the changes in the muscular thickness have already been mentioned in some nutritional experiments in different species (broilers, rabbits and rats) the explanation of this phenomena it is yet poorly understood. In our study, muscular layer thickness showed significant difference between A_2 and B_2 groups. Fish fed B_2 probiotic diet had the thinnest muscular layer among treatments denoting a possible influence of the type of probiotic inclusion in the intestine morphology.

Goblet cells are specialized cells that secrete mucins, glycoprotein compounds. They are important in gut immunology binding pathogenic microorganisms and

reducing their adherence to the intestinal mucosa (Blomberg et al., 1993). The probiotic used in our experiment are foreign bacteria and the host may react by producing more mucus in order to get rid of the probiont and reducing the protective adherent to gut microbiota, however in our study, no differences were observed in the number of goblet cells.

Serum peroxidase, lysozyme and ACH50 activities, are commonly used as indicators of non-specific immune status in fish. Panigrahi et al. (2004), Kim and Austin (2006a) and Newaj-Fyzul et al. (2007) showed that dietary probiotic supplementation increased serum lysozyme activity in fish. Conversely, Balcázar et al. (2007a), Merrifield et al. (2010a) and Merrifield et al. (2010b) did not find significant effects of probiotics on serum lysozyme activity. In our study, the dietary probiotic supplementation did not affect the immune parameters. Nevertheless, fish fed dietary probiotic supplementation showed a tendency to increase lysozyme and ACH50 activities. This trend was also observed by Díaz-Rosales et al. (2009) working with Senegalese sole and Kim and Austin (2006a) in rainbow trout, where probiotics induced a slightly increase in some of the studied immune parameters. In fish, macrophage activity and NO production plays an important role in the non-specific cellular defence mechanisms (Buentello and Gatlin III, 1999; Neumann et al., 1995; Tafalla and Novoa, 2000). Thus, probiotics as immunomodulators may have a stimulatory effect, increasing the production of reactive oxygen species by macrophages in fish species (Díaz-Rosales et al., 2006a; Salinas et al., 2006). However, we cannot forget the potential influence of the probiotic supplementation duration time. Díaz-Rosales et al. (2009) noted that only the phagocytes of Senegalese sole treated with probiotic for 60 days showed a significant increase of ROS, while such effect was not detected in sole treated for 30 days. It is plausible to infer that our trial duration was not long enough to observe the full effects of dietary probiotic administration, although previous studies observed effects of probiotic supplementation applying similar trial duration of probiotic administration (Brunt et al., 2007; Newaj-Fyzul et al., 2007).

Under the current experimental conditions, the immune status did not vary significantly between control and probiotic groups. Nevertheless, probiotic supplementation tended to increased growth homogeneity, as a function of DGI and weight gain, showing diet A_1 the best possible alternative to decrease costs associated to size grading. Gut morphology did not vary for the villus height and

number of goblet cells, but the muscular layer thickness was influenced by the type of probiotic included.

2.5. Conclusions

The beneficial effects of probiotics application in Senegalese sole remain with few answers and further studies are needed, regarding doses, supplementation period and type of bacteria to use. In addition, gut microbiota needs to be better evaluated using PCR-DGGE techniques and the effect of dietary probiotic supplementation should be tested against acute stress conditions, such as nutritional, environmental, or infections by pathogen agent.

2.6. Acknowledgements

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CHAPTER 3

Changes in intestinal microbiota, immune- and stress-related transcript levels in Senegalese sole (*Solea senegalensis*) fed plant ingredients diets intercropped with probiotics or immunostimulants

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Changes in intestinal microbiota, immune- and stress-related transcript levels in Senegalese sole (*Solea senegalensis*) fed plant ingredients diets intercropped with probiotics or immunostimulants

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Abstract

Senegalese sole (*Solea senegalensis*) is a highly valued flatfish that grows well with diets containing plant ingredients but their effects on immune competence is still a matter of debate. The current study aimed to examine changes in innate immune parameters and gut microbiota in Senegalese sole fed with 35% or 72% of plant ingredients with or without probiotic or yeast supplementation. Overall, fish fed diets with 72% of plant ingredients showed lower transcript levels of key immune- and stress-related genes in distal intestine, rectum and head-kidney than the 35% diets. In particular, *hsp90b* mRNA levels in distal intestine were down-regulated by 70% and 60% with the use of high content of plant ingredients in the diet containing the multispecies probiotic and autolyzed yeast, respectively. Denaturing gradient gel electrophoresis showed lower similarity values for distal intestine than rectum. Also fish fed high content of plant ingredients displayed lower similarity values, pointing to a difference in the microbial populations between fish fed different plant ingredients content on the diet. Our data revealed that inclusion of plant ingredients was associated with differences in gene expression and a more diverse microbiota profile but without a significant effect on growth performance. Moreover, probiotic supplementation resulted in up-regulation of *hsp90b*, *gpx*, *cat* and *apoa1* transcript levels in distal intestine concomitantly with a growth rate reduction compared to non-supplemented fish.

3.1 Introduction

Significant advances have been made in alternative protein diet formulations, effectively replacing a large proportion of fishmeal in aquafeeds. Formulated commercial feeds are composed of several ingredients, mixed in various proportions to complement each other, and form a nutritionally complete compounded diet. In Europe, the commercial aquafeeds are largely dependent on soybean products and corn or wheat products (Rana et al., 2009). A number of different plant ingredients have been successfully used as fishmeal replacement in fish feed (Kaushik et al., 1995) without reducing growth performance. The ability of Senegalese sole (*Solea senegalensis*) to efficiently use diets with high levels of plant protein (PP) sources has been widely reported (Cabral et al., 2011; Silva et al., 2009; Valente et al., 2011) and is an important asset towards the intensification and commercialization of this species. Recent studies showed that sole could effectively use practical diets containing up to 75% of PP sources, without affecting feed intake, growth performance or nutrient utilization whilst preserving the nutritional fillet value (Cabral et al., 2013). Nevertheless, the extent to which high levels of plant ingredients may affect health conditions of fish, including the anti-oxidant and immune capacity remains controversial. The suppression of the innate immune response upon feeding high amounts of plant protein ingredients has been reported in rainbow trout (Burrells et al., 1999) and in gilthead seabream, *Sparus aurata* (Sitjà-Bobadilla et al., 2005). Conversely, another study using soybean in rainbow trout (Rumsey et al., 1994) showed an increase in the innate immune parameters examined. There are limited studies focused on the immune responses of the Senegalese sole, particularly using a multifactorial approach; i.e. interactions between biotic and abiotic stressors stressors (reviewed by Morais et al. (2014)).

Prebiotics and probiotics are increasingly used as preventive therapy, with high success rates in reducing the incidence of diseases and increasing fish resistance to infection (Gaggìa et al., 2010). The use of probiotics has increased in the aquaculture sector due to its success in livestock production (Fulton et al., 2002) and in human health (Gill, 2003). The probiotic genera commonly used in animal feeding are *Saccharomyces*, *Lactobacillus*, *Enterococcus* and *Bacillus* (Gaggìa et al., 2010). *Lactobacillus* are common bacteria in the gut microbiota of humans and animals, whereas their occurrence and number are host dependent. *Enterococcus* as well as *Lactobacillus* belong to the lactic acid bacteria (LAB) group and are found

naturally in food products. *Bacillus* are Gram-positive spore-forming microorganisms and are normally allochthonous microbes to the intestinal tract as a result of ingestion (Gaggìa et al., 2010).

The use of a multispecies probiotic, from one or preferentially more genera, has provided superior results over monostrain probiotic preparations (Timmerman et al., 2004). Paubert-Braquet et al. (1995) tested mono and multistrain *Lactobacillus* in mice challenged with *Salmonella typhimurium* and observed a clear protective effect of the multistrain probiotic preparation. Modulation of the innate humoral and cellular defences is one of the benefits of probiotic treatment described in fish (Nayak, 2010), since the innate immune system is the first line of host defense against pathogenic organisms (Sinyakov et al., 2002). The inter-relationship between gut mucosal epithelial cells, mucus, antimicrobial products, and organisms resident in the gut and immune cells in the mucosa/sub-mucosa are vital for the health and well-being of the fish (Merrifield, et al., 2010b). Several studies have demonstrated the immunological and haematological stimulation of fish defence mechanisms by probiotic bacteria (Brunt et al., 2008; Pieters et al., 2008). In fish most of the beneficial effects, have been recorded within a dietary probiotics feeding regime of 1-10 weeks (Nayak, 2010). The time course for optimum induction of immune response differs with respect to probiotic strain and also with the type of immune parameter (Choi and Yoon, 2008). Batista et al. (2014) studied the effects of dietary probiotic supplementation (monospecies and multispecies) in Senegalese sole (*Solea senegalensis*) juveniles during a 1-month trial and this time duration was not able to produce any effect on growth or humoral innate immune response. The short-time probiotic feeding may lead to failure of the probiotic strains to establish themselves in the fish gut and therefore long-time administration should be investigated.

As the use of plant protein sources to replace fish meal is a major trend in aquafeeds, it is important to gather health-related information to confirm the suitability of the newly developed plant-based diets in farmed fish. Therefore, the aim of this study is to evaluate the effects of two dietary plant protein levels intercropped with probiotic supplementation on the growth performance, innate immune response and intestine microbiota diversity in sole (*Solea senegalensis*).

3.2 Materials and methods

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

3.2.1 Feeding experiment

The Senegalese sole feeding experiment took place at the CIIMAR facilities (University of Porto, Porto, Portugal). Fish were fed for 73 days, in triplicate, with six isoproteic (55% crude protein) and isolipidic (8% lipid) diets with similar amino-acid composition (Table 1 and supplementary table S1). Two practical diets were formulated to contain either low or high plant protein (PP) inclusion levels (35% and 72%, respectively). Each of these diets were supplemented with either PROB or YEAST, and compared with the unsupplemented ones (UN).

The duration of the trial was selected as the best option to ensure the establishment and multiplication of probiotic strains in the fish gut while allowing diets to impact on fish growth. Diets were extruded (3 mm pellet) by the use of a pilot-scale twin-screw extruder (Clextral BC45). PROB was a multispecies probiotic bacteria (AquaStar® Growout: *Bacillus* sp, *Pediococcus* sp, *Enterococcus* sp, *Lactobacillus* sp) incorporated at 1.34×10^{10} CFU kg^{-1} diet (CFU – colony forming unit) in the extruded pellets by means of vacuum coating (Dinnisen Pegasus vacuum mixer, PG-10VCLAB) using fish oil as a carrier. YEAST was autolyzed yeast (Levabon® Aquagrow E: *Saccharomyces cerevisiae*) supplemented in the mixture at 4g kg^{-1} diet. Diets were prepared according to the normatives ISO (6887-1:1999, 7218:2007, 6498:2012) for the microbiological examinations. Thereafter, the isolation and enumeration of bacteria in the diets followed the European standards for *Enterococcus* spp. (EN 15788:2009), *Lactobacillus* spp. (EN 15787:2009), *Pediococcus* spp. (EN 15786:2009) and *Bacillus* spp. (EN 15784:2009).

Senegalese sole were obtained from a commercial fish farm (Aquacria S.A., Portugal) and transported to the rearing facilities of CIIMAR. After 5-week acclimation, fish of similar weight and length were distributed into 18 fibreglass tanks of 50 L, supplied by a recirculation system.

Table 1 - Feed ingredients and proximate composition of the experimental basal diet

	Diets	
	PP35	**PP72**
Feed ingredients (%)		
Fishmeal 70 L	24.5	5.5
Fishmeal 60	27.0	0.0
CPSP[a]	5.0	5.0
Squid meal	5.0	5.0
Pea (Lysamine GP)	0.0	14.0
Soycomil PC	0.0	6.0
Soybean meal	12.5	9.0
Potato concentrate	0.0	6.0
Wheat gluten	0.0	7.0
Corn gluten	0.0	9.0
Extruded peas (Aquatex G2000)	11.0	11.5
Wheat meal	11.0	9.6
Fish oil	2.0	5.7
Vitamin[b] and Mineral Premix[c]	1.0	1.0
Di-calcium phosphate	0.0	4.0
L-Lysine	0.0	0.5
DL-Methionine	0.0	0.2
Binder (Alginate)	1.0	1.0
Proximate composition		
Crude protein (%DM)	54.43	53.31
Crude fat (%DM)	11.75	11.98
Gross Energy (kj g−1DM)	21.04	21.98

DM, dry matter; [a]Soluble fish protein hydrolysate (75% crude protein, Sopropêche, France); [b]Vitamins (mg or IU kg^{-1}diet): Vitamin A (retinyl acetate), 20000 IU; vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1 (thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B12 (cyanocobalamin), 0.1 mg; vitamin B5 (pantothenic acid), 100 mg; vitamin B3 (nicotinic acid), 200 mg; vitamin B9 (folic acid), 15 mg; vitamin H (biotin), 3mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1000 mg; vitamin C (stay C), 1000 mg; vitamin E, 100 mg; [c]Minerals (% or mg kg^{-1}diet): Mn (manganese oxide), 9.6 mg; I (potassium iodide), 0.5 mg; Cu (cupric sulfate), 9 mg; Co (cobalt sulfate), 0.65 mg; Zn (zinc oxide), 7.5 mg; Se (sodium selenite), 0.01 mg; Fe (iron sulfate), 6 mg; Cl (sodium chloride), 2.41%; Ca (calcium carbonate), 18,6%; NaCl (sodium), 4%.

Senegalese sole were obtained from a commercial fish farm (Aquacria S.A., Portugal) and transported to the rearing facilities of CIIMAR. After 5-week acclimation, fish of similar weight and length were distributed into 18 fibreglass tanks of 50 L, supplied by a recirculation system. Fish were at a density of 5.8 kg m^{-2} (31 fish per tank with a mean initial body weight of 33.1 ± 0.2 g). Of the 18 tanks, nine received 35% PP (PP35) the PP35 diet and the other nine 72% PP (PP72) diet. Of the nine from each diet, three received the PROB supplementation, three YEAST and three were unsupplemented. Rearing conditions, such as salinity (32.0 ± 0.1 ppm), temperature (19.7 ± 0.5 ºC), ammonia (0.33 ± 0.27 mg L^{-1} NH_3), nitrite (0.14 ± 0.07 mg L^{-1} NO_2) and flow rate of 1.5 L min^{-1}, were monitored during the feeding

entire trial. The recirculation system was equipped with UV lights and ozone generator to hamper bacteria growth in the rearing water. Fish were fed to apparent satiety based on visual observation of acceptance and refusal of feed using temporized automatic feeders. To prevent stressful conditions, high stocking densities and repeated handling stress were avoided.

3.2.2 Sampling procedures

Prior to sampling, fish were fasted for 24 h and then sacrificed with an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222, 200 mg L^{-1}). All fish were individually weighed and measured at the beginning and at the end of the experiment in order to determine growth performance. Ten fish from the initial stock and two fish from each tank at the end of the trial (6 fish per treatment) were sampled and stored at -20 ºC for body composition analyses. For the evaluation of humoral innate immune parameters (lysozyme, peroxidase and alternative complement pathway – ACH50) blood was withdrawn from the caudal vein of 9 fish per treatment using heparinised syringes and centrifuged at 5000 × g for 10 min at 4 ºC to collect the plasma. The liver and viscera weights were recorded to calculate HSI (hepatosomatic index) and VSI (viscerosomatic index). The posterior intestine was aseptically sampled and divided in two sections (distal intestine and rectum). The intestinal microbiota, including the probiotic bacteria tested, were determined in both intestine sections, separately. The expression of immune-related genes, were quantified (RT-qPCR) in the head-kidney, distal intestine and rectum. All samples were stored at -80 ºC for further analyses.

3.2.3 Chemical analyses of diets and body composition

All chemical analyses were carried in duplicate according to AOAC (2006). Fish were minced without thawing using a meat mincer, pooled and used to determine their dry matter content (105 °C for 24 h). Fish were then freeze-dried and analysed for dry matter, ash (Nabertherm L9/11/B170; Germany; 550ºC for 6 h), crude protein (N×6.25, Leco N analyser, Model FP-528, Leco Corporation, St. Joseph, USA), crude lipid (petroleum ether extraction, 40–60ºC, Soxtherm, Gerhardt, Germany) and gross energy (adiabatic bomb calorimeter, Werke C2000, IKA, Germany).

3.2.4 Humoral innate immune parameters

All measurements were done in triplicate on a Power-Wave™ microplate spectrophotometer (BioTek Synergy HT, USA). Plasma lysozyme activity (EU ml^{-1}) was determined using a turbidimetric assay adapted to microtitre, as described by Hutchinson and Manning (1996). One lysozyme enzyme unit (EU) was defined as the amount of lysozyme that caused a decrease in absorbance of levels per min. Plasma peroxidase activity (EU ml^{-1}) was measured following the procedure adapted to *Solea senegalensis* by Costas et al. (2011), defining that one unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway (ACH50) was based on the lysis of rabbit red blood cells (2.8×10^8 cells mL^-1; RaRBC), as reported by Sunyer and Tort (1995). ACH50 units were defined as the concentration of plasma giving 50% lysis of RaRBC.

3.2.5 Immune- and stress-related gene expression

Except for lysozyme c (*lyzc*), target and reference genes used in this study were based on published information (Table 2). The immune-related genes selected are: lysozymes (*lyzc* and *lyzg*), heat shock proteins (*hsp90a*, *hsp90b* and *hsp90b1*), iron chelating proteins (*ftm* and *apoa1*), complement factors (*c3a1* and *c3a2*), the cysteine protease *casp3* and oxidative enzymes (*cat* and *gpx*).

3.2.5.1 RNA extraction and cDNA synthesis

Senegalese sole tissues (head-kidney, distal intestine or rectum) were homogenised with Lysing matrix D beads for 20 s at 5500 rpm on a MagNA Lyser instrument (Roche) and used for RNA extraction following the QIAzol protocol (Qiagen). Assessment of RNA quality, RNA quantification and cDNA synthesis followed the methodology described by Campos et al. (2010).

Table 2 – List of the reference and target gene primers used for real-time PCR

Gene	Accession	Reference	Forward sequence (5'→ 3') Reverse sequence (5'→ 3')	Product size (bp)	Ta (ºC)	Efficiency
Reference genes						
eef1a1	AB326302	(Campos et al., 2010)	ATTGGCGGCATTGGAACA CATCTCCACAGACTTGACCTC	117	60	2.03
rps4	AB291557	(Campos et al., 2010)	CTGCTGGATTCATGGATGTG GGCAGTGATGCGGTGGAC	103	60	2.10
Target genes						
lyzc	EU380795	N/A	ATCAGAGCCTGGGTTGCC TTAAACTCCACATCCTCTCACG	42	60	1.92
lyzg	AB428773	(Salas-Leiton et al., 2010)	ACTGCTCGCGGTGAATGGGACA CCTGAAAATTTATTACGGATTCGGCCAATG	96	60	1.99
hsp90a	AB367526	(Manchado et al., 2008)	GACCAAGCCTATCTGGACCCGCAAC TTGACAGCCAGGTGGTCCTCCCAGT	79	70	2.18
hsp90b	AB367527	(Manchado et al., 2008)	TCAGTTTGGTGTGGGTTTCTACTCGGCTTA GCCAAGGGGCTCACCTGTGTCG	148	60	1.88
hsp90b1	FJ263549	(Osuna-Jiménez et al., 2009)	GAGTCTTCTCCCTTTGTTGAGCGGCTG TGATGCCTTCCTTTGCCACGTTCTG	142	70	2.00
ftm	FF682434	(Prieto-Alamo et al., 2009)	ATGGAGTCTCAAGTGCGTCGGAACTACCAC CATGCTCCTTCTCCTCCTCGCTGTTCTC	171	60	1.80
apoa1	FF283994	(Tingaud-Sequeira et al., 2009)	TTGAGGCTAATCGTGCCAAA CCTGCGTGCTTGTCCTTGTA	76	60	1.97
c3-1	FJ345403	(Makridis et al., 2009)	TATAAGAACAAGGATCACGATG GGTAGATGATCAATGAACCTC	106	54	2.01
c3-2	FF682240	(Prieto-Alamo et al., 2009)	ACCTTAGACTGCCCTACTCTGCTGTCCGTG GCACTGCACACATCATCCGTCTCAGAC	147	60	2.26
casp3	HQ115741	(Costa et al., 2011)	CATCATCAACAACAAGAACTTTGACG ATGGTCTTCCTCCGAGGCTT	182	60	1.83
gpx	HM068301	(Costa et al., 2011)	ATGAACGAGCTGCACTGTCG AGATAGACAAACAAGGGGTGTG	208	60	2.10
cat	GU946411	(Costa et al., 2011)	TGAGCAGGCTGAAAAGTTCC GGCATGTTACTTGGGTCAAAG	163	60	1.90

3.2.5.2 Quantitative real-time PCR (qPCR)

Quantification of mRNA levels was done by qPCR in LightCycler 480 (Roche) using SYBR green (Qiagen), as detailed elsewhere (Campos et al., 2010; Fernandes et al., 2008). Briefly, 10 µL reactions were prepared in 96-well plates and included 4 µL of 25 ×-diluted cDNA template, 1 µL of each primer pair at 5 µM and 5 µL of QuantiTect SYBR Green containing ROX as reference dye (Qiagen). Samples were denatured (15 min at 95 ºC) and then amplified (45 cycles), according to the following thermocycling profile: denaturation for 10 s at 95 ºC, annealing for 20s at 60 ºC and extension for 20 s at 72 ºC. Six-point standard curves of a 2-fold dilution series (1:1–1:32) from pooled cDNA were used for PCR efficiency calculation. Specificity of the qPCR reactions was determined by melting curve analysis and further confirmed by Sanger sequencing of the PCR product, as reported by Campos et al. (2010). Data were analysed by the relative quantification method after normalization using the geometric mean of the best reference genes (Fernandes et al., 2008).

3.2.6 PCR amplification and denaturing gradient gel electrophoresis (PCR + DGGE)

The DGGE analysis of PCR-amplified genes coding for 16S rRNA was carried out in the distal intestine and rectum (2 fish / treatment) to make a qualitative study of the microbial community in the intestine. DNA was extracted using cetyltrimethylammoniumbromide (Zhou et al., 1996). For the detection of the multispecies bacteria PROB, the PCR amplification of DNA was carried out using the primer combination of 341F with GC clamp, CCTACGGGAGGCAGCAG and 907R, CCGTCAATTCMTTTGAGTTT (Muyzer et al., 1995). Yeast was detected in animals by PCR amplification using *Saccharomyces cerevisiae* specific primers combination of SCIF, GTGCTTTTGTTATAGGACAATT and SI5R, AGAGAAACCTCTCTTTGGA (Chang et al., 2007). PCR amplification by a modified touchdown protocol was performed as described Dhanasiri et al. (2011), except for the temperature of the initial denaturation step (96ºC). DGGE was performed on the PCR products from DNA samples using a 16 × 16 cm gel on the Bio-Rad DCode ™ system (Bio-Rad, USA), as described by Dhanasiri et al. (2011), but using a 30 to 55% (w/v) urea and formamide denaturing gradient. PCR products from distal

intestine and rectum sections were loaded into two separated gels (6 fish/ treatment) and electrophoresis was performed with 0.5% TAE buffer, at 20 V for 15 minutes and then at a constant voltage of 75 V for 17 hours. DGGE profile analysis (Quantity One® version 4.6.3 software, Bio-Rad) was performed for gels A and B (Fig. 1) to determine similarity matrices (Dhanasiri et al., 2011). Bands of interest were excised, purified and sequenced. The results were subjected to BLAST sequence similarity search using BLASTN from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov).

3.2.7 Calculations of growth performance

Feed conversion ratio was calculated as: dry feed intake (g) / wet weight gain (g), and the daily growth index (% BW day^{-1}) as: $100 \times [(W_1)^{1/3} - (W_0)^{1/3}]$ / days, where W_0 and W_1 are the initial and the final fish mean weights in grams and days is the trial duration. Voluntary feed intake (% BW day^{-1}) was calculated as: 100 × (dry feed intake (g) / average BW (g) / days), where average BW was calculated as: (W_1+W_0) / 2. The protein efficiency ratio was calculated as weight gain (g) / protein ingested (g). The hepatosomatic index was calculated as: 100 × [liver weight (g) / whole body weight (g)] and the viscerosomatic index as 100 × [viscera weight (g) / whole body weight (g)].

3.2.8 Statistical analysis

Statistical analyses were performed with the software SPSS (IBM SPSS STATISTICS, 17.0 package, IBM Corporation, New York, USA). Results are expressed as mean ± standard deviation (SD_{pooled} as weighted average of each group's standard deviation) and the level of significance used was $P \leq 0.05$. Data were analysed for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) and were log-transformed whenever necessary. Data were analysed by a two-way ANOVA with diet and probiotic as main factors. When significant differences were obtained from the ANOVA, Tukey's post hoc tests were carried out to identify significantly different groups. When data did not meet the ANOVA assumptions, a non-parametric Kruskal–Wallis test was performed for each factor. Evaluation of expression stability of reference genes was done using the statistical application geNorm (Vandesompele et al., 2002). Expression of target genes was evaluated by the relative quantification method as reported in Fernandes et al.

(2008). Heat maps of transcript levels were produced using PermutMatrix software, with the Euclidean distance clustering algorithm and gene expression normalized for rows.

3.3 Results

3.3.1 Growth performance

Data from growth performance are presented in table 3. Fish grew from 33.1 ± 0.20 g to 50.6 ± 1.2g (PP72_UN). Growth performance did not differ between PP35 and PP72 groups. PROB groups had significantly lower final body weight (45.0 ± 1.9) and daily growth index (0.5 ± 0.1) compared to UN groups (50.5 ± 2.0 and 0.7 ± 0.1, respectively). Additionally, UN groups had significantly better feed conversion ratio (1.5 ± 0.1) and higher protein efficient (1.3 ± 0.1) than the probiotic supplemented groups. Voluntary feed intake was also lower in UN groups (0.8 ± 0.0), and differed significantly from YEAST groups (0.9 ± 0.1). Visceral somatic index and hepatosomatic index did not differ between treatments.

3.3.2 Humoral innate immune parameters

After the 73-day feeding trial, humoral immune parameters did not present any significant differences between treatments (supplementary table S2). Lysozyme, peroxidase and ACH50 varied between 1225.9 ± 251.0 and 2061.7 ± 366.2 EU mL^{-1}, 22.5 ± 9.0 and 50.3 ± 14.1 EU mL^{-1} and 88.2 ± 14.0 and 110.3 ± 14.9 U mL^{-1}, respectively.

3.3.3 Immune- and stress-related gene expression

Expression of immune-related genes is presented in Fig. 2 and supplementary tables S3, S4 and S5. Distal intestine transcript levels were significantly affected by plant ingredients content (*hsp90b* and *apoa1*) and by probiotic supplementation (*hsp90b1* and *gpx*). PP72 groups have lower values for *hsp90b* (1.4 ± 0.8) and *apoa1* (9.6 ± 5.4) genes, compared to PP35 groups (3.2 ± 1.9 and 14.9 ± 7.3, respectively). YEAST groups presented higher values for *hsp90b* (3.4 ± 1.9) compared to PROB (2.1 ± 1.8) and UN (1.5 ± 0.8) groups. However, for *hsp90b1* and *gpx* expression, PROB groups (4.1 ± 2.4 and 1.1 ± 0.4 respectively) had higher values compared to YEAST (1.8 ± 0.5 and 0.6 ± 0.3 respectively) and UN (2.2 ± 1.0

and 0.7 ± 0.3 respectively) groups. Considering the distal intestine *cat* expression, PP35_PROB (21.3 ± 12.1) was significantly higher than PP72_YEAST (7.0 ± 2.3) treatment. No significant differences were detected in distal intestine gene expression for lysozymes (*lyzc* and *lyzg*), *c3* complement components (*c3-1* and *c3-2*), *hsp90a, ftm* and *casp3* genes.

Similarly to distal intestine, some rectum genes transcript levels were affected by the use of plant ingredients. PP72 groups (*casp3*: 2.0 ± 0.6, *gpx*: 0.1 ± 0.0 and *cat*: 2.3 ± 0.6) showed significantly lower values compared to PP35 groups (*casp3*: 2.6 ± 0.6, *gpx*: 0.2 ± 0.1 and *cat*: 2.9 ± 0.8). Rectum *hsp90a* expression was two-fold higher for PROB (0.04 ± 0.02) groups compared to YEAST (0.02 ± 0.01) groups. Rectum *ftm* expression was higher for UN (3.3 ± 0.6) groups compared to PROB (2.5 ± 0.8) groups but not different to YEAST (2.7 ± 0.7) groups. *apoa1* expression was significantly higher in PP35_YEAST (1.3 ± 1.1) in rectum, compared to PP35_UN (0.0 ± 0.1), PP35_PROB (0.1 ± 0.2) and PP72_YEAST (0.2 ± 0.2). No significant differences in transcript levels for lysozymes (*lyzc* and *lyzg*), heat shock proteins (*hsp90b* and *hsp90b1*), complement components (both *c3* analysed) were detected in rectum.

In head-kidney, only mRNA levels of *hsp90b1* were significantly lower in PP72 (0.4 ± 0.2) than in PP35 (0.6 ± 0.2) groups.

3.3.4 Probiotic detection and gut microbiota profiles

The marker bands (PROB bacteria profile) are present in samples of fish that have been fed the multispecies bacteria probiotic (Fig. 1A). A comparison of the DGGE profiles (Table 4) between distal intestine and rectum tissues revealed lower similarity values for distal intestine (34.4 – 54.9%) than rectum (47.9 – 72.4%), showing higher microbiota diversity in distal intestine. We also observed that fish fed PP72 diets have a tendency to display a lower similarity value compared with fish fed PP35 diets (34.4 – 54.9% for distal intestine and 47.9 – 72.4% for rectum).

In distal intestine, comparison of the profiles between PP35_UN *vs* PP72_UN revealed similarity values less than 34.4%, pointing to a difference in the microbial populations between fish fed different plant ingredients content on the diet. However, when adding the probiotic to the diet, similarity values, in distal intestine, increase (54.9% for PROB and 46.3% for YEAST).

Table 3 – Growth performance of Senegalese sole juveniles after the 73-day feeding trial

	Dietary treatments							p value		
	PP35			PP72			SD_{pooled}	Diet	Suplementation	Diet*Suplementation
	UN	PROB	YEAST	UN	PROB	YEAST				
Growth performance										
IBW (g)	33.03	33.16	33.02	33.16	33.04	33.25	0.21	0.44	0.95	0.35
FBW (g)	50.49^{x}	45.73^{y}	45.82^{xy}	50.58^{x}	44.21^{y}	47.73^{xy}	2.91	0.91	0.02	0.60
DGI (g kg^{-1} BW day^{-1})	0.67^{x}	0.50^{y}	0.51^{xy}	0.67^{x}	0.45^{y}	0.56^{xy}	0.10	0.98	0.02	0.68
FCR (g g^{-1})	1.46^{y}	2.04^{x}	2.11^{x}	1.49^{y}	2.42^{x}	2.02^{x}	0.34	0.53	0.01	0.51
VFI (g kg^{-1} BW day^{-1})	0.83^{y}	0.88^{xy}	0.90^{x}	0.85^{y}	0.94^{xy}	0.94^{x}	0.33	0.13	0.02	0.80
PER (g g^{-1})	1.27^{x}	0.91^{y}	0.89^{y}	1.27^{x}	0.79^{y}	0.97^{y}	0.16	0.87	0.00	0.62
Somatic indexes (g Kg^{-1})										
VSI	2.34	2.04	2.08	2.18	2.48	2.06	0.19	0.35	0.18	0.05
HSI	0.89^{AB}	0.82^{B}	0.84^{A}	0.89^{AB}	0.78^{B}	1.05^{A}	0.08	0.18	0.03	0.05

In each line, different superscript letters indicate significant differences (P<0.05): for a particular diet, differences caused by probiotic inclusion are indicated using x, y; for a particular probiotic inclusion, differences among the diets are shown using A, B. Dietary treatments are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or with the immunostimulant yeast. The other abbreviations are: IBW = Initial body weight; FBW = Final body weight; DGI = Daily growth index; FCR = Feed conversion ratio; VFI = Voluntary feed intake; PER = Protein efficiency ratio; VSI = Viscerosomatic index; HIS = Hepatosomatic index. SD_{pooled} = pooled standard deviation. Values represent mean ± SD_{pooled}, n=3.

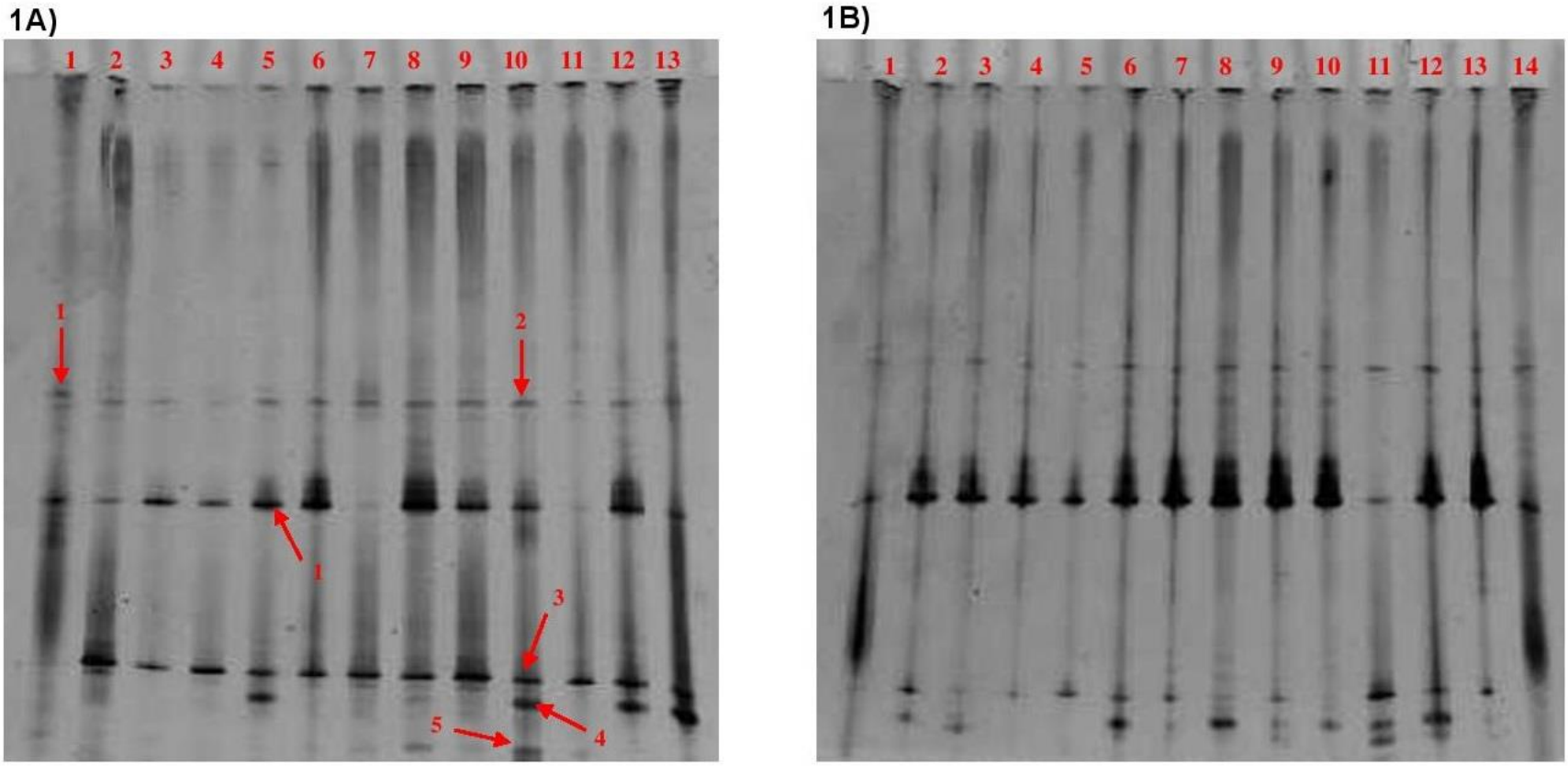

Figure 1 – DGGE images showing bands corresponding to the intestinal bacteria present in the distal intestine wall (A) and rectum wall (B) samples of Senegalese sole juveniles that were fed for 73 days with diets with or without probiotic/ yeast supplementation. Lanes: 1 and 14 – probiotic bacteria marker; 2 and 3 – PP35_UN; 4 and 5 – PP35_PROB; 6 and 7 – PP35_ YEAST; 8 and 9 – PP72_UN; 10 and 11 – PP72_PROB; 12 and 13 – PP72_ YEAST. Arrowheads with numbers indicate the bands that were excised and sequenced for molecular identification (1 - *Enterococcus* sp; 2 - *Lactobacillus* sp; 3 - *Bacillus* sp; 4 - *Enterococcus* sp; 5 – *Pediococcus* sp), n=2 fish per treatment group.

Table 4 – Average percentage of similarity obtained for the DGGE profiles of the distal intestine wall (A) and rectum wall (B) samples of Senegalese sole juveniles after the 73-day feeding trial

A)

		Percentage of similarity (%)[a]					
		PP35			PP72		
		UN	PROB	YEAST	UN	PROB	YEAST
PP35	UN	48.2					
	PROB	**55.7**	**55.6**				
	YEAST	43.6	**54.0**	**52.5**			
PP72	UN	34.4	45.2	**56.9**	43.0		
	PROB	47.2	**54.9**	**54.9**	42.4	42.6	
	YEAST	35.8	44.2	46.3	40.4	40.3	20.6

B)

		Percentage of similarity (%)[a]					
		PP35			PP72		
		UN	PROB	YEAST	UN	PROB	YEAST
PP35	UN	**61.4**					
	PROB	**63.4**	**68.5**				
	YEAST	**58.6**	**62.4**	48.4			
PP72	UN	**72.4**	**63.9**	**67.9**	**81.4**		
	PROB	**52.8**	47.9	49.5	**61.3**	25.5	
	YEAST	**62.3**	47.1	**51.3**	**65.5**	**51.2**	**51.3**

[a] Percentage of similarity computed using Quantity One® program. If the lanes are identical to each other, the percentage of similarity is 100. Similarity values higher than 50% are presented in bold. Dietary treatments (n=2) are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or with the immunostimulant yeast.

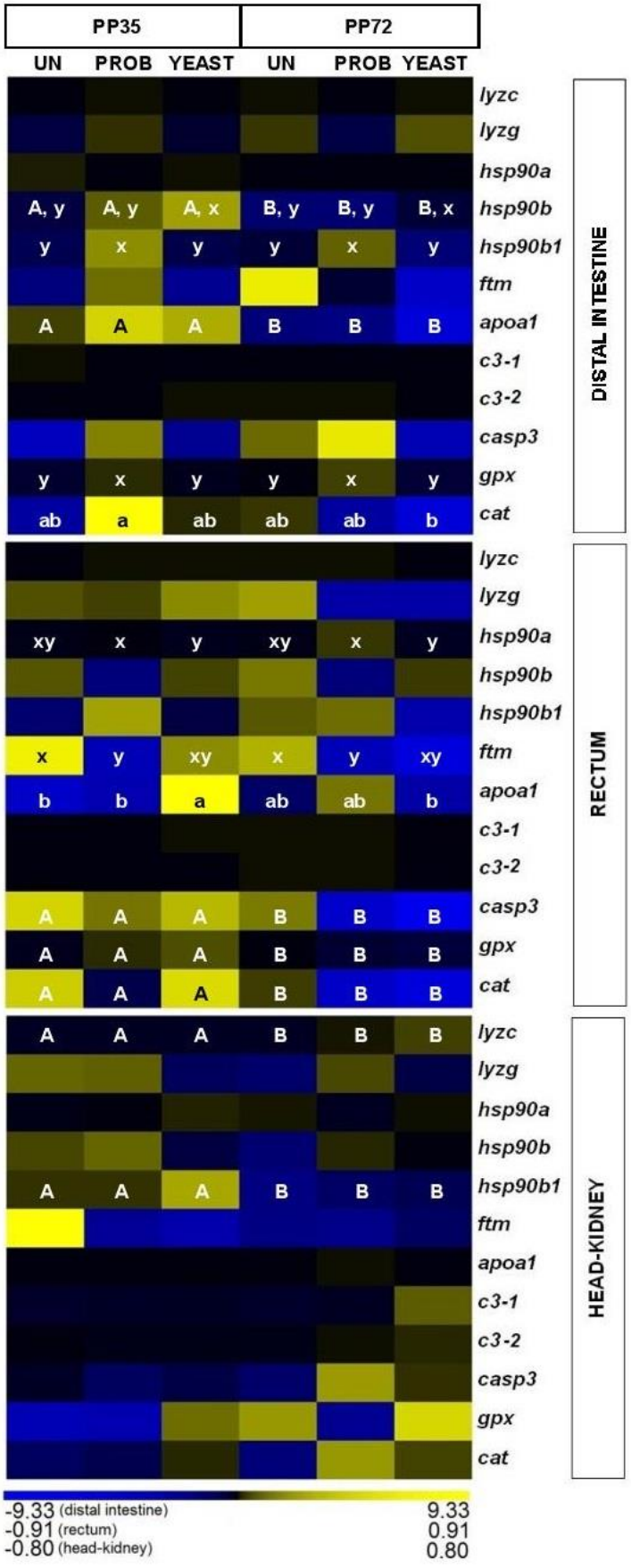

Figure 2 – Heat maps showing the normalized mRNA levels of selected genes in the distal intestine, rectum and head kidney tissues of Senegalese sole juveniles after the 73-day feeding trial. Each block represents the mean mRNA level of 6 fish quantified by qPCR. Letters indicate significant differences ($P<0.05$).

3.4 Discussion

It is well recognised that plant ingredients have to be increasingly employed in aquafeeds to cater to the demand of the industry. However, the application of these ingredients at relatively high levels in the diets of some carnivorous fish species may cause nutritional imbalances and influence the immune response as they may contain anti-nutritional factors (Hardy, 2010). In order to cope with the need to depend on the plant materials in fish feeds, efforts have to be made to alleviate the negative influence of these components may have on fish. Our approach in this direction has been to exploit the potential of probiotic organisms to counter any negative influence that may arise upon increasing the levels of plant components in fish diets. Senegalese sole that received plant diets supplemented with the selected probiotics seems to have altered immune and stress responses compared to fish receiving plant diets that lacked the probiotics. Our findings are discussed mainly based on the expression of the immune and stress-related genes in the intestinal segment which is considered as an immunologically relevant region in fish (Rombout et al., 2011) where the applied diets can have a direct impact on the elicited responses.

In fish, the induction of heat shock proteins (*hsp*) is a component of the cellular stress response against a diversity of stressors, such as osmotic stress, heat shock or infections (Basu et al., 2002). *hsp90b1* (also known as *gp96* or *grp94*) is the primary chaperone of the endoplasmic reticulum and has crucial immunological functions, serving as a natural adjuvant for priming innate and adaptive immunity (Strbo and Podack, 2008). No differences in *hsp90a* expression were detected between treatments in the present study, regardless of the tissues examined. Distal intestine *hsp90b* and head-kidney *hsp90b1* mRNA levels were significantly affected by diets, with lower expression in fish fed higher plant ingredients inclusion level. In contrast, high levels of plant protein in the feeds did not affect the expression of *hsp70* and *hsp90* in Atlantic cod (*Gadus morhua* L.) (Hansen et al., 2006). Interestingly, considering the effect of the probiotic, Senegalese sole fed yeast supplemented diets had higher *hsp90b* expression in the distal intestine, while fish fed multispecies probiotic displayed higher *hsp90b1* and *hsp90a* transcript levels in distal intestine and rectum, respectively.

In addition to their well-known role in reverse cholesterol transport and lipid metabolism, apolipoproteins display anti-inflammatory, antimicrobial and antioxidant

activities (Barter et al., 2004; Tada et al., 1993). The main high density lipoprotein, Apoa1, has antioxidant properties (Barter et al., 2004) and is involved in regulation of fish complement (Magnadóttir, 2006).

Rectum *apoa1* expression, from fish fed PP35 diet, was up-regulated by the yeast supplementation (PP35_YEAST). However, the same gene *apoa1* was down-regulated in the distal intestine from fish fed PP72 diet compared with PP35. Borges et al. (2013) reported that *apoa1* expression in sole is not regulated by dietary lipid levels and our data indicate that the use of different protein sources may affect *apoa1* expression.

Some probiotics are known to be effective in enhancing the natural complement activity in fish (Choi and Yoon, 2008; Panigrahi et al., 2007; Salinas et al., 2008). In our study, no significant differences were observed in transcript levels of two *c3* paralogues in distal intestine, rectum or head-kidney, related to the dietary treatment. Expression of the key effector caspase *casp3* did not change with treatment in distal intestine and head-kidney, but in rectum, the transcript level were significantly lower for PP72 groups compared to PP35 groups, suggesting that plant ingredients could be associated with a reduction in apoptotic activity. Nevertheless, in mice, van Breda et al. (2005) observed that 7 genes involved in apoptosis were up-regulated by consumption of a 40% plant protein diet.

Certain nutrients or immunostimulants, including probiotics, can be supplemented in the feed to modulate serum lysozyme activity in fish (Kim and Austin, 2006a). In our study, none of tissues analysed presented treatment-related differences for *lyzc* and *lyzg* transcript levels. Synthesis of ferritin is repressed under conditions of iron deprivation (Torti and Torti, 2002) and the inclusion of probiotic PROB in the diet down-regulated *ftm* in Senegalese sole rectum, compared to unsupplemented diets. It is plausible that oxidative stress in the rectum, which may have indirectly mobilised iron (Pantopoulos and Hentze, 1995), accounting for the observed *ftm* down-regulation. Further the up-regulation of *gpx1* and *cat* transcript levels in the distal intestine could also be indicating an antioxidative effect of dietary probiotic supplementation. Catalases are a class of enzymes that facilitates the dismutation of hydrogen peroxide to oxygen and water (Nicholls, 2012) and *gpx1* is as an enzyme counteracting oxidative stress due to its hydroperoxide-reducing capacity (Brigelius-Flohe and Maiorino, 2013). Rueda-Jasso et al. (2004) suggested a relationship between *cat* activity and diet composition (lipid level and starch type).

Nutritional imbalances and diet composition may play a role in oxidation processes and antioxidation defense mechanisms (Rueda-Jasso et al., 2004). In Senegalese sole, dietary plant ingredients were associated with a decrease in *cat* and *gpx1* transcript levels in rectum, compared to control groups, thus indicating that plant ingredients may have an impact on antioxidation defense mechanisms.

The stimulatory effect of probiotics on the innate immune system of fish has been reported (Nayak, 2010). However, complement, lysozyme and peroxidase activities did not differ between probiotic and control groups in the present study. These results suggest that the immunostimulatory effect of probiotics varies greatly among fish species and probiotic strains, and is in agreement with previous reports in Senegalese sole (Batista et al., 2014) and rainbow trout (Merrifield et al., 2010a). The use of probiotics or dietary raw materials that could modulate the microbiota to prevent pathogen colonisation has the added advantage of enhancing animal health (Tuohy et al., 2005) but this is still poorly understood in flatfish, including Senegalese sole. In the present study, the presence of the bacterial probiotic consortium and the yeast *Saccharomyces cerevisiae* in the intestine was confirmed intestine of animals fed PROB and YEAST treatments, respectively. It should be noted that a band for *Lactobacillus* sp was found in all gut samples regardless of probiotic supplementation, corroborating a previous report that lactobacilli are part of the natural gut flora in Senegalese sole (Tapia-Paniagua et al., 2015).

It was observed that fish fed PP72 diets had a higher number and diversity of bacteria in their gut compared to the PP35 diets. Microbial diversity was also affected by soybean meal in gilthead sea bream (*Sparus aurata*) (Dimitroglou et al., 2010) and Atlantic salmon (*Salmo salar*) (Bakke-McKellep et al., 2007) where fish fed the soybean meal diet had higher total number as well as a more diverse population composition of adherent bacteria in the distal intestine. Intestinal microbiota protects against infections and actively exchanges regulatory signals with the host to prime mucosal immunity (Gaggìa et al., 2010). A recent report on Atlantic salmon, demonstrated the ameliorating effect of a microbial additive on combating intestinal inflammation and establishing intestinal homeostasis (Vasanth et al., 2015). In Senegalese sole, the distal intestine contained a higher microbiota diversity compared to rectum, which may indicate variable immune properties across the different parts of the intestine (Inami et al., 2009). In addition to ascertaining microbial diversity by molecular methods, we used a conventional

microbiological approach to identify culturable bacteria in the gastrointestinal tract. Dietary probiotic supplementation caused an increase in the proportion of *Bacillus* sp, concomitantly with a reduction in *Vibrio* sp content (Batista, et al., unpublished). Growth performance did not differ between from PP35 and PP72 groups, showing that Senegalese sole copes well with diets in which animal protein is replaced by plant source, in accordance to the literature (Silva et al., 2009). It is noteworthy that the use of dietary probiotic supplementation produced a significant decrease in growth performance. In fact, there are contradictory reports on the effect of probiotics and prebiotics on growth performance in fish. For example, the use of lyophilized probiotic positively influenced the growth performance, promoting the feed efficiency and growth performance in Atlantic cod (Lauzon et al., 2010), rainbow trout (Merrifield et al., 2010a; Ramos et al., 2015), Japanese flounder (Taoka et al., 2006a) and sea bream (Dawood et al., 2015). In contrast, Gunther and Jimenez-Montealegre (2004) and García de la Banda et al. (2012) showed that lyophilized probiotic supplementation did not improve growth performance in Nile tilapia and in Senegalese sole, respectively.

3.5 Conclusion

Our data revealed that inclusion of probiotics and plant ingredients in the diet was associated with differences in immune- and stress-related gene expression. Overall, fish fed PP72 diets showed lower transcript levels than the PP35 diets. In particular, multispecies bacteria supplementation resulted in up-regulation of genes involved in the antioxidative stress response (*gpx* and *cat*) in distal intestine, concomitantly with the down-regulation of *ftm* mRNA in rectum. Moreover, the distal intestine of *S. senegalensis,* showed higher microbiota variability than rectum. Inclusion of plant ingredients was associated with a more diverse microbiota profile with no effect on growth performance.

3.6 Acknowledgements

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3.7 Supplementary material

Table S1 – Amino acid composition (g 16 g^{-1}N) of experimental diets

	PP35	***PP72***
His	1.40	1.17
Arg	3.65	3.12
Thr	2.21	1.96
Val	2.64	2.41
Met + Cys	1.96	2.11
Ile	2.24	2.05
Leu	3.68	4.20
Phe + Tyr	3.90	4.07
Lys	4.00	3.50
Trp	0.55	0.53
Total AA	26.23	25.13

Table S2 – Effects on humoral innate immune parameters of Senegalese sole juveniles after the 73-day feeding trial

	Dietary treatments							**p value**		
	PP35			**PP72**			**SD_{pooled}**	**Diet**	**Suplementation**	**Diet*Suplementation**
	UN	**PROB**	**YEAST**	**UN**	**PROB**	**YEAST**				
Lysozyme (EU mL^{-1})	2061.73	1511.73	1630.25	1612.96	1225.93	1439.51	798.70	0.32	0.29	0.60
Peroxidase (EU mL^{-1})	26.16	22.48	33.17	30.38	50.32	33.97	26.97	0.16	0.71	0.42
ACH50 (units mL^{-1})	110.34	108.18	100.79	88.15	104.29	98.04	43.32	0.42	0.86	0.76

Absence of superscript letters indicates no significant difference or interaction (P<0.05). Dietary treatments are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or with the immunostimulant yeast. The other abbreviations are: EU = Enzyme Unit; ACH50 = Alternative complement pathway activity; SD_{pooled} = pooled standard deviation. Values represent mean ± SD_{pooled}, n=9.

Table S3 – Gene relative expression in distal intestine of Senegalese sole juveniles after the 73-day feeding trial

	Dietary treatments							p value		
	PP35			PP72			SD_{pooled}	Diet	Suplementation	Diet*Suplementation
	UN	PROB	YEAST	UN	PROB	YEAST				
Lysozymes										
lyzc	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.84	0.33	0.07
lyzg	1.81	2.48	2.00	2.57	1.76	2.88	1.07	0.64	0.93	0.44
Heat shock proteins										
hsp90a	0.14	0.05	0.07	0.04	0.06	0.04	0.08	0.51	0.43	0.56
hsp90b	$1.87^{A;y}$	$3.14^{A;y}$	$4.73^{A;x}$	$1.11^{B;y}$	$1.06^{B;y}$	$1.97^{B;x}$	1.20	0.00	0.00	0.39
hsp90b1	2.05^{y}	4.61^{x}	2.21^{y}	2.43^{y}	3.68^{x}	1.43^{y}	1.55	0.37	0.00	0.19
Iron chelating proteins										
ftm	9.45	12.10	8.98	17.35	10.66	7.05	5.74	0.44	0.08	0.08
apoa1	12.64^{A}	16.85^{A}	15.07^{A}	10.77^{B}	10.60^{B}	7.31^{B}	6.58	0.02	0.61	0.53
Complement components										
c3-1	0.02	0.00	0.00	0.01	0.00	0.00	0.01	1.00	0.22	0.39
c3-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.66	0.56	0.04*
Cysteine protease										
casp3	9.08	14.24	10.63	13.72	18.63	9.51	7.99	0.34	0.16	0.62
Oxidative Enzymes										
gpx	0.56^{y}	1.01^{x}	0.72^{y}	0.80^{y}	1.24^{x}	0.49^{y}	0.33	0.49	0.00	0.15
cat	9.54^{ab}	21.29^{a}	12.12^{ab}	12.28^{ab}	9.52^{ab}	7.02^{b}	6.79	0.06	0.32	0.04

Transcripts were quantified by qPCR and normalized using the geometric average of suitable reference genes. In each line, different superscript letters indicate significant differences (P<0.05): for a particular diet, differences caused by probiotic inclusion are indicated using x, y; for a particular probiotic inclusion, differences among the diets are shown using A, B; and among treatments (a, b). Dietary treatments are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or with the immunostimulant yeast. SD_{pooled} = pooled standard deviation. *Without significant differences after post hoc analysis. Values represent mean ± SD_{pooled} in arbitrary units, n=6.

Table S4 – Gene relative expression in rectum of Senegalese sole juveniles after the 73-day feeding trial

	Dietary treatments							p value		
	PP35			PP72			SD_{pooled}	Diet	Suplementation	Diet*Suplementation
	UN	PROB	YEAST	UN	PROB	YEAST				
Lysozymes										
lyzc	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.92	0.76	0.09
lyzg	1.36	1.34	1.47	1.53	1.04	1.04	0.36	0.16	0.25	0.15
Heat shock proteins										
hsp90a	0.03^{xy}	0.03^{x}	0.02^{y}	0.02^{xy}	0.06^{x}	0.02^{y}	0.01	0.18	0.01	0.08
hsp90b	1.29	1.09	1.27	1.36	1.08	1.26	0.25	0.73	0.11	0.83
hsp90b1	1.05	1.41	1.12	1.24	1.28	0.89	0.37	0.64	0.10	0.36
Iron chelating proteins										
ftm	3.51^{x}	2.51^{y}	3.01^{xy}	3.13^{x}	2.50^{y}	2.33^{xy}	0.68	0.14	0.02	0.51
apoa1	0.04^{b}	0.14^{b}	1.34^{a}	0.33^{ab}	0.56^{ab}	0.18^{b}	0.60	0.81	0.05	0.00
Complement components										
c3-1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.42	0.32	0.29
c3-2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.32	0.63	0.18
Cysteine protease										
casp3	2.77^{A}	2.44^{A}	2.63^{A}	2.45^{B}	1.90^{B}	1.68^{B}	0.60	0.01	0.13	0.42
Oxidative Enzymes										
gpx	0.14^{A}	0.16^{A}	0.20^{A}	0.14^{B}	0.12^{B}	0.11^{B}	0.06	0.03	0.78	0.10
cat	3.01^{A}	2.54^{A}	3.09^{A}	2.63^{B}	2.21^{B}	2.09^{B}	0.73	0.03	0.36	0.47

Transcripts were quantified by qPCR and normalized using the geometric average of suitable reference genes. In each line, different superscript letters indicate significant differences ($P<0.05$): for a particular diet, differences caused by probiotic inclusion are indicated using x, y; for a particular probiotic inclusion, differences among the diets are shown using A, B; and among treatments (a, b). Dietary treatments are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or with the immunostimulant yeast. SD_{pooled} = pooled standard deviation. Values represent mean ± SD_{pooled} in arbitrary units, n=6.

Table S5 – Gene relative expression in head-kidney of Senegalese sole juveniles after the 73-day feeding trial

	Dietary treatments							p value		
	PP35			PP72			SD_{pooled}	Diet	Suplementation	Diet*Suplementation
	UN	PROB	YEAST	UN	PROB	YEAST				
Lysozymes										
lyzc	0.00^{B}	0.00^{B}	0.00^{B}	0.00^{A}	0.02^{A}	0.05^{A}	0.03	0.02	0.49	0.08
lyzg	0.46	0.46	0.31	0.28	0.42	0.33	0.14	0.23	0.16	0.25
Heat shock proteins										
hsp90a	0.02	0.02	0.04	0.03	0.01	0.03	0.02	0.49	0.66	0.37
hsp90b	0.97	1.01	0.89	0.82	0.94	0.93	0.22	0.47	0.67	0.69
hsp90b1	0.55^{A}	0.55^{A}	0.76^{A}	0.39^{B}	0.45^{B}	0.47^{B}	0.21	0.02	0.29	0.58
Iron chelating proteins										
ftm	1.59	0.60	0.54	0.64	0.63	0.71	0.51	0.91	0.17	0.16
apoa1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.22	0.98	0.37
Complement components										
c3-1	0.00	0.00	0.01	0.00	0.01	0.09	0.06	0.35	0.07	0.20
c3-2	0.00	0.00	0.00	0.00	0.01	0.02	0.02	0.14	0.31	0.24
Cysteine protease										
casp3	0.53	0.47	0.50	0.45	0.74	0.56	0.16	0.16	0.31	0.08
Oxidative Enzymes										
gpx	0.62	0.64	1.00	1.08	0.73	1.31	0.59	0.19	0.21	0.78
cat	0.34	0.37	0.43	0.30	0.61	0.46	0.18	0.26	0.12	0.18

Transcripts were quantified by qPCR and normalized using the geometric average of suitable reference genes. In each line, different superscript letters indicate significant differences ($P<0.05$): for a particular diet, differences caused by probiotic inclusion are indicated using x, y; for a particular probiotic inclusion, differences among the diets are shown using A, B; and among treatments (a, b). Dietary treatments are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or with the immunostimulant yeast. SD_{pooled} = pooled standard deviation. Values represent mean ± SD_{pooled} in arbitrary units, n=6.

CHAPTER 4

Innate immune response, intestinal morphology and microbiota changes in Senegalese sole fed plant protein diets with probiotics or autolyzed yeast

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Innate immune response, intestinal morphology and microbiota changes in Senegalese sole fed plant protein diets with probiotics or autolyzed yeast

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Abstract

The effects of using plant ingredients in Senegalese sole (*Solea senegalensis*) diet on immune competence and intestine morphology and ecology are still controversial. Probiotics or immunostimulants can potentially alter the intestinal microbiota in a way that protects fish against pathogens. The current study aimed to examine the intestine histology and microbiota, and humoral innate immune response in juvenile sole fed diets with low (35%) or high (72%) content of plant protein (PP) ingredients supplemented with a multispecies probiotic bacteria or autolyzed yeast. Fish fed the probiotic diet had lower growth performance. Lysozyme and complement activities were significantly higher in fish fed PP72 diets than in their counterparts fed PP35 diets after 17 days and 38 days of feeding. At 2 days of feeding, fish fed unsupplemented PP72 showed larger intestine section area and longer villus than fish fed unsupplemented PP35. At 17 days of feeding, fish fed unsupplemented PP72 showed more goblet cells than the other dietary groups, except the group fed yeast supplemented PP35 diet. Short-term feeding with high dietary PP level may enhance the immune system and improve surface area for nutrient absorption. However, this effect was reversed with long-time feeding (73 days), suggesting a habituation to dietary treatments and/or immunosuppression, with a reduction in the number of the goblet cells. Fish fed for 38 days with diets supplemented with autolyzed yeast showed longer intestinal villus, also suggesting an improvement on the surface area for nutrients absorption.

The predominant bacteria found in sole intestine were *Vibrio* sp and dietary probiotic supplementation caused a reduction in *Vibrio* content, regardless of the PP level.

4.1 Introduction

Aquaculture is a growing sector of the food-production in the world (FAO) and much effort has been done during the last years on increasing fish growth efficiency by manipulation of dietary formulations. Senegalese sole (*Solea senegalensis*) is a promising flatfish species in the European aquaculture, due to its high commercial value and nutritional properties. The ability of Senegalese sole to efficiently use novel plant-based diets (Silva et al., 2009) is an important asset towards the intensification and commercialization of this species. Currently, diseases outbreaks remain a primary constraint to aquaculture expansion. One of the newest scientific area of high interest to improve fish growth efficiency and to prevent and/or control fish diseases is the application of probiotics and microbial feed additives as alternatives to antibiotics (Dimitroglou et al., 2011; Magnadottir, 2010). These feed additives or dietary raw materials favourably affect animal performance and welfare, particularly through the modulation of the intestinal microbiota which plays a critical role in maintaining host health (Tuohy et al., 2005) and through the modulation of the immune system, influencing various humoral and cellular defences (Nayak, 2010).

Among promising probiotic candidates, the Bacilli class has been widely assayed in fish and numerous studies have demonstrated that its administration enhances immune responses and disease resistance (Aly et al., 2008; Kumar et al., 2008; Newaj-Fyzul et al., 2007; Salinas et al., 2005). On the other hand, the use of some selectively fermented ingredients like yeast, allows specific changes in the composition and/or activity of the gastrointestinal microflora that confers benefits upon the host's wellbeing and health (Marcel, 2008). Natural immunostimulants are valuable for activating the fish immune system and protecting fish against adverse conditions (Anderson, 1992; Sakai, 1999). Among them the yeast *Saccharomyces cerevisiae* has been found to be a good enhancer of the trout immune system (Siwicki et al., 1994) and it can also add nutritional value in fish diets as a possible alternative to commonly used fish meal protein (Rumsey et al., 1992).

The microbial flora present in fish intestine plays an important role on breaking down ingested food or inhibiting the colonization of the intestine by pathogens (Manzano et al., 2012). The composition of bacterial communities in fish can be affected by several factors, including the environment and their interaction with diet. A balanced intestinal microbiota constitutes as an efficient barrier against pathogen

colonization, producing metabolic substrates (e.g. vitamins and short-chain fatty acids) and stimulating the immune system (Gaggìa et al., 2010). In this context probiotics and some immunostimulants may play a significant role to improve the resistance to pathogenic bacteria colonization and enhancement of host mucosa immunity, thus reducing pathogen load and an enhancing health status of the animals (Choct, 2009). Bacterial diversity has an important role in the function of ecosystems (Bell et al., 2005). Biodiversity protects ecosystems against decline in their functionality and allows for adaptation to changing conditions, as the coexistence of many bacterial species provides an effective fail-safe measures to help keep the pathogen bacteria from spreading (Yachi and Loreau, 1999). The complex microbial ecology of the intestinal tract (GI) provides nutritional benefit and protection against pathogens. Moreover, microbial ecology may play an important role in modulating interactions between environment and the immune system (Balcázar et al., 2007a). The potential pathogens present within the GI tract should be reduced in order to avoid mucosal damage and improve absorptive surface area (Merrifield et al., 2010c). Rawls et al. (2004) observed that microbiota stimulated intestinal epithelial proliferation and influenced enterocyte morphology.

The aim of the current study was to evaluate the effects of dietary plant protein levels intercropped with a multispecies probiotic (from the *Bacilli* class) or an autolysed yeast supplementation on growth performance, innate immune response, intestine morphology and microbiota diversity in Senegalese sole (*Solea senegalensis*).

4.2 Materials and methods

The feeding experiment took place at the CIIMAR facilities (University of Porto, Portugal). The current study was conducted under the supervision of an accredited expert in laboratory animal science by the Portuguese Veterinary Authority (1005/92, DGV-Portugal, following FELASA category C recommendations) and according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE. The current study is an extension of a submitted work by Batista et al. (2016).

4.2.1 Feed and fish

Six isoproteic (55%) and isolipidic (8%) diets with similar amino-acid composition were formulated to contain 35% (PP35) or 72% (PP72) of plant ingredients (Table

1), each supplemented with PROB and YEAST (commercial products) or unsupplemented (UN) in a 2 × 3 factorial design. PROB (AquaStar® Growout - Biomin® Holding GmbH, Herzogenburg, Austria) was a multispecies probiotic (*Bacillus* sp, *Pediococcus* sp, *Enterococcus* sp, *Lactobacillus* sp) incorporated at 1.34×10^{10} CFU kg^{-1} diet (CFU – colony forming unit) in the extruded pellets by means of vacuum coating (Dinnisen Pegasus vacuum mixer, PG-10VCLAB) using fish oil as a carrier. YEAST (Levabon® Aquagrow - Biomin®) was autolyzed yeast (*Saccharomyces cerevisiae*) supplemented in the mixture at 4g kg^{-1} diet. Diets were prepared according to the normatives ISO (6887-1:1999, 7218:2007, 6498:2012) for the microbiological examinations. Thereafter, the isolation and enumeration of bacteria in the diets followed the European standards for *Enterococcus* spp (EN 15788:2009), *Lactobacillus* spp (EN 15787:2009), *Pediococcus* spp. (EN 15786:2009) and *Bacillus* spp (EN 15784:2009).

Senegalese sole were obtained from a commercial fish farm (Aquacria S.A., Portugal) and transported to the rearing facilities of CIIMAR. After 5-week acclimation period, fish were distributed into 18 fibreglass tanks of 50 L each, at a density of 5.8 kg m^{-2} (31 fish per tank, 33.1 ± 0.2 g each) and fed the experimental diets in triplicate. The tanks were supplied with seawater (32.0 ± 0.1 ppm NaCl, 19.7 ± 0.5 ºC, 0.33 ± 0.27 mg L^{-1} NH_3, 0.14 ± 0.07 mg L^{-1} NO_2) at a flow rate of 1.5 L min^{-1}, in a controlled semi-closed recirculation system under a constant photoperiod (12 h light:12 h darkness). Rearing conditions were monitored during the entire trial. The recirculation system was equipped with UV lights and ozone generator to hamper bacteria growth in the rearing water. The trial lasted 73 days and fish were fed to apparent satiety based on visual observation of acceptance and refusal of feed using temporized automatic feeders.

Table 1 - Feed ingredients, proximate composition and amino acid (g 16 g^{-1}N) of the experimental diets.

	PP35			PP72		
	UN	**PROB**	**YEAST**	**UN**	**PROB**	**YEAST**
Feed ingredients (%)						
Fishmeal 70 L[a]	24.5	24.5	24.5	5.5	5.5	5.5
Fishmeal 60[b]	27.0	27.0	27.0	0.0	0.0	0.0
CPSP[c]	5.0	5.0	5.0	5.0	5.0	5.0
Squid meal	5.0	5.0	5.0	5.0	5.0	5.0
Pea (Lysamine GP)	0.0	0.0	0.0	14.0	14.0	14.0
Soycomil PC[d]	0.0	0.0	0.0	6.0	6.0	6.0
Soybean meal[e]	12.5	12.5	12.5	9.0	9.0	9.0
Potato concentrate	0.0	0.0	0.0	6.0	6.0	6.0
Wheat gluten	0.0	0.0	0.0	7.0	7.0	7.0
Corn gluten	0.0	0.0	0.0	9.0	9.0	9.0
Extruded peas (Aquatex G2000)[f]	11.0	11.0	11.0	11.5	11.5	11.5
Wheat meal	11.0	11.0	11.0	9.6	9.6	9.6
Fish oil	2.0	2.0	2.0	5.7	5.7	5.7
Vitamin[g] and Mineral Premix[h]	1.0	1.0	1.0	1.0	1.0	1.0
Di-calcium phosphate	0.0	0.0	0.0	4.0	4.0	4.0
L-Lysine	0.0	0.0	0.0	0.5	0.5	0.5
DL-Methionine	0.0	0.0	0.0	0.2	0.2	0.2
Binder (Alginate)[i]	1.0	1.0	1.0	1.0	1.0	1.0
Proximate composition						
Crude protein (%DM)	54.26	54.37	54.66	53.16	53.31	53.46
Crude fat (%DM)	11.90	11.41	11.93	12.21	11.45	12.27
Gross Energy (kj g−1DM)	20.84	21.18	21.12	21.84	22.05	22.04

[a]Peruvian fishmeal LT (71% crude protein, 11% crude fat, EXALMAR, Peru); [b]Fair Average Quality (FAQ) fishmeal (62% crude protein, 12% crude fat, COFACO, Portugal); [c]Soluble fish protein hydrolysate (87% crude protein, 6.5% crude fat, Sopropêche, France); [d]Soycomil-P (soy protein concentrate, 65% crude protein, 0.7% crude fat, ADM, The Netherlands); [e]Dehulled solvent extracted soybean meal (micronized); [f]Aquatex G2000 (Dehulled, grinded pea grits, 24% crude protein, 0.4% crude fat, SOTEXPRO, France); [g]Vitamins (mg or IU kg^{-1}diet): Vitamin A (retinyl acetate), 20000 IU; vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1 (thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B12 (cyanocobalamin), 0.1 mg; vitamin B5 (pantothenic acid), 100 mg; vitamin B3 (nicotinic acid), 200 mg; vitamin B9 (folic acid), 15 mg; vitamin H (biotin), 3mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1000 mg; vitamin C (stay C), 1000 mg; vitamin E, 100 mg; [h]Minerals (% or mg kg^{-1}diet): Mn (manganese oxide), 9.6 mg; I (potassium iodide), 0.5 mg; Cu (cupric sulfate), 9 mg; Co (cobalt sulfate), 0.65 mg; Zn (zinc oxide), 7.5 mg; Se (sodium selenite), 0.01 mg; Fe (iron sulfate), 6 mg; Cl (sodium chloride), 2.41%; Ca (calcium carbonate), 18,6%; NaCl (sodium), 4%; [i]Diatomaceous earth: Kielseguhr: LIGRANA GmbH, Germany.

4.2.2 Sampling procedures

Prior to sampling, fish were fasted for 24 h and then sacrificed with an overdose of ethyl 3-aminobenzoate methanesulfonate (MS-222, 200 mg L^{-1}). All fish were individually weighed and measured at the beginning and at the end of the experiment in order to determine growth performance. Ten fish from the initial stock and two fish from each tank at the end of the trial (6 fish per treatment) were sampled and stored at -20 ºC for body composition analyses. Total feed consumption was

registered during the entire feeding trial. The innate immune response and intestine histology were analysed at days 3, 17, 38 and 73 of the feeding trial.

For the evaluation of humoral innate immune parameters (lysozyme, peroxidase and alternative complement pathway – ACH50) blood was withdrawn from the caudal vein of 9 fish per treatment using heparinised syringes and centrifuged at 5000 × g for 10 min at 4 ºC. Liver and intestine of each fish was sampled, cut and fixed in buffered formalin to histology analysis. To examine intestinal microbiota, the whole intestine of other 9 fish per treatment was aseptically removed at 17, 38 and 73 days of feeding the experimental diets. The intestinal contents were sampled by stripping and pooled by tank (3 fish per tank) and homogenized in 1 ml sterile phosphate-buffered saline (PBS - pH 7.2).

4.2.3 Chemical analyses of diets and body composition

All chemical analyses were carried in duplicate according to AOAC (2006). Fish were minced without thawing using a meat mincer, pooled and used to determine their dry matter content (105 °C for 24 h). Fish were then freeze-dried and analysed for dry matter, ash (Nabertherm L9/11/B170; Germany; 550ºC for 6 h), crude protein (N×6.25, Leco N analyser, Model FP-528, Leco Corporation, St. Joseph, USA), crude lipid (petroleum ether extraction, 40–60ºC, Soxtherm, Gerhardt, Germany) and gross energy (adiabatic bomb calorimeter, Werke C2000, IKA, Germany).

4.2.4 Humoral innate immune parameters

All measurements were done in triplicate on a Power-Wave^{TM} microplate spectrophotometer (BioTek Synergy HT, USA). Plasma lysozyme activity (EU ml^{-1}) was determined using a turbidimetric assay adapted to microtitre, as described by Hutchinson and Manning (1996). One lysozyme enzyme unit (EU) was defined as the amount of lysozyme that caused a decrease in absorbance per min. Plasma peroxidase activity (EU ml^{-1}) was measured following the procedure adapted to *Solea senegalensis* by Costas et al. (2011), defining that one unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway (ACH50) activity was based on the lysis of rabbit red blood cells (2.8×10^8 cells mL^{-1}), as reported by Sunyer and Tort (1995). ACH50 units were defined as the concentration of plasma giving 50% lysis of rabbit red blood cells.

4.2.5 Histological evaluation

The samples (liver and a segment with 1.5cm length from the proximal intestine) were fixed in phosphate buffered formalin (4%, pH 7, VWR, Portugal) for 24 hours. They were subsequently dehydrated and embedded in paraffin according to standard histological procedures. Thereafter, 3 micra transversal sections were obtained and stained with haematoxylin and eosin (H&E - Merck, Portugal) and Periodic Acid-Schiff (PAS - Merck, Portugal) before examination under a light microscope (Olympus BX51, cell^B software, GmbH, Hamburg, Germany).

Three different intestine sections of each animal were used for quantitative measurements. The intestine section area (mm^2), villus height (µm), muscular layer thickness (from serosa to submucosa; µm) and goblet cells (number per section) positive to PAS staining of intestinal wall were measured according Batista et al. (2014). The villus width (µm) was measured across the base of the villus at the luminal surface of 10 selected *villi* per section.

In the liver, the occurrence of possible pathological damage (presence or absence of defined vacuoles indicator of fat degeneration) was evaluated. For each sampled fish, the cytoplasm vacuolization degree (H&E) and glycogen content (PAS) of the hepatocytes were analysed (Figure 1). Observations were consistently made using a combination of low magnification (objective of 10×) for notice the general aspects and then with higher magnification (objective of 40×) for categorizing. Ten randomly sampled fields were analysed per section. Evaluation of the hepatocyte vacuolization degree was made using a semi-quantitative approach, according to the following three grades and criteria: 0 (none) – less than 1/3 of the hepatocyte cytoplasm shows vacuoles; 1 (moderate) – between 1/3 and less than 2/3 of the hepatocyte cytoplasm shows vacuolization; 2 (high) – more than 2/3 of the hepatocyte cytoplasm shows vacuolization. Assessment of hepatocyte carbohydrates (namely glycogen) content also followed a semi-quantitative approach, with three degrees: 0 (none) – when hepatocytes in PAS sections did not stain positively or had a very weak colour; 1 (moderate) – PAS sections presented a median staining; 2 (high) – PAS sections present hepatocytes with a very strong staining.

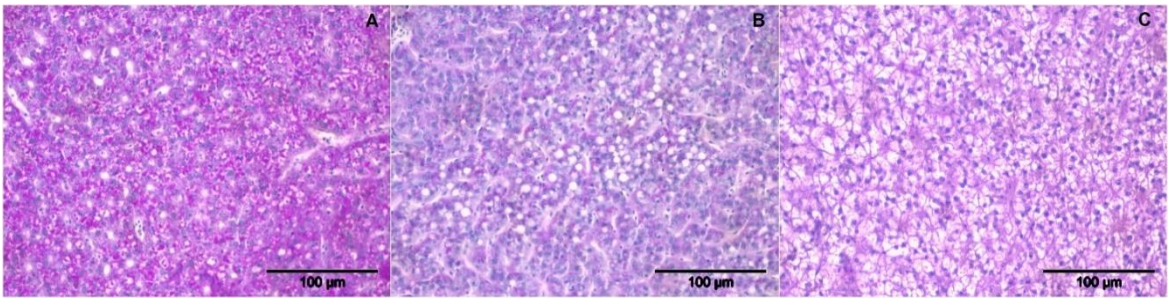

Figure 1 – Liver histology of Senegalese sole for quantification of vacuoles and glycogen content. A) high content of glycogen and reduce vacuolization B) Moderate content of glycogen and vacuoles. C) High content in vacuoles and small amounts of glycogen (PAS stained, Bar = 100µm).

4.2.6 Intestinal microbiota

Microbial populations were isolated from the intestinal content as described by Merrifield et al. (2009). Intestinal content samples were serially diluted to 10^{-2} with PBS and 100 µL were spread onto duplicate tryptone soy agar (TSA – 0.9% NaCl) plates. Plates were incubated at 25 ºC for 48 hours and colony forming units (CFU) g^{-1} was calculated from statistically viable plates (i.e. plates containing 30-300 colonies). One hundred colonies of one of these plates were randomly collected from each dietary treatment assayed and cultured on TSA to obtain pure cultures. Then the colonies were identified by the amplification and sequencing of a fragment of 16S rDNA. Briefly, the colonies isolated were suspended in 100µL of sterile milli-Q water. Samples were boiled at 100ºC for 15 minutes and adjusted to 1mL. These suspensions were centrifuged at 12000 rpm for 5 minutes and 1-5µL of the supernatant was used to carry out the PCR reactions. The fragment 16S rDNA was amplified using the universal primers SD-Bact-0008-a-S20 (5' AGAGTTTGATCCTGGCTCAG 3') and SD-Bact-1492-a-A-19 (5' GGTTACCTTGTTACGACTT 3') (Kim and Austin, 2006a). Polymerase chain reactions were carried out in a 50 µl reaction mixture that included 5 pmol of each primer, 200 µM dNTPs, 1 x PCR buffer, 2 mM $MgCl_2$, 1 U BIOTAQ DNA Polimerase (Bioline, London, UK) and 1 µl of a boiled colony suspension. Thermal cycling consisted of an initial step of 2 min at 95ºC and 35 cycles of 30 s at 95ºC, 30s at 52ºC and 40s at 72ºC and a final extension step 5 min at 72ºC. Polymerase chain reaction products were electrophoresed on 1% agarose gel, visualized via ultraviolet transillumination and then they were purified with a QiaQuick PCR purification kit

(QIAGEN, Hilden, Germany). The PCR products were sequenced by cycling sequencing using SD-Bact-0008-a-S20 and SD-Bact-1492-a-A-19 by Macrogen (Corea). The resulting sequences (~500 bp) were compared with the sequences from the National Center for Biotechnology Information (NCBI) or Greengenes DNA sequence database using the BLAST sequence algorithm (Altschul et al., 1990).Chimeric sequences were identified by using the CHECK CHIMERA program of the Ribosomal Database Project (Maidak et al., 1999) and the sequences reported in this study have been deposited in the GenBank database under the following accession numbers: KU725824 to KU725849.

4.2.7 Calculations of growth performance

Feed conversion ratio (FCR) was calculated as: dry feed intake (g) / wet weight gain (g), and the specific growth rate (SGR, % BW day^{-1}) as: $100 \times (\ln W_0 - \ln W_1)$ / days, where W_0 and W_1 are the initial and the final fish mean weights in grams and days is the trial duration. Voluntary feed intake (VFI, % BW day^{-1}) was calculated as: 100 × (dry feed intake (g) / ABW (g) / days), where ABW (average body weight) was calculated as: $(W_1+W_0) / 2$. The net protein utilization (NPU) was calculated as (PBF – PBI) / protein fed, where PBF is the final protein content of fish and PBI is the initial protein content of fish.

4.2.8 Statistical analysis

Statistical analyses were performed with the software SPSS (IBM SPSS STATISTICS, 17.0 package, IBM Corporation, New York, USA). Results are expressed as mean ± standard deviation (SD_{pooled} as weighted average of each group's standard deviation) and the level of significance used was $P \leq 0.05$. Data were analysed for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) and were log-transformed whenever necessary. Data were analysed by a two-way ANOVA with diet and probiotic as main factors. When significant differences were obtained from the ANOVA, Tukey's post hoc tests were carried out to identify significantly different groups. When data did not meet the ANOVA assumptions, a non-parametric Kruskal–Wallis test was performed.

4.3 Results

4.3.1 Growth performance

Fish grew from 33.1 ± 0.20 g to 50.6 ± 1.2 g after feeding the experimental diets for 73 days (Table 2). Growth performance did not differ between PP35 and PP72 groups. PROB groups had significantly lower final body weight (FBW – 45.0 ± 1.9) and daily growth index (SGR – 0.42 ± 0.1) compared to UN groups (50.5 ± 2.0 and 0.58 ± 0.0 respectively). Additionally, UN groups had significantly better feed conversion ratio (FCR – 1.5 ± 0.1) and net protein utilization (NPU – 19.07 ± 2.3) than the PROB (2.2 ± 0.4 and 10.4 ± 1.9 respectively) and YEAST (2.1 ± 0.4 and 13.0 ± 4.6 respectively) supplemented groups. Voluntary feed intake (VFI) was also lower in UN groups (0.8 ± 0.0), and differed significantly from YEAST groups (0.9 ± 0.1).

4.3.2 Humoral innate immune parameters

The values of humoral innate parameters analyzed are present in Table 3. Peroxidase activity (EU mL^{-1}) was not significantly different ($P<0.05$) among dietary groups for any sampling time. Lysozyme activity and ACH50 were not affected by probiotic administration, but were significantly changed by the dietary PP level at 17 and 38 days of feeding. At 17 days, fish from PP72 groups had higher lysozyme (359 ± 201 EU mL^{-1}) and ACH50 (204 ± 55 Units ml^{-1}) comparing to fish from PP35 groups (177 ± 106 EU mL^{-1} and 169 ± 19 Units ml^{-1}, respectively). At 38 days of feeding, fish from PP72 groups showed higher lysozyme (908 ± 399 EU mL^{-1}) and ACH50 (258 ± 64 Units ml^{-1}) activities, comparing to fish from PP35 groups (647 ± 457 EU mL^{-1} and 183 ± 49 Units ml^{-1}, respectively).

Table 2 – Growth performance of Senegalese sole juveniles after the 73 days of feeding the experimental diets.

	Dietary treatments							p value		
	PP35			PP72			SD_{pooled}	PP	S	PP*S
	UN	PROB	YEAST	UN	PROB	YEAST				
Growth performance										
FBW (g)	50.49^{x}	45.73^{y}	45.82^{xy}	50.58^{x}	44.21^{y}	47.73^{xy}	2.91	0.91	0.02	0.60
SGR	0.58^{x}	0.44^{y}	0.45^{xy}	0.58^{x}	0.40^{y}	0.49^{xy}	0.08	0.99	0.02	0.68
FCR	1.46^{y}	2.04^{x}	2.11^{x}	1.49^{y}	2.42^{x}	2.02^{x}	0.34	0.53	0.01	0.51
VFI	0.83^{y}	0.88^{xy}	0.90^{x}	0.85^{y}	0.94^{xy}	0.94^{x}	0.33	0.13	0.02	0.80
NPU	20.11^{x}	10.48^{y}	10.83^{y}	18.03^{x}	10.31^{y}	15.26^{y}	3.05	0.62	0.00	0.21

In each line, different superscript letters indicate significant differences (P<0.05): for a particular PP level, differences caused by probiotic inclusion are indicated using x, y; for a particular probiotic inclusion, differences among PP level are shown using A, B. Dietary treatments are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or with the immunostimulant yeast. The other abbreviations are: PP = Pant protein level factor; S = Supplementation factor; FBW = Final body weight; SGR = Specific growth rate; FCR = Feed conversion ratio; VFI = Voluntary feed intake; NPU = Net protein utilization; SD_{pooled} = pooled standard deviation. Values represent mean ± SD_{pooled}, n=3.

Table 3 – Effects on humoral innate immune parameters of Senegalese sole juveniles after the 2, 17, 38 and 73 days of feeding the different experimental diets.

	Dietary treatments							p value		
	PP35			PP72			SD_{pooled}	PP	S	PP*S
	UN	PROB	YEAST	UN	PROB	YEAST				
Lysozyme (EU mL^{-1})										
2 days	190.98	290.74	440.12	379.32	628.70	457.64	288.07	0.07	0.06	0.43
17 days	124.21^{B}	225.35^{B}	175.79^{B}	410.32^{A}	338.09^{A}	287.04^{A}	156.58	0.00	0.76	0.27
38 days	612.50^{B}	695.99^{B}	628.39^{B}	761.11^{A}	1047.62^{A}	950.69^{A}	439.55	0.02	0.70	0.69
73 days	2061.73	1511.73	1630.25	1612.96	1225.93	1295.15	787.12	0.23	0.25	0.51
Peroxidase (EU mL^{-1})										
2 days	122.11	97.44	101.56	105.12	59.09	92.82	48.68	0.28	0.06	0.15
17 days	141.65	111.80	110.51	100.71	71.51	122.82	61.59	0.25	0.19	0.16
38 days	59.90	80.03	48.99	54.39	78.59	31.21	45.32	0.86	0.06	0.77
73 days	26.16	22.48	33.17	30.38	50.32	33.97	26.97	0.15	0.61	0.30
ACH50 (units mL^{-1})										
2 days	112.29	113.85	103.17	97.97	119.27	113.59	47.47	0.98	0.88	0.86
17 days	161.34^{B}	177.01^{B}	167.67^{B}	221.55^{A}	195.88^{A}	193.10^{A}	51.21	0.02	0.82	0.45
38 days	174.86^{B}	197.38^{B}	177.52^{B}	270.95^{A}	247.59^{A}	253.50^{A}	58.78	0.00	0.92	0.52
73 days	110.34	108.18	100.79	88.15	104.29	98.04	43.32	0.42	0.86	0.76

In each line, different superscript letters indicate significant differences ($P<0.05$): for a particular probiotic inclusion, differences among PP level are shown using A, B. Dietary treatments are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or the immunostimulant yeast. The other abbreviations are: PP = Pant protein level factor; S = Supplementation factor; EU = Enzyme Unit; ACH50 = Alternative complement pathway activity. SD_{pooled} = pooled standard deviation. Values represent mean ± SD_{pooled}, n=9.

4.3.3 Histological evaluation

Histological measurements of the intestinal mucosa of fish are present in Table 4 Muscular layer thickness did not vary significantly, regardless of the dietary treatment and sampling time. Intestine section area was significantly different at 2 days of feeding trial, showing PP72_UN group (2.75 ± 0.66 mm^2) with higher area comparing to PP35_YEAST (1.93 ± 0.30 mm^2) and PP35_UN (1.99 ± 0.42 mm^2) groups. Villus was significantly wider ($P<0.05$) at 73 days in fish fed PP35_YEAST group (91.01 ± 1.49 µm) than fish fed PP72_YEAST (79.45 ± 7.28 µm) (Figure 2). Villus length was significantly different ($P<0.05$) at 2, 38 and 73 days of feeding the experimental diets (Figure 2). At 2 days of feeding, fish fed PP72_UN diet had longest villus (418 ± 30 µm) when compared to fish fed PP35_UN (357 ± 41 µm) and PP72_YEAST (361 ± 13 µm) diets. At 38 days of feeding, fish from YEAST groups had a significant longer villus (413 ± 45 µm) than fish from UN groups (372 ± 44 µm). The number of goblet cells was significantly different ($P<0.05$) at 17 and 73 days of feeding dietary treatments (Figure 3). At 17 days of feeding, fish fed PP72_UN diet had a significantly ($P<0.05$) higher number of goblet cells (379 ± 119) comparing to the others dietary treatments, except fish fed PP35_YEAST diet. At 73 days of feeding, both villus length and number of goblet cells were affected by dietary PP level, with fish from PP35 groups having larger villus (455 ± 52 µm) and higher number of goblet cells (363 ± 210) than fish from PP72 groups (395 ± 57 µm and 260 ± 129, respectively).

Liver histology revealed different degrees of vacuolization at 2 and 73 days of feeding. Fish from the PP72 groups showed a higher vacuolization (grade 2) compared to fish from PP35 groups (grade 0).

At 2 days of feeding, fish fed diets supplemented with YEAST showed higher hepatocytes glycogen content (more than 2/3 of the liver positively stained) when comparing with the fish fed the diets supplemented with PROB. At 17 days of feeding, fish fed PP72 presented hepatocytes with a weak PAS staining, indicating a very low content of carbohydrates (namely glycogen).

Table 4 - Intestinal morphology of Senegalese sole after 2, 17, 38 and 73 days of feeding the different experimental diets.

	Dietary treatments							p value		
	PP35			PP72			SD_{pooled}	PP	S	PP*S
	UN	PROB	YEAST	UN	PROB	YEAST				
Intestine section area (mm²)										
2 days	1.99^{b}	2.34^{ab}	1.93^{b}	2.75^{a}	2.05^{ab}	2.15^{ab}	0.44	0.09	0.14	0.01
17 days	1.88	1.89	1.76	1.82	1.75	2.01	0.55	0.92	0.94	0.55
38 days	1.78	1.79	2.18	1.85	2.00	2.12	0.42	0.56	0.10	0.67
73 days	2.80^{A}	2.85^{A}	2.47^{A}	2.25^{B}	2.16^{B}	2.13^{B}	0.55	0.00	0.47	0.02
Muscular layer thickness (µm)										
2 days	77.83	93.53	81.05	92.65	95.77	84.93	16	0.14	0.11	0.50
17 days	87.46	81.10	82.65	80.91	88.13	79.62	20	0.88	0.86	0.60
38 days	84.12	89.09	108.18	79.48	88.08	80.48	19	0.13	0.62	0.28
73 days	85.22	103.11	93.57	96.80	99.43	96.43	21	0.54	0.37	0.58
Villus length (µm)										
2 days	356.92^{b}	384.17^{ab}	375.21^{ab}	418.14^{a}	371.91^{ab}	361.00^{b}	35	0.27	0.33	0.01
17 days	340.42	341.58	351.17	345.19	311.72	351.50	56	0.60	0.41	0.61
38 days	384.80^{y}	366.11^{xy}	414.20^{x}	352.39^{y}	400.39^{xy}	411.84^{x}	42	0.99	0.02	0.10
73 days	461.08^{A}	476.89^{A}	431.90^{A}	409.32^{B}	391.20^{B}	385.70^{B}	55	0.00	0.28	0.53
Villus Width (µm)										
2 days	82.33	86.00	82.87	85.41	82.00	80.78	6	0.36	0.24	0.35
17 days	76.64	75.02	76.95	75.42	76.18	78.90	9	0.81	0.72	0.88
38 days	79.34	80.46	84.61	75.45	79.33	81.37	6	0.15	0.07	0.81
73 days	90.18^{ab}	89.17^{ab}	91.01^{a}	82.21^{ab}	82.61^{ab}	79.45^{b}	6	0.00	0.90	0.01
Goblet cells (number per section)										
2 days	148.60	221.33	230.40	214.62	187.60	170.29	95	0.74	0.78	0.17
17 days	192.93^{b}	231.83^{b}	278.28^{ab}	378.81^{a}	173.29^{b}	179.63^{b}	90	0.71	0.04	0.00
38 days	277.06	195.56	245.56	158.58	277.74	336.63	172	0.34	0.78	0.62
73 days	383.24^{A}	326.04^{A}	384.56^{A}	242.43^{B}	256.42^{B}	280.04^{B}	179	0.04	0.80	0.85

In each line, different superscript letters indicate significant differences (P<0.05): for a particular PP level, differences caused by probiotic inclusion are indicated using x, y; for a particular probiotic inclusion, differences among PP level are shown using A, B; and among treatments (a, b). Dietary treatments are abbreviated as PP35 and PP72 for diets with low and high content of plant ingredients, respectively and UN, PROB and YEAST to diets not supplemented or supplemented with probiotic or the immunostimulant yeast. The other abbreviations are: PP = Pant protein level factor; S = Supplementation factor; SD_{pooled} = pooled standard deviation. Values represent mean ± SD_{pooled}, n=9.

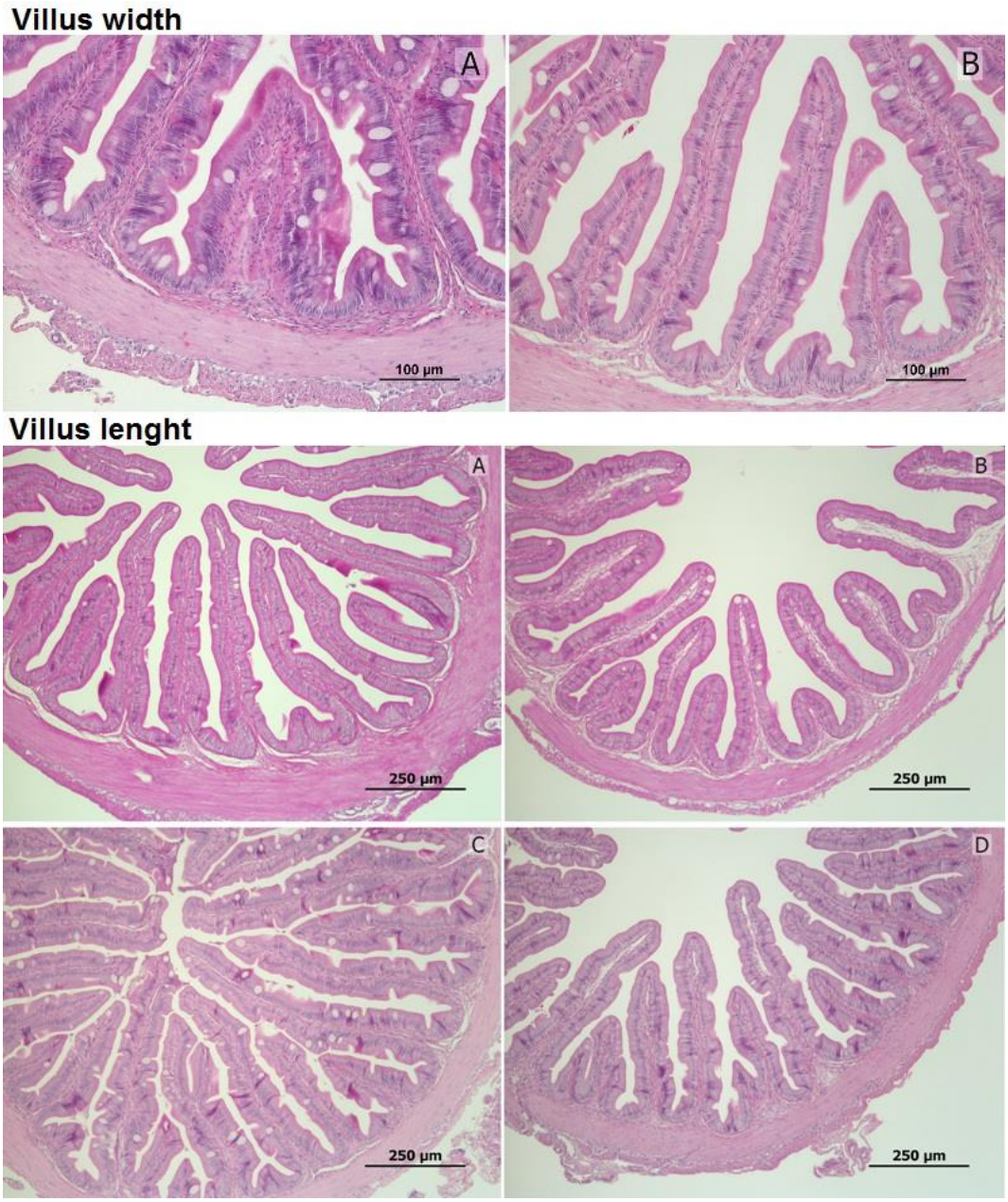

Figure 2 - Histological sections of anterior intestines of Senegalese sole for villus width (VW) and for villus length (VL). VW: A) PP35_YEAST diet showing higher VW than B) that correspond to PP72_YEAST diet (H&E, Bar = 100µm); VL: A and B represents 2 days samples time, and C and D 73 days sampling time. Note the difference between the villus dimensions in the images A and C showing higher villus length when comparing with B and D. A) PP35 diet and B) PP72_YEAST diet; C) PP35 diet and D) PP72_YEAST diet (H&E, Bar = 250µm).

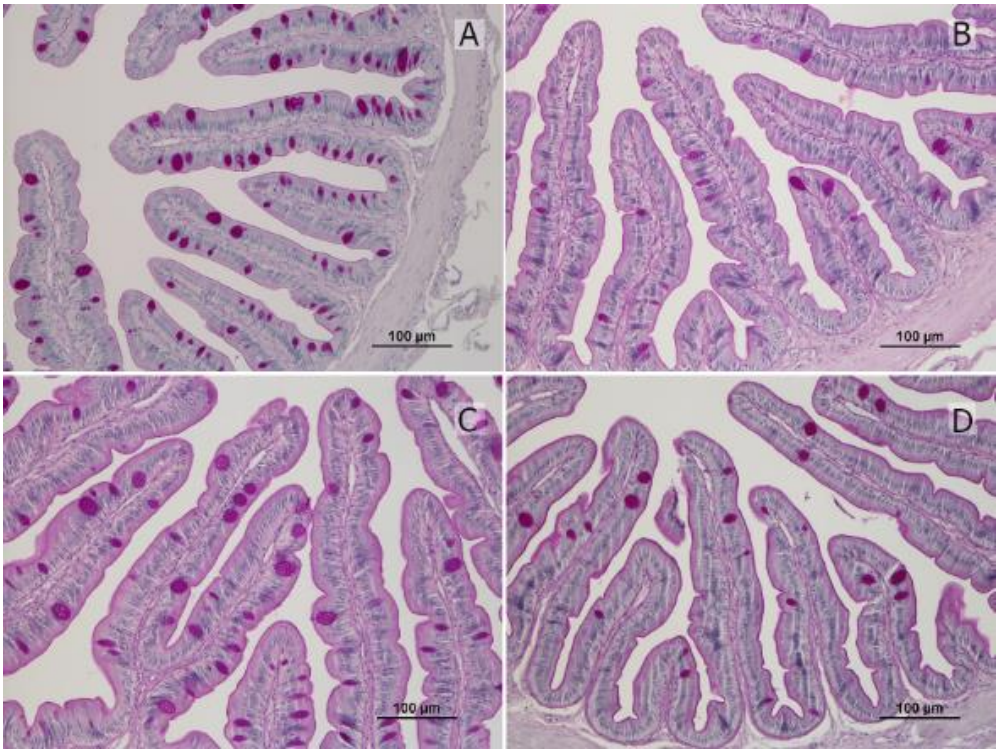

Figure 3 - Histological sections of anterior intestines of Senegalese sole for number counting of goblet cells (GC). A) PP72 diet and B) PP72_PROB diet, both at 17 days sampling time; C) PP35 diet and D) PP72_YEAST diet at 73 days sampling time. Note the difference observed between the images A and C which showing higher number of GC, comparing with B and D (PAS stained, Bar = 100µm).

4.3.4 Intestinal microbiota

The intestinal bacteria counts varied from $8.2x10^5$ to $2.4x10^7$ CFU ml^{-1}, as an effect of the diet and sampling time (Table 5). In general, the highest relative percentage corresponded to *Vibrio* sp (24% to 99%). Probiotic supplementation greatly reduced the concentration of intestinal *Vibrio* sp (Table 5), especially in fish fed low dietary PP level (PP35_PROB). The duration of the dietary treatment had a clear effect on the reduction of intestinal *Vibrio* sp concentration in fish fed PP35_PROB. *V. harveyi* and *V. owensii*, were identified in fish fed PP72_PROB and PP72_YEAST and not identified in fish fed PP72_UN (Table 6). In addition, *S. saprophyticus* was observed only in the intestine of fish fed PROB diets (tables 6 and 7).

Table 5 – Concentration of microorganisms (CFU/ml) and relative percentage of *Vibrio*, *Staphylococcus* and other bacteria identified from the bacterial isolates characterized from group of specimens of *Solea senegalensis* at 17, 38 and 73 days of feeding the different experimental diets. The values of standard deviation were never higher than 20%.

	Dietary treatments					
	PP35			**PP72**		
	UN	**PROB**	**YEAST**	**UN**	**PROB**	**YEAST**
CFU/ ml						
17 days	$7.7x10^6 \pm 1.2x10^6$	$6.9x10^6 \pm 1.3x10^6$	$3.4x10^6 \pm 5.8x10^5$	$1.7x10^7 \pm 3x10^6$	$5.5x10^6 \pm 5.1x10^5$	$1.1x10^6 \pm 1.7x10^5$
38 days	$2.1x10^6 \pm 2.8x10^5$	$1.5x10^6 \pm 1.9x10^5$	$6.3x10^6 \pm 1.2x10^6$	$2.4x10^7 \pm 4.8x10^6$	$3.4x10^6 \pm 6x10^5$	$4.0x10^6 \pm 7.6x10^5$
73 days	$3.1x10^6 \pm 4.5x10^5$	$2.5x10^6 \pm 5.3x10^5$	$8.2x10^5 \pm 1.3x10^5$	$1x10^6 \pm 1.6x10^5$	$1.8x10^6 \pm 1.6x10^5$	$4.2x10^6 \pm 8.3x10^5$
***Vibrio sp.* (%)**						
17 days	95	42	92	99	24	99
38 days	99	36	63	83	59	62
73 days	98	16	97	97	50	94
***Staphylococus sp.* (%)**						
17 days	0	30	0	0	14	0
38 days	0	12	0	0	0	0
73 days	0	24	0	0	0	0
Others (%)						
17 days	5	28	8	1	62	1
38 days	1	52	37	17	41	38
73 days	2	60	3	3	50	6

CFU – colony forming unit

Table 6 - Closest matches of GenBank database sequences to isolates from intestinal samples of *Solea senegelensis* fed PP72 diets for 17, 38 and 73 days.

			PP72_UN			PP72_PROB			PP72_YEAST		
Closest relative	**Similarity (%)**	**Accession Number**	**17 days**	**38 days**	**73 days**	**17 days**	**38 days**	**73 days**	**17 days**	**38 days**	**73 days**
γ-Proteobacteria											
Vibrio alfacsensis	100	KU725824	+	+	+	-	+	+	+	-	-
Vibrio alginolyticus	99	KU725825	+	-	-	-	-	-	-	+	-
Vibrio communis	100	KU725826	-	-	+	+	-	+	+	+	+
Vibrio harveyi	98	KU725827	-	-	-	+	-	+	+	+	+
Vibrio ichthyoenteri	100	KU725828	+	-	+	+	+	+	-	-	+
Vibrio owensii	100	KU725830	-	-	-	+	-	-	+	-	-
Vibrio scophthalmi	98	KU725831	-	-	+	+	+	+	-	+	-
Vibrio tapetis	99	KU725833	+	+	-	+	-	-	-	-	-
Vibrio sp	99	KU725834	+	+	+	+	+	-	-	-	-
Vibrio sp	97	KU725837	-	-	-	-	-	-	-	+	-
Vibrio sp	*98*	KU725838	+	-	-	-	-	-	-	-	-
Vibrio sp	100	KU725836	+	-	-	-	-	-	-	-	-
Firmicutes											
Staphylococcus saprophyticus	100	KU725839	-	-	-	+	-	-	-	-	-

Presence (+); Absence (-)

Table 7 - Closest matches of GenBank database sequences to isolates from intestinal samples of *Solea senegelensis* fed PP35 diets for 17, 38 and 73 days.

			PP35_UN			PP35_PROB			PP35_YEAST		
Closest relative	**Similarity (%)**	**Accession Number**	**17 days**	**38 days**	**73 days**	**17 days**	**38 days**	**73 days**	**17 days**	**38 days**	**73 days**
γ-Proteobacteria											
Vibrio alfacsensis	100	KU725824	+	+	+	-	+	+	-	+	-
Vibrio alginolyticus	98	KU725825	-	-	-	-	-	-	-	-	+
Vibrio communis	99	KU725826	+	-	-	-	-	-	-	-	-
Vibrio harveyi	98	KU725827	-	-	-	-	+	-	+	-	-
Vibrio ichthyoenteri	98	KU725828	-	-	+	-	-	+	-	-	+
Vibrio mytilili	100	KU725829	-	-	-	-	-	-	-	-	-
Vibrio owensii	100	KU725830	+	-	-	-	-	-	-	-	-
Vibrio scophthalmi	98	KU725831	+	+	-	+	-	-	-	-	-
Vibrio sinaloensis	100	KU725832	-	-	-	-	-	-	+	+	+
Vibrio tapetis	99	KU725833	-	+	-	-	-	-	-	-	-
Vibrio sp	99	KU725834	+	+	+	+	+	-	+	-	+
Vibrio sp	99	KU725846	-	-	+	-	-	-	-	-	-
Vibrio sp	100	KU725835	-	+	-	-	-	-	-	+	-
Vibrio sp	97	KU725836	-	-	+	-	-	-	-	-	-
Vibrio sp	98	KU725840	+	-	-	-	-	-	-	-	-
Vibrio sp	99	KU725841	-	-	-	-	-	-	+	+	-
Vibrio sp	98	KU725842	-	-	-	-	-	-	+	-	-
Vibrio sp	99	KU725843	-	+	-	-	-	-	-	-	-
Firmicutes											
Staphylococcus saprophyticus	100	KU725839	-	-	-	+	+	+	-	-	-

Presence (+); Absence (-)

4.4 Discussion

Probiotics confer beneficial actions to the host or to their environment through different modes of action. Their application in aquaculture has been regarded as a sustainable and promising strategy, not only in the context of disease control but also in nutrition, growth and immunity (Lazado and Caipang, 2014a).

The capacity of Senegalese sole to cope with diets in which the marine-derived protein was replaced by plant ingredients has been previously reported (Cabral et al., 2011; Cabral et al., 2013; Silva et al., 2009). Similarly, to previous studies, the current study demonstrated that growth performance of Senegalese sole was not affected by high content of plant ingredients. Final body weight and FCR was, however, negatively affected by dietary probiotic supplementation (PROB diet). In fact, the effects of probiotics and prebiotics on fish growth performance are often contradictory. Probiotic improved the feed efficiency and growth performance in Senegalese sole (García de la Banda et al., 2012), Japanese flounder (Taoka et al., 2006a) and Atlantic cod (Lauzon et al., 2010). Nevertheless, Ferguson et al. (2010) showed no clear effect on growth performance in Nile tilapia fed with *Pediococcus acidilactici* supplementation, whereas Gunther and Jimenez-Montealegre (2004) observed a growth depression in tilapia fed *Bacillus subtilis*.

All humoral immune parameters analysed, plasma lysozyme, peroxidase and alternative complement (ACH50) activities were not significantly altered by PROB or YEAST. This trend was also observed by Batista et al. (2014) working with sole fed the same multispecies bacteria and Díaz-Rosales et al. (2009) working with sole fed with two different *Shewanella* sp. However, ACH50 was enhanced when rainbow trout were fed for 4 weeks with diets supplemented with heat-inactivated multispecies bacteria (Choi and Yoon, 2008) and gilthead seabream fed for 3 weeks (Salinas et al., 2008). In addition, serum lysozyme activity increased significantly from 438 ± 75 U ml^{-1} (control) to 1269 ± 134 U ml^{-1} in rainbow trout fed for 14 days with diets supplemented with a autochthonous intestinal *Bacillus subtilis* (Newaj-Fyzul et al., 2007). These changes in the innate immune responses were associated to the inhibitory effect of that probiotic against the pathogenic *Aeromonas sp*.

Previous studies have demonstrated that oral administration of fungal carbohydrates enhance fish innate immune response and protection against infections (Efthimiou, 1996; Esteban et al., 2001; Siwicki et al., 1994), although this effect seems to be temporary (reversible) and dependent on its inclusion level and

feeding duration (Ortuño et al., 2002). In the current study, innate immune parameters were affected by the increase of dietary plant ingredients content. At 17 and 38 days of feeding trial, fish from PP72 groups had higher values of lysozyme and ACH50 activities than fish from PP35 groups, suggesting a stimulation of the innate immune activity by dietary high PP content. Also Geay et al. (2011) observed an increase in the ACH50, while the lysozyme was lower, in fish fed a PP diet when compared to fish fed fishmeal based diet. On the other hand, Sitjà-Bobadilla et al. (2005) reported that high level of fishmeal replacement by plant protein sources had no significant effect on lysozyme activity. Jalili et al. (2013) showed that rainbow trout fed diets with high PP levels (70 and 100%) resulted in a decrease on the ACH50, as reported by Sitjà-Bobadilla et al. (2005) in gilthead seabream. Hepatocytes provide the major source of complement factors (Abelseth et al., 2003) and the progressive fat degeneration of liver with the PP inclusion could decrease complement proteins synthesis (Sitjà-Bobadilla et al., 2005). According to our results, this effect could be improved by a long-term feeding the diets with high plant ingredients content. Liver from fish fed PP75 diets showed a higher level of vacuolization comparing to fish fed PP35 diets. The use of high levels of plant ingredients is recognised to have several disadvantages, particularly related to the amino acid profiles and unsaturated fatty acid imbalances but especially due to their levels of anti-nutritional factors (Geay et al., 2011). In the current study, high PP level seems to result in an acute stimulation of lysozyme and ACH50, since at 2 and 17 days fish fed PP72 diets showed higher values than fish fed PP35 diets. Nevertheless, at 73 days of feeding trial such effects disappeared. Food legumes and cereals may have adverse effects on some fish species after long-term intake, resulting in morphological problems in liver and intestine that could lead to metabolic failure (Russell et al., 2001). PP72 diet, when compared to PP35, was formulated to contain a low amount of soybean meal, but high content of mixed plant ingredients, including insoluble pea protein, as protein source replacing fishmeal. Thus, the dietary incorporation level of plant protein sources used in the current study may have provided a high level of antinutritional factors.

Intestinal microbiota is confined within a highly specialized barrier defenses, composed by the stratified mucous layer, an epithelium and a lamina propria with several innate and adaptive immune cells (Maynard et al. 2012). An increase in intestine section area, villus length and villus width are directly related to an increase

of surface area, which may indicate improvement of intestinal absorptive capacity nutrients (Caspary, 1992). At 2 days of feeding, fish fed PP72_UN showed larger intestine section area and longer villus than fish fed PP35_UN. At 17 days of feeding, fish fed PP72_UN showed more goblet cells than the other dietary groups, except the group fed PP35_YEAST. At 73 days, however, villus length and width, and goblet cells were significantly increased in fish fed low PP diets compared to high PP diets. The latter may indicate short-term feeding fish with diets formulated with high plant ingredients level may enhance the immune defence and improve surface area for absorption. However, when animals are fed high PP diets for longer periods the effects were reversed, probably due to habituation of fish and/or to a negative effect of chronic ingestion of high PP diets and presence of antinutritional factors. Intestinal epithelium is covered by a layer of mucus continuously produced by goblet cells and being the first line of defense against microbes (Maynard et al., 2012). So, the reduced number of goblet cells, after 73 days of feeding fish with the PP72 diets, causes a decrease in mucous production, thus probably reducing intestinal protection capacity of those fish.

After 38 days of feeding the experimental diets, villus length was significantly increased in YEAST supplemented groups, comparing to unsupplemented (UN) groups. Yeast cells have been reported to be a source of nucleotides, which contribute for the intestinal maintenance in aquatic animals by improving mucosal flora and mucosal surfaces with relative elongation of the intestinal tract (Li et al., 2007). Also, (Abu-Elala et al., 2013) observed that fish treated with *S. cerevisiae*, showed yeast colonization in intestine, accompanied by an increase in the length and density of the intestinal villus. Such morphological alterations are often associated with an improve in food digestion and absorption. After 2 days feeding the YEAST diets, fish had a higher hepatocyte glycogen content comparing with fish fed diets supplemented with PROB, showing a stimulation of the glycogen liver storage, the first line of energy source when blood glucose concentration falls during stress. *S. cerevisiae* used in YEAST diets is rich in bioactive ingredients and nutrients such as cell wall carbohydrates. According to Wilson (1994), fish fed with diets rich in digestible carbohydrates, showed high hepatic glycogen reserves. However, when fish is fed YEAST for longer duration (73 days), such energy benefit content disappeared.

Fish intestinal microbiota changes with the diet and this effect is of particular interest considering that the effects of high fishmeal replacement by plant ingredients are still poorly understood, especially in flatfish. Molecular techniques have facilitated culture-independent studies, becoming a valid support to traditional techniques (Ercolini, 2004). In the current study, from all bacteria isolated from sole intestine, approximately 20% were not identified. The intestinal tract of fish harbours a high density of non-culturable bacteria and the composition of which has not yet been reported, leading to differences between viable and total microbial counts (Shiina et al., 2006). In this study, it has been possible to demonstrate the ability to modulate the intestinal microbiota of Senegalese sole by feeding diets supplemented either with probiotic or autolyzed *Saccharomyces cerevisiae*. The results obtained are in accordance with those reported by Standen et al. (2015), who observed in tilapia (*Oreochromis niloticus*) the modulation of the intestinal microbiota and morphology caused by feeding the fish with same multispecies probiotic used in the current study. The modulation exerted on the intestinal microbiota by viable and inactivated cells of *Saccharomyces cerevisiae* have been reported in tilapia and sturgeon (*Huso huso*) (Hoseinifar et al., 2011; Waché et al., 2006). In addition, the modulation of intestinal microbiota has also been demonstrated in fish treated with *S. cerevisiae* fermentation products (He et al., 2011) and glucans obtained from yeast (Kuhlwein et al., 2013). In the current study, the predominant bacteria found in *Solea senegalensis* intestine were *Vibrio* sp.*,* which is in agreement with other studies carried out with the same flatfish cultured under extensive, semi-extensive or intensive production systems (Martin-Antonio et al., 2007). This trend was also detected in farmed Senegalese sole fed fresh or lyophilized *Shewanella putrefaciens* (Tapia-Paniagua et al., 2010; 2015).

Fish fed dietary probiotic supplementation (PP35_PROB and PP72_PROB) had reduced intestinal bacteria related to *Vibrio* species*.* Previous studies observed that strains of probiotic *Bacillus* (Liu et al., 2015; Luis-Villasenor et al., 2015; Wu et al., 2014), *Enterococcus* (Lin et al., 2013) and *Pediococcus* and *Lactobacillus* (Munoz-Atienza et al., 2013) showed an antibacterial activity against fish pathogens, including *Vibrio*.

V. harveyi was detected in sole fed probiotic supplemented and especially in fish fed yeast supplemented diets, and not detected in fish fed unsupplemented diets. These *Vibrio* species are included in the Harveyi Clade and are well-known as

pathogenic for marine farmed organisms (Austin and Zhang, 2006; Cano-Gomez et al., 2011; Gomez-Gil et al., 2004), including *Solea senegalensis* in the case of *V. harveyi* (Arijo et al., 2005a; Zorrilla et al., 2003). However, it is frequent the presence of virulent and non-virulent strains of *V. harveyi* (Rico et al., 2008; Zorrilla et al., 2003), and for this reason more studies are necessary to evaluate the potential virulence of the strains of these species and how diet formulation may modulate bacterial presence in the intestine. On the other hand, the dietary probiotic supplementation in PP35 diet (PP35_PROB) induced the presence of *S. saprophyticus. S. saprophyticus* is associated to microorganisms that play a critical role in fish fermentation and are known to be resistant to different antibiotics (Sergelidis et al., 2014; Zhang et al., 2015). In the current study, *S. saprophyticus* were detected solely in fish fed probiotic diets, especially in low PP groups. Although there are no reports that *S. saprophyticus* caused diseases in fish, Sun et al. (2011) speculate that may be a potential harmful bacterium as it is often implicated in human urinary tract infections (Kuroda *et al.* 2005). Its presence in the intestinal microbiota of fish must be considered as a route for the transmission of antibiotic resistance (Chajecka-Wierzchowska et al., 2015).

4.5 Conclusion

Senegalese sole was able to cope with high plant ingredient content in replacement of marine-derived ingredients. The short-term feeding with high PP content may enhance the immune defence and improve the intestinal surface area, beneficial for increasing intestinal absorptive capacity in fish. However, it seems that this is a temporary (acute) effect that was reversed in long-term feeding high PP diets. Fish fed PP72 showed reduced number of the goblet cells and low hepatic glycogen content, indicating a reduced capacity to overcome stress situations, since they seem to have less immune competence and energy storage. Fish fed for 38 days with YEAST diets had longer villus than unsupplemented groups.

It has been possible to demonstrate the ability to modulate the intestinal microbiota of Senegalese sole by dietary supplementation with probiotic or autolyzed *Saccharomyces cerevisiae*. The predominant bacteria present in sole intestine, were *Vibrio sp.*, and there was an inhibitory effect of dietary probiotic supplementation on *Vibrio sp.* colonization, regardless of the dietary PP level.

4.6 Acknowledgements

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CHAPTER 5

Effects of autochthonous intestine bacteria on growth, disease resistance, intestinal morphology and microbiota in Senegalese sole infected with *Photobacterium damselae* sp. *piscicida*

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Effects of autochthonous intestine bacteria on growth, disease resistance, intestinal morphology and microbiota in Senegalese sole infected with *Photobacterium damselae* sp. *piscicida*

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Abstract

In fish farms, the control of bacterial pathogens is achieved by the administration of chemotherapeutic agents, which could lead to the emergence of drug-resistant microorganism and antibiotic residues. Probiotics used in aquaculture may be useful feed additives to combat diseases, improve growth and stimulate immunity response of fish. A growth trial followed by a bacterial challenge were carried out in order to evaluate the benefits of using autochthonous fish bacteria as a dietary probiotic treatment in Senegalese sole (*Solea senegalensis*). *Enterococcus raffinosus* (PB1) and *Pseudomonas protegens* (PB2) were isolated from sole intestine and incorporated in the experimental diets at 1.6×10^{10} and 1.3×10^{10} CFU kg feed^{-1}, respectively. A total of 264 sole (22.65 ± 0.15 g) were distributed into the three dietary treatments: a control diet without probiotics (CTRL) and two probiotic diets (PB1 and PB2). Fish were fed the experimental diets for 36 days during the growth trial and then for the following 17 days of bacterial challenge. Growth performance, disease resistance, immune responses, intestinal morphology and changes in intestinal microbiota were evaluated. Growth performance was not affected by dietary probiotic supplementation. Fish fed PB1 diet had an increase in muscular layer thickness (115.3 ± 5.1 µm) when compared to PB2 diet (88.8 ± 5.2 µm) ($p < 0.01$), and an increase in goblet cell number (240.2 ± 35.1) when compared to control (117.6 ± 25.8) and PB2 (110.6 ± 26.1) diets ($p < 0.01$). Intestinal microbiota of posterior (66.0 ± 13.5%) and distal (62.3 ± 16.8%) intestine were similar in fish fed probiotic diets. In contrast, fish fed CTRL diet showed low similarity with that of fish fed PB1 (proximal: 48.1 ± 18.3%; distal: 49.3 ± 30.3%) and PB2 (proximal: 49.1 ± 18.7%; distal: 45.7 ± 28.7%) diets, indicating significant differences in the microbial populations between CTRL and the two probiotic groups. At the end of the challenge, non-infected fish fed CTRL diet had an increase in villus length (609.3 ± 13.2 µm) compared to fish fed PB1 infected (488.1 ± 22.4 µm) or non-infected (381.5 ± 88.1 µm) groups ($p < 0.01$). Cumulative mortality achieved 14.3%, 13.8% and 7.1% in PB2, control and PB1 groups, respectively. At 15 days post-challenge, non-infected fish showed higher peroxidase activity (48.5 ± 21.1 EU mL^{-1}) than infected fish (30.3 ± 15.5 EU mL^{-1}) ($p < 0.01$), regardless of the diet.

In the present study, it was revealed the influence of the diet and the *Photobacterium damselae* sp. *piscicida* infection in modulating the intestinal microbiota. Dietary

supplementation with *E. raffinosus* had an apparent benefit on protection against photobacteriosis.

5.1. Introduction

The high number of disease outbreaks continues to compromise the intensification of sole (*Solea senegalensis*) aquaculture, leading to significant mortalities and economic losses in Mediterranean countries (Arijo et al., 2005a; Romalde, 2002; Zorrilla et al., 1999). Photobacteriosis is caused by *Photobacterium damsela* ssp. *piscicida* (Phdp), and is the most important disease affecting various marine species (Bakopoulos et al., 2003; Cerezuela et al., 2012c; Mosca et al., 2014; Toranzo et al., 2005; Xing et al., 2013), including sole (Barroso et al., 2014; Costas et al., 2013; Toranzo et al., 2005; Zorrilla et al., 1999) at the early grow-out stages. Phdp is a rod-shaped gram-negative bacterium (Magariños et al., 1996; Romalde, 2002) which can induce whitish tubercles in the internal organs and haemorrhages on head and/or fins in chronically infected fish.

Conventionally, antibiotics and chemical disinfectants have been used as the first defence line against Phdp. However, diseased fish eat poorly the medicated food and the emerging of drug-resistant pathogens, limit their use substantially. Moreover, only a limited number of effective drugs/chemicals are available for treatment in aquaculture. In addition, recent studies on the use of probiotics to control *Photobacteriosis* have given encouraging results (García de la Banda et al., 2012; Tapia-Paniagua et al., 2012).

In this context, probiotics represent alternative prophylactic tools, increasingly used in aquaculture systems, both in water and feed, as biological control agents. Probiotics are defined as live microorganisms which contribute to the proper microbiological balance of host digestive tract, promoting their immune defences and nutritional/ physiological development (Gaggìa et al., 2010). Probiotics treatment may improve fish performance and reduce bacterial pathogen outbreaks, by multiple ways, including pathogen inhibition via production of antagonistic compounds, competition for attachment sites or nutrients, alteration of enzymatic activity of pathogens, immuno-stimulatory functions, improvement of feed digestibility and utilization. Previous studies, have reported several advantages associated to probiotics, like the in vitro tests of inhibition of pathogens, and / or *in vivo* tests that evidenced gut microflora profiles and changes on immune response in different fish species. *Roseobacter* (27-4 strain) isolated from the tank walls of healthy turbot (*Scophthalmus maximus* L.) was show to have a protective effect against *Vibrio anguillarum* in turbot larvae (Planas et al., 2006), leading to improved

survival. Queiroz and Boyd (1998) reported increased survival and growth performance of channel catfish (*Ictalurus punctatus*), when a blend of *Bacillus sp*. was added to the pond rearing water. *Carnobacterium* sp., previously isolated from Atlantic salmon (*Salmo salar*) intestine, was effective in reducing infections caused by *Aeromonas salmonicida*, *V. ordalii*, and *Yersinia ruckeri* in juvenile salmonids (Robertson et al., 2000). An *in vitro* study carried out by Nikoskelainen et al. (2001b) showed that *Lactobacillus rhamnosus* and *L. bulgaricus* were effective in reducing the growth of *A. salmonicida,* isolated from rainbow trout (*Oncorhynchus mykiss*) mucus. A reduction in *V. anguillarium* and *V. ordalii* virulence degree and an increase in phagocytic activity of leukocytes was also reported in trout after oral administration of *Clostridium butyricum* (Sakai et al., 1995), indicating immunostimulation.

Probiotics were also shown to modulate the fish immune system in several fish species resulting in beneficial effect to the host (Aly et al., 2008; Cerezuela et al., 2012b; Irianto and Austin, 2002b). In addition, the effect of probiotics in intestinal structure and gut microbiota has been reported in fish (Lazado and Caipang, 2014b). For instance, Nile tilapia (*Oreochromis niloticus*) fed a diet supplemented with *L. rhamnosus*, showed a significant increase in villus height compared to fish fed the control diet (Pirarat et al., 2011). By contrast, gilthead seabream (*Sparus aurata*) fed *B. subtillis* showed a significant reduction in microvilli height compared to fish fed the control diet (Cerezuela et al., 2012a).

Several probiotic have already been studied as feed additive in Senegalese sole, *Solea senegalensis*. There are recent *in vivo* studies testing commercial probiotics (Barroso et al., 2014; Batista et al., 2014) in Senegalese sole juveniles. Additional studies were carried out testing bacteria recovered from skin mucus of gilthead sea bream in sole diet (Lobo et al., 2014; Tapia-Paniagua et al., 2014; Tapia-Paniagua et al., 2015). To our knowledge, this is the first *in vivo* study testing autochthonous intestinal bacteria as potential dietary probiotics in Senegalese sole juveniles. Their effectiveness to modulate growth performance, disease resistance, intestinal morphology and ecology will be evaluated.

5.2. Materials and methods

All procedures were approved by the Ethics and Animal Welfare Committee of Institute of Aquaculture Torre de la Sal (Spain) and supervised by the Ethics experts

of the TNA selection panel of the AQUAEXCEL project. It was carried out in a registered installation (code 36271-42-A) in accordance with the principles published in the European animal directive (2010/63/EU) and Spanish laws (Royal Decree RD53/2013) for the protection of animals used in scientific experiments. In all lethal samplings, all efforts were made to minimize suffering. Fish were fasted for 24 h and then were decapitated after an over-exposure with 3-aminobenzoic acid ethyl ester (MS-222, 100 μg mL^{-1}).

5.2.1. Screening procedure for candidate strain probiotics

The autochthonous intestine bacteria tested in the current study as candidate probiotics for Senegalese sole were selected using *in vitro* antagonism tests based on the production of inhibitory compounds against common fish pathogens. 250 bacteria strains, isolated from the intestine microbiota of farmed healthy sole, were tested for their antimicrobial activity against several common fish pathogens. Thereafter, the 16 bacteria strains with the best results were tested for their stability and viability during feed processing. Among all bacteria tested, *Enterococcus raffinosus* and *Pseudomonas protegens* were selected due to their *in vitro* antimicrobial activity (not published results) and their capacity to survive during freeze-drying and encapsulation (not published results).

5.2.2. Experimental diets and probiotics isolation

A basal diet (57% crude protein, 9% crude lipid) formulated by SPAROS S.A. (Olhão, Portugal) was used as the control (CTRL) without probiotic supplementation. Two probiotic diets (PB1 and PB2) were also formulated by supplementing the basal diet with *E. raffinosus* or *Ps. protegens*, respectively (Table 1). *E. raffinosus* and *Ps. protegens* were previously isolated from Senegalese sole intestinal microbiota and identified by the amplification and sequencing of a fragment of 16S rDNA gen. Bacterial strains were revived in 10 mL of LB broth (Luria Bertani Broth, Invitrogen) and subsequently mass cultured in flasks containing 5 L of LB broth for 36 h at room temperature (20-22 °C) with continuous shaking. Upon incubation, cultures were harvested by centrifugation at 5000 rpm, at 4ºC for 20 minutes (Thermo Heraeus Multifuge X3 FR, Thermo Scientific), and washed three times with sterile saline water (0.85%, w/v, NaCl, Panreac). In aquaculture, the dose of probiotics usually varies from 10^{6-10} CFU g $feed^{-1}$ (Panigrahi et al., 2004). The

harvested bacteria were suspended in 150 mL of saline solution to obtain a dose of 10^{10} colony forming units (CFU) mL^{-1}. To verify the dose, appropriate dilutions of each bacterial culture, were spread onto plates of LB agar (Luria Bertani Agar, Invitrogen) following Miles and Misra method (Miles et al., 1938) and incubated at 25ºC for 24 h. Then, each bacteria suspension was incorporated in the extruded pellets by means of vacuum coating (Dinnisen Pegasus vacuum mixer, PG-10VCLAB) using fish oil as a carrier. The viability of the incorporated strains was assessed by vortexing 10 g of each diet in 90 mL of peptone water and preparing serial dilutions, which were spread on LB agar (Luria Bertani Agar, Invitrogen) plates as above. The bacterial counts were able to confirm the established dietary incorporation of each strain and their survival and viability in the diet preparations for at least 4 weeks. *E. raffinosus* was incorporated in the diets at 1.6×10^{10} CFU kg^{-1} feed and *Ps. protegens* at 1.3×10^{10} CFU kg^{-1} feed. During the growth and challenge trials, diets were stored at 4ºC and new diet batches of the diets was prepared every 4 weeks.

5.2.3. Fish and rearing conditions during growth trial

Both trials were carried out at the indoor experimental facilities of the Pathology Unit of IATS-CSIC (Castellón, Spain). These facilities ensured the adequate water quality and the correct disinfection of the effluent water and the material in contact with the pathogen bacteria used during the bacterial challenge. Non vaccinated Senegalese sole were obtained from a commercial fish farm (Aquacria S.A., Portugal) and acclimatized for 20 days to IATS experimental facilities. Fish were fed during the acclimatization period with the CTRL diet in a flow-through system of flat-bottomed tanks with aerated seawater.

After acclimation, 264 fish (22.65 ± 0.15 g body weight) were randomly distributed (22 fish / tank, 22.6 ± 0.2 g each) into 12 90 L-fibreglass tanks, supplied with seawater in a flow-through system. Fish were fed for 36 days the experimental diets (n=4 tanks / treatment) to apparent satiety with automatic feeders. Visual observation of acceptance and refusal of feed was used to adjust daily ration, as previously described by (Borges et al., 2009). Water parameters were daily monitored with salinity at 37.5 ± 0.1 ppm, temperature at 18.0 ± 1.1 ºC, oxygen levels always higher than 85% saturation and unionized ammonia below toxic levels

(< 0.05 mg L^{-1}). The tanks were under natural photoperiod at our latitude (40°5´N; 0°10´E).

Table 1 - Feed ingredients and proximate composition of the experimental diet

	DIET
Feed ingredients (%)	
Fishmeal 70 L[a]	24.5
Fishmeal 60 (COFACO)[b]	27.0
CPSP90[c]	5.0
Squid meal	5.0
Soybean meal (micro)[e]	12.5
Extruded peas (Aquatex G2000)[f]	11.0
Wheat meal	11.0
Fish oil	2.0
Vitamin[g] and Mineral Premix[h]	1.0
Binder (Alginate)[i]	1.0
Proximate composition	
Dry Matter (DM, %)	91.02
Crude protein (%DM)	57.49
Crude fat (%DM)	8.82
Ash	16.41
Gross Energy (kj g−1DM)	20.32

[a]Peruvian fishmeal LT (71% crude protein, 11% crude fat, EXALMAR, Peru); [b]Fair Average Quality (FAQ) fishmeal (62% crude protein, 12% crude fat, COFACO, Portugal); [c]Soluble fish protein hydrolysate (87% crude protein, 6.5% crude fat, Sopropêche, France); [d]Soycomil-P (soy protein concentrate, 65% crude protein, 0.7% crude fat, ADM, The Netherlands); [e]Dehulled solvent extracted soybean meal (micronized); [f]Aquatex G2000 (Dehulled, grinded pea grits, 24% crude protein, 0.4% crude fat, SOTEXPRO, France); [g]Vitamins (mg or IU kg^{-1}diet): Vitamin A (retinyl acetate), 20000 IU; vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1 (thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B12 (cyanocobalamin), 0.1 mg; vitamin B5 (pantothenic acid), 100 mg; vitamin B3 (nicotinic acid), 200 mg; vitamin B9 (folic acid), 15 mg; vitamin H (biotin), 3mg; betaine, 500 mg; inositol, 500 mg; choline chloride, 1000 mg; vitamin C (stay C), 1000 mg; vitamin E, 100 mg; [h]Minerals (% or mg kg^{-1}diet): Mn (manganese oxide), 9.6 mg; I (potassium iodide), 0.5 mg; Cu (cupric sulfate), 9 mg; Co (cobalt sulfate), 0.65 mg; Zn (zinc oxide), 7.5 mg; Se (sodium selenite), 0.01 mg; Fe (iron sulfate), 6 mg; Cl (sodium chloride), 2.41%; Ca (calcium carbonate), 18,6%; NaCl (sodium), 4%; [i]Diatomaceous earth: Kielseguhr: LIGRANA GmbH, Germany.

5.2.4. Pathogen challenge

5.2.4.1. Pathogen inoculum preparation and challenge dose validation

Phdp strain Lab 2 used in this study was kindly provided by Dr. Belén Fouz and Skretting Spain, isolated from diseased European seabass (*Dicentrarchus labrax*), was cultured in tryptic soy agar (TSA, Pronadisa, Madrid, Spain) supplemented with NaCl at a final concentration of 1% (TSA-1) and sheep blood at a final concentration of 5% (TSAB-1), at 22 °C for 48 hours.

To identify the suitable infective dose for the bacterial challenge, four different doses were tested in a pre-challenge experiment. Juvenile sole, held in 90 L tanks at an average temperature of 19 °C, were intracoelomically (i.c.) injected with 0.1 ml of four different bacterial suspensions (D1, – 3.0×10^7, D2 – 6.0×10^6, D3 – 1.4×10^6 and D4 – 7.0×10^5 CFU mL^{-1}) in phosphate-buffered saline (pH 7.4) at a ratio of 8 fish/dose, according to Azeredo et al. (2015) and to Fouz et al. (2010). Negative control fish were i.c. injected 0.1 ml of PBS (non-infected group). Mortality was recorded daily during 3 weeks and the cause of death was considered due to *Phdp* if the inoculated bacterium was recovered in pure culture from internal organs (kidney and liver) streaked onto TSAB-1 plates. Identification of the pathogen was carried out by means of an agglutination test with the corresponding antiserum. The lethal dose causing 30% of mortality (LD_{30}) was chosen in order to follow the ethics specifications of the AQUAEXCEL TNA selection committee that considered LD_{30} to be sufficient to answer the question of whether fish fed the supplemented diet resist disease better than controls.

5.2.4.2. Bacterial challenge

At the end of the growth trial, a total of 85 fish (29 from CTRL, 28 from PB1 and 28 from PB2) were i.c. injected with the LD_{30} of Phdp. Infected fish were returned to the original tanks (13-15 fish $tank^{-1}$), and kept under the same rearing conditions as the growth trial. Similarly, 84 fish from each dietary treatment (28, 27 and 29 from CTRL, PB1 and PB2, respectively) were i.c. injected with PBS and then returned to the original tanks (non-infected groups). Fish were fed daily the experimental diets during the post-challenge (p.c.) period at a fixed rate of 1% body weight.

Fish mortality was monitored daily until no more mortalities were recorded for a minimum of two consecutive days. Post-mortem examination was performed by standard microbiological methods, based on pathogen culturing and isolation steps as described above. Cumulative mortality (CM) was calculated per dietary treatment as follows: CM = “number of dead fish” “initial total number of fish”$^{-1}$ × 100.

5.2.5. Sampling procedures

Prior to sampling, fish were fasted for 24 h and then sacrificed with an overdose of MS-222. Fish were individually weighed and measured at the beginning and at the end of the growth trial, in order to determine growth performance. Eight fish from the

initial stock and two fish from each tank at the end of the growth trial (8 fish per treatment) were sampled and stored at -20 ºC for whole body composition analyses. Total feed consumption was registered during the whole trial. Liver and viscera from other 12 fish per treatment were sampled to calculate hepatosomatic (HSI) and viscerosomatic (VSI) indexes. HSI was calculated as: 100 × [liver weight (g) / whole body weight (g)] and VSI as 100 × [viscera weight (g) / whole body weight (g)].

The innate immune response was assessed at the end of the growth trial and at 2 and 15 days post-challenge (p.c.). Blood was withdrawn from the caudal vein of 12 fish (end of the growth trial) or 6 fish (2 and 15 days p.c.) per treatment using heparinised syringes and centrifuged at 5000 × g for 10 min at 4 ºC for plasma collection. Humoral innate immune parameters, lysozyme, peroxidase and alternative complement pathway (ACH50) activities, were analysed in plasma. Intestine histology and microbiota diversity were assessed at the end of the growth trial (8 fish per treatment) and 15 days of p.c. (6 fish per treatment). The intestine of each fish was collected, and a segment with 1.5 cm length from the proximal intestine was cut and stored in buffered formalin for the histological analyses. PI and DI segments were aseptically removed and stored at -80 ºC for examine intestinal microbiota diversity (DGGE) and for PCR-based analyses for *E. raffinosus* and *Ps. protegens* detection.

5.2.6. Proximate composition

Frozen whole fish from each tank were minced without thawing, using a meat mincer, pooled and moisture content was determined. Fish were subsequently freeze-dried before further analysis. Feed and whole body samples were analysed for dry matter (105°C for 24 h), ash (Nabertherm L9/11/B170; Germany; 550ºC for 6 h), crude protein (N × 6.25, Leco N analyser, Model FP-528, Leco Corporation, St. Joseph, USA), crude lipid (petroleum ether extraction, 40 - 60ºC, Soxtherm, Gerhardt, Germany) and gross energy (adiabatic bomb calorimeter, Werke C2000, IKA, Germany).

5.2.7. Detection of *E. raffinosus* and *Ps. protegens* in sole intestine

A PCR-based method was used to detect the presence / absence of *E. raffinosus* and *Ps. protegens* in the PI and DI of fish collected at the end of the growth trial. DNA was extracted using cetyltrimethylammoniumbromide (Zhou et al., 1996). A

specific combination of primers for the detection of both bacteria was designed using the Beacon Designer™ software. The sequences with the accession numbers Y18296 and AJ278812 were used for *E. raffinosus* and *Ps. protegens*, respectively. A set of primers (0.2 µM each) were then used for the amplification by PCR using the EmeraldAmp® Max PCR Master Mix (Takara Bio Inc., Otsu, Japan) in a 50 µL reaction. The primer pair F - GCGTGAGTGAAGAAGGT and R – CTGGTTAGATACCGTCAA was used for *E. raffinosus* detection and the primer pair F – ACCTCACGCTATTAGATG and R – ATCCTCTCAGACCAGTTA was used for *Ps. protegens* detection. Samples were denatured for 5 min at 96°C and then subjected to 45 cycles of amplification (1 min at 94ºC, 1 min at 56ºC and 2 min at 72ºC) and an extension for 10 min at 72°C. Then samples were loaded into a 1.5% (w/v) agarose gel containing SYBR safe DNA gel stain (Invitrogen, Carlsbad, CA, USA) for gel electrophoresis. Identity of the amplicons were confirmed by Sanger sequencing.

5.2.8. Humoral innate immune parameters

All measurements were done in triplicate on a Power-Wave^{TM} microplate spectrophotometer (BioTek Synergy HT, USA). Plasma lysozyme activity (EU ml^{-1}) was determined using a turbidimetric assay adapted to microtitre, as described by Hutchinson and Manning (1996). One lysozyme enzyme unit (EU) was defined as the amount of lysozyme that caused a decrease in 0.001 absorbance units per min. One lysozyme enzyme unit (EU) was defined as the amount of lysozyme that caused a decrease in absorbance of levels per min. Plasma peroxidase activity (EU ml^{-1}) was measured following the procedure adapted to *Solea senegalensis* by Costas et al. (2011), defining that one unit of peroxidase produces an absorbance change of 1 OD. Alternative complement pathway activity (ACH50) was based on the lysis of rabbit red blood cells (2.8×10^8 cells mL^{-1}), and measured as reported by Sunyer and Tort (1995). ACH50 units were defined as the concentration of plasma giving 50% lysis of rabbit red blood cells.

5.2.9. Intestine morphological evaluation

Proximal intestine segments (1.5 cm length) were fixed in phosphate buffered formalin (4%, pH 7, VWR, Portugal) for 24 hours. Samples were subsequently dehydrated and embedded in paraffin according to standard histological

procedures. Thereafter, 3 µm-thick sections were obtained and stained with haematoxylin and eosin (H&E - Merck, Portugal) and Periodic acid-Schiff (PAS - Merck, Portugal) before examination under a light microscope (Olympus BX51, cell^B software, GmbH, Hamburg, Germany). The intestine section area (mm^2), villus height (µm), villus width (µm), muscular layer thickness (µm) and PAS-positive goblet cells (GC - number per section) were determined according described to Batista et al. (2016).

5.2.10. Intestinal microbiota composition

The DGGE analysis of PCR-amplified 16S rRNA genes was carried out in the proximal and distal intestine of 2 - 4 fish per treatment, to determine the similarity indices (100% similarity, represents complete identity) of intestinal microbiota diversity between treatments. DNA was extracted using cetyltrimethylammoniumbromide (Zhou et al., 1996). The PCR amplification of DNA was carried out using the primer combination of 341F with GC clamp, CCTACGGGAGGCAGCAG and 907R, CCGTCAATTCMTTTGAGTTT (Muyzer et al., 1995), by a modified touchdown protocol as described by Dhanasiri et al. (2011), except for the temperature of the initial denaturation step (96ºC). DGGE was performed on the PCR products from DNA samples using a 16 × 16 cm gel on the Bio-Rad DCode ™ system (Bio-Rad, USA), as described by Dhanasiri et al. (2011) but using a 30 to 55% (w/v) urea and formamide denaturing gradient. PCR products from proximal and distal intestine were loaded into separated gels and electrophoresis was performed with 0.5% TAE buffer, at 20 V for 15 minutes and then at a constant voltage of 75 V for 17 hours. DGGE profile analysis (Quantity One® version 4.6.3 software, Bio-Rad) was performed in gels to determine similarity matrices (Dhanasiri et al., 2011).

5.2.11. Calculations of growth performance

Feed conversion ratio (FCR) was calculated as: feed intake (g) / wet weight gain (g), and the daily growth index (DGI, % BW / day) as: $100 \times [(W_1)^{1/3} - (W_0)^{1/3}] / t$, where W_0 and W_1 are the initial and the final body weights (g) and t is the trial duration. Voluntary feed intake (VFI, % BW / day) was calculated as: 100 × (feed intake (g) / ABW (g) / days), where ABW (average body weight) was calculated as:

(W_1+W_0) / 2. The protein efficiency ratio (PER) was calculated as weight gain (g) / protein intake (g).

5.2.12. Statistical analysis

Statistical analyses were performed with the software SPSS (IBM SPSS STATISTICS, 17.0 package, IBM Corporation, New York, USA). Data were analysed for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) and were log-transformed whenever necessary. Data were analysed by a one-way ANOVA (at 36-days of growth trial and in the end of the challenge) considering diet as factor and two-way ANOVA (at 2 and 15 days p.c.) with dietary treatment and infection as main factors. When significant differences were obtained from the ANOVA, Tukey's post hoc tests were carried out to identify significant differences between groups. When data did not meet the ANOVA assumptions, a non-parametric Kruskal–Wallis test was performed for each factor and the pairwise multiple comparison of mean ranks were carried out to identify significant differences between groups. In all cases, the minimum level of significance used was set at $p \leq 0.05$.

5.3. Results

5.3.1. Growth performance and body composition

Fish grew from 22.6 ± 0.2 g to 39.9 ± 1.6 g after 36 days of feeding the experimental diets (Table 2). Daily growth index (1.53 ± 0.06% BW day^{-1}), feed conversion ratio (1.43 ± 0.12), voluntary feed intake (2.07 ± 0.06% BW day^{-1}), and protein efficiency ratio (1.23 ± 0.06) did not differ ($p < 0.05$) between dietary treatments. HSI (1.1 ± 0.0%) and VSI (2.7 ± 0.1 %) indexes did not vary among the dietary groups. Whole body crude protein (17.78 ± 0.39 %), crude lipid (5.09 ± 0.33 %) and gross energy (6.13 ± 0.16 kJ/g) were not significantly affected by the dietary treatments ($p < 0.05$).

5.3.2. Detection of *E. raffinosus* and *Ps. protegens* in the intestine

E. raffinosus (96 bp) and *Ps. protegens* (92 bp) PCR products were detected in the distal and posterior intestine. The image gels from proximal (A) and distal (B) intestine tissues assayed for *E. raffinosus* detection are depicted in Figure 1. All proximal intestine samples (24), regardless of the dietary treatment, presented a 96

bp amplification product (100% present) (Figure 1A). In the distal intestine (Figure 1B), 7 out of 8 fish (88%) fed the PB1 diet presented the 96 bp amplification product. All fish from CTRL (8) and PB2 (8) groups had 100% of presence. Figure 2 shows the image gels from proximal (A) and distal (B) intestine tissues assayed for *Ps. protegens* detection. For both PI and DI samples, 100% of the samples from fish fed PB2 diet presented the amplification product of 92 bp. However, for PI (Figure 2A), *Ps. protegens* was detected in 75% and 88% of samples form fish fed CTRL and PB1 diets, respectively. Also in DI (Figure 2B), only 63% and 50% of samples from fish fed CTRL and PB1 diets, respectively, contained *Ps. protegens*.

Table 2 – Growth performance, nutrient utilization and whole body composition of Senegalese sole juveniles after the 36-day growth trial.

	Dietary treatments			
	CTRL	**PB1**	**PB2**	**p value**
Growth				
IBW (g)	22.62 ± 0.17	22.66 ± 0.18	22.67 ± 0.16	0.92
FBW (g)	39.9 ± 1.6	38.3 ± 1.8	38.1 ± 1.9	0.35
DGI	1.63 ± 0.14	1.50 ± 0.14	1.48 ± 0.15	0.31
FCR	1.34 ± 0.15	1.48 ± 0.11	1.50 ± 0.10	0.20
PER	1.30 ± 0.14	1.19 ± 0.08	1.16 ± 0.07	0.17
VFI	2.05 ± 0.10	2.10 ± 0.12	2.10 ± 0.19	0.84
HSI	1.11 ± 0.07	1.05 ± 0.14	1.10 ± 0.20	0.87
VSI	2.80 ± 0.38	2.65 ± 0.21	2.67 ± 0.31	0.76
Whole body composition (% WW)				
Dry matter	25.08 ± 0.25	24.76 ± 0.79	24.64 ± 0.25	0.29
Ash	2.16 ± 0.08	2.16 ± 0.08	2.17 ± 0.17	0.99
Protein	17.88 ± 0.30	17.76 ± 0.58	17.70 ± 0.32	0.58
Lipid	5.32 ± 0.34	4.99 ± 0.36	4.98 ± 0.22	0.27
Energy (kj)	6.24 ± 0.12	6.09 ± 0.22	6.05 ± 0.09	0.22

The results were not significantly different among the dietary treatments ($p > 0.05$). Abbreviations are: CTRL = Diet with no supplementation; PB1 = Diet supplemented with *Enterococcus raffinosus*; PB1 = Diet supplemented with *Pseudomonas protegens*; IBW = Initial body weight; FBW = Final body weight; SGR = Specific growth rate; FCR = Feed conversion ratio; VFI = Voluntary feed intake; DGI = Daily growth index; PER = Protein efficiency ratio; HIS = Hepatosomatic index; VSI = Viscerosomatic index. Values represent mean ± SD, n = 4, except for HIS and VSI with n = 8.

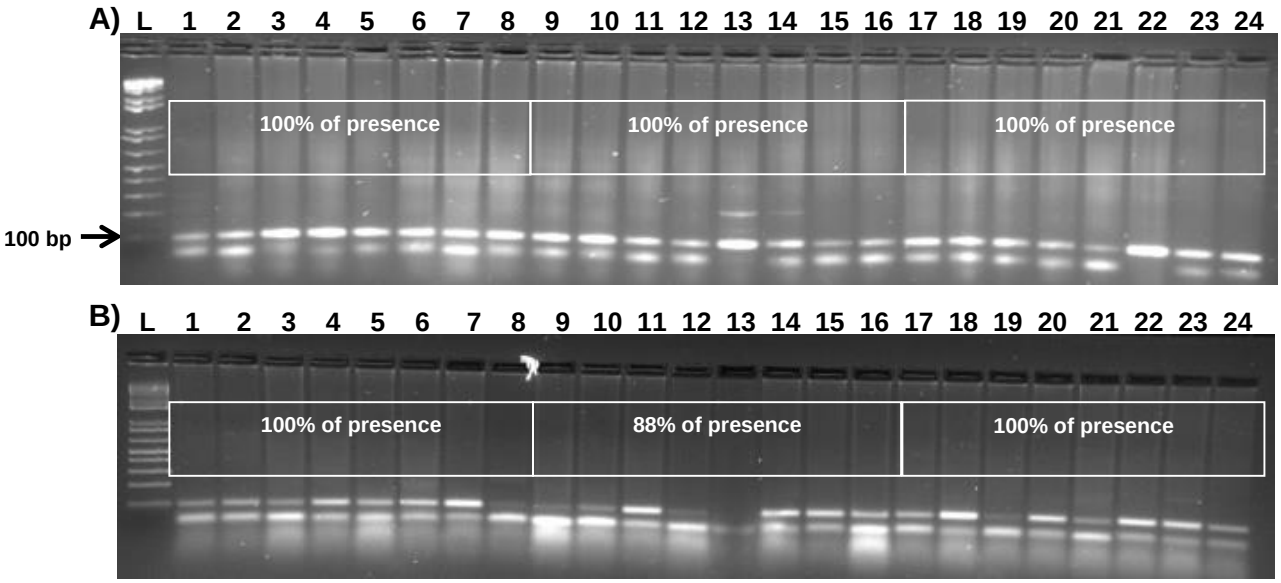


Figure 1 - Agarose electrophoresis of PCR products obtained from the DNA extracted from proximal (A) and distal (B) intestine tissues for *Enterococcus raffinosus* detection (n = 8). Samples collected after the 36-days growth trial. Lanes: L - molecular weight marker 1 Kb Plus Ladder; 1 to 8 – Fish fed CTRL diet; 9 to 16 – Fish fed PB1 diet; 17 to 24 – Fish fed PB2 diet. The 100 bp band is indicated. *E. raffinosus* strain should yeald a single amplicon of 96 bp.

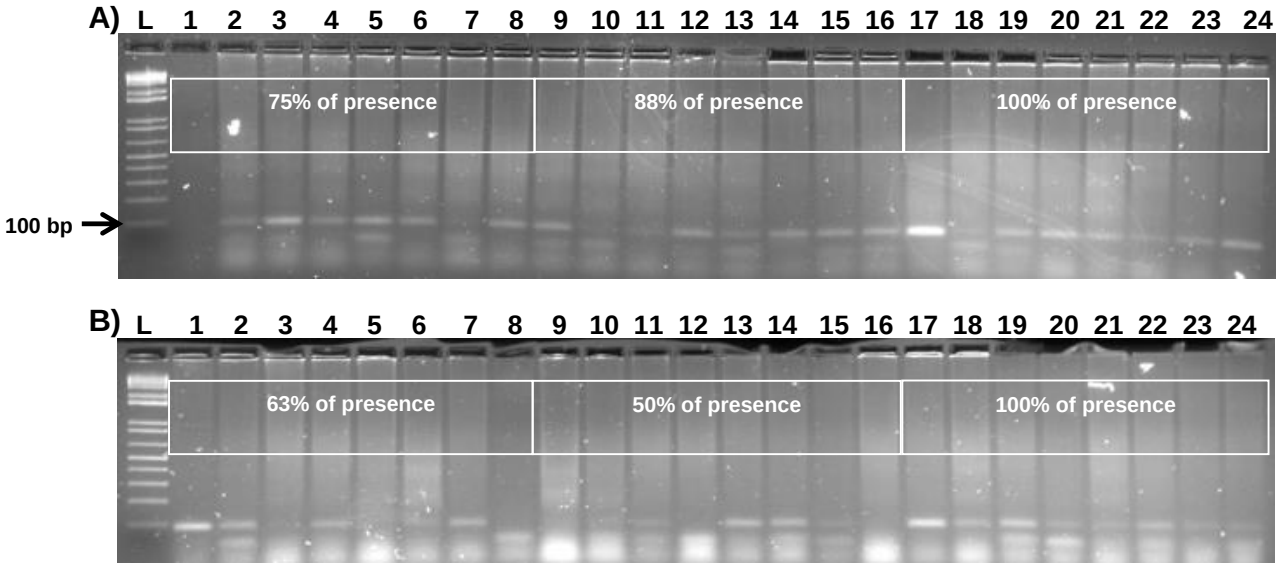


Figure 2 - Agarose electrophoresis of PCR products obtained from the DNA extracted from proximal (A) and distal (B) intestine tissues for *Pseudomonas protegens* detection (n = 8). Samples collected after the 36-days growth trial. Lanes: L - molecular weight marker 1 Kb Plus Ladder; 1 to 8 – Fish fed CTRL diet; 9 to 16 – Fish fed PB1 diet; 17 to 24 – Fish fed PB2 diet. The 100 bp band is indicated. *Ps. protegens* strain should yeald a single amplicon of 92 bp.

5.3.3. Cumulative mortality after challenge

The dose chosen for the infection trial was 1.4×10^6 CFU mL^{-1} as the dose causing 30% mortality (LD_{30}). The mean cumulative mortality of each group during the bacterial challenge is shown in Figure 3. Fish mortality started in PB1 and PB2 groups at day 7 p.c. and in CTRL group at day 8. Cumulative mortality was 14.4%, 10.5% and 7.7% in PB2, CTRL and PB1 groups, respectively at the end of the trial (17 days p.c.). Nevertheless, cumulative mortality was not statistically different among the dietary treatments. No mortality was observed in non-infected groups, regardless of the dietary treatment (data not shown).

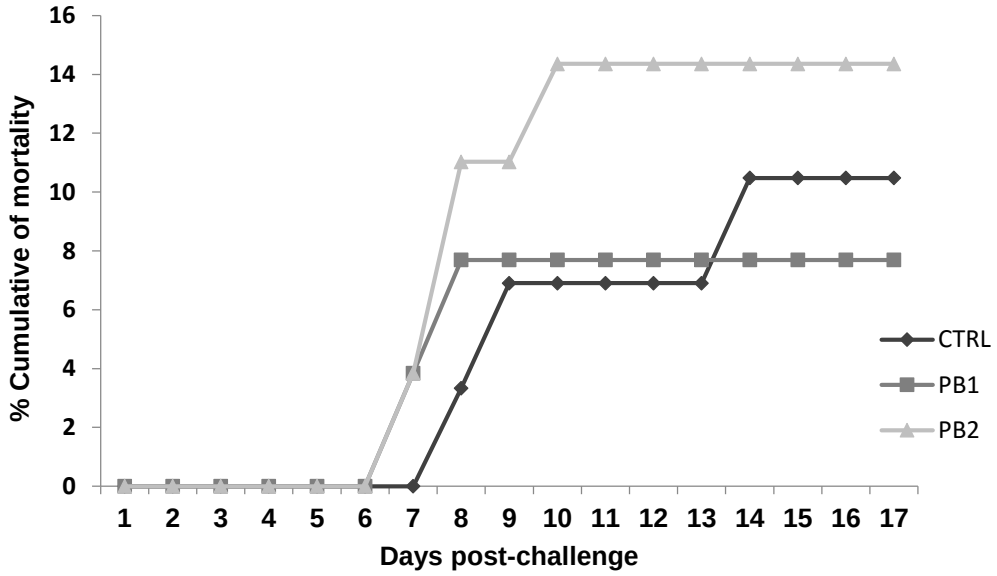


Figure 3 – Cumulative mortality (%) of sole fed the experimental diets for 36 days followed by a challenge with *Photobacterium damselae* subsp *piscicida* (3×10^6 CFU $fish^{-1}$). Values are means of duplicate tanks (28-29 fish/tank). CTRL, PB1 and PB2 represent diets without supplementation, supplemented with *Enterococcus raffinosus* and supplemented with *Pseudomonas protegens*, respectively.

5.3.4. Humoral innate immune parameters

Humoral innate parameters analysed are shown in Table 3. After the 36 days of the growth trial and 2 days p.c., lysozyme, peroxidase and ACH50 activities did not present significant differences ($p < 0.05$) between diets, varying between 886.1 ± 41.6 and 1308.3 ± 154.7 EU mL^{-1}, 53.7 ± 12.74 and 95.1 ± 27.6 EU mL^{-1} and 18.0 ± 2.4 and 30.0 ± 8.4 U mL^{-1}, respectively. At 15 days p.c., non-infected fish showed

significantly higher peroxidase activity (48.5 ± 21.1 EU mL^{-1}) than the infected ones (30.3 ± 15.5 EU mL^{-1}) ($p < 0.01$).

5.3.5. Intestinal morphological evaluation

Morphological evaluation results of the intestinal wall are depicted in Table 4. At the end of the growth trial, fish fed PB1 diet had a significantly thicker muscular layer (115.3 ± 5.1 µm) than PB2 fed fish (88.8 ± 5.2 µm) ($p < 0.01$), and a significantly higher number of PAS-positive GC (240.2 ± 35.1) than control (117.6 ± 25.8) and PB2 (110.6 ± 26.1) fed fish ($p < 0.01$). The analysis performed 15 days p.c. showed that fish fed control diet had a higher villus length (609.3 ± 13.2 µm) than PB1 fed fish, in both infected (488.1 ± 22.4 µm) and non-infected (381.5 ± 88.1 µm) groups ($p < 0.01$).

5.3.6. Assessment of intestinal microbiota

Percentages of similarity obtained for the DGGE profiles of PI and DI of sole at the end of the growth trial are shown in Figure 4A and Figure 4B, respectively. Fish fed the PB1 and PB2 diets had high similarity values for both PI (66.0 ± 13.5 %, mean ± deviation) and DI (62.3 ± 16.8%), however only in PI that difference was statistical significant ($p < 0.05$). Fish fed CTRL diet showed low similarity values with fish fed PB1 (PI: 48.1 ± 18.3%; DI: 49.3 ± 30.3%) or PB2 (PI: 49.1 ± 18.7%; DI: 45.7 ± 28.7%) diets, indicating differences in the microbial populations between CTRL and the two probiotic groups.

At day 15 p.c., the DGGE images generated from the PI (Figure 5) and DI (Figure 6) samples were analysed, considering both infected and non-infected animals. In the PI samples, higher similarity values were observed within the infected fish (Figure 5B), compared to the non-infected fish (Figure 5A). The mean similarity value within the infected fish was more than 60%. In the DI samples, the opposite occurred, showing in this case that non-infected fish (Figure 6A) had higher similarity values between treatments, comparing to the infected ones (Figure 6B). However, this similarity was less evident (> 50%) than the infected fish in the PI segment (> 60%).

Table 3 – Humoral innate immune parameters of Senegalese sole juveniles after the 36-day growth trial and after 2 and 15 days p.c. with *Photobacterium damselae* sp. p*iscicida.*

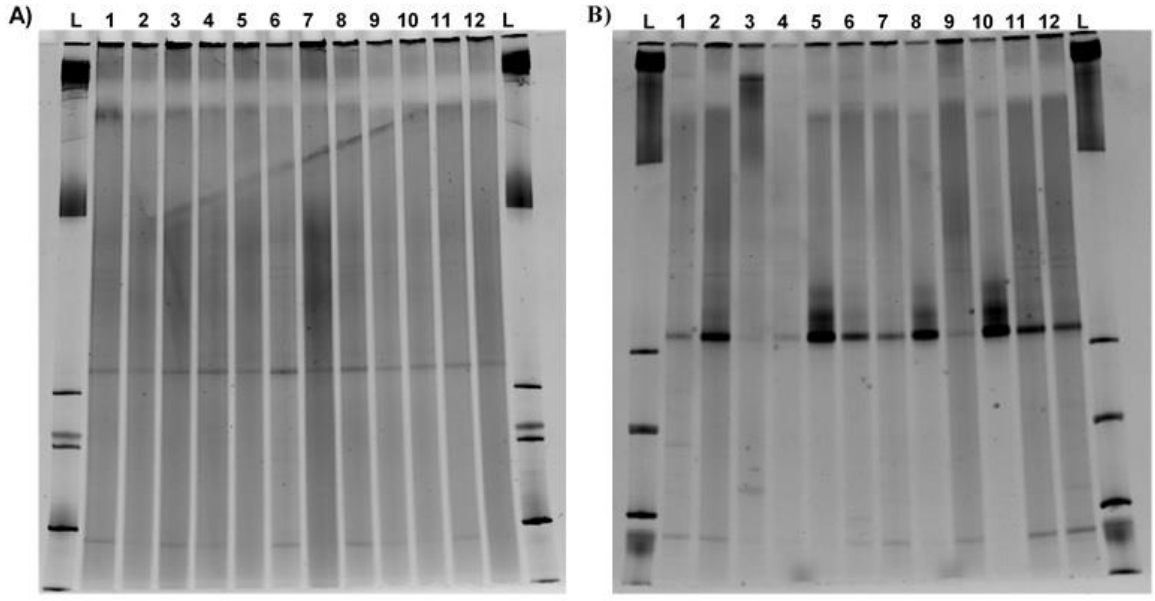
	Dietary treatments								
	CTRL		PB1		PB2		p value		
	NON-INFECTED	***INFECTED***	***NON-INFECTED***	***INFECTED***	***NON-INFECTED***	***INFECTED***	**D**	**I**	**D*I**
36-day growth trial									
Lysozyme (EU mL^{-1})	1164.9 ± 133.3	-	886.1 ± 41.6	-	1308.3 ± 154.7	-		0.17	
Peroxidase (EU mL^{-1})	95.1 ± 27.6	-	61.0 ± 10.4	-	53.7 ± 12.74	-		0.86	
ACH50 (units mL^{-1})	19.7 ± 1.7	-	18.0 ± 2.4	-	30.0 ± 8.4	-		0.29	
2 days post-challenge									
Lysozyme (EU mL^{-1})	1168.5 ± 49.8	1118.5 ± 118.3	1327.8 ± 24.4	1139.4 ± 173.1	1209.3 ± 107.4	1442.6 ± 157.0	0.33	0.99	0.23
Peroxidase (EU mL^{-1})	65.8 ± 28.1	48.2 ± 15.9	30.5 ± 4.1	24.1 ± 2.4	22.9 ± 2.4	34.5 ± 5.3	0.66	0.72	0.68
ACH50 (units mL^{-1})	13.8 ± 1.2	18.9 ± 1.6	21.1 ± 3.7	22.6 ± 4.1	18.1 ± 0.6	20.4 ± 3.7	0.19	0.23	0.82
15 days post-challenge									
Lysozyme (EU mL^{-1})	1223.6 ± 135.7	1413.0 ± 188.4	995.4 ± 107.6	1218.5 ± 209.5	1112.5 ± 104.4	1234.7 ± 234.0	0.46	0.21	0.96
Peroxidase (EU mL^{-1})	58.0 ± 6.5^{x}	35.4 ± 8.8^{y}	38.5 ± 10.3^{x}	20.4 ± 2.5^{y}	49.0 ± 8.0^{x}	35.0 ± 6.8^{y}	0.09	0.01	0.86
ACH50 (units mL^{-1})	18.5 ± 4.2	17.6 ± 1.7	22.7 ± 2.0	22.7 ± 1.8	18.1 ± 2.0	19.1 ± 2.5	0.14	0.98	0.92

In each line, different superscript letters (x, y) indicate significant differences (P<0.01) within a particular diet, caused by infection. Dietary treatments are abbreviated as CTRL, PB1 and PB2 for diets without supplementation, supplemented with *Enterococcus raffinosus* and supplemented with *Pseudomonas protegens*, respectively. Abbreviations are: D = Diet factor; I = Infection factor; EU = Enzyme Unit; ACH50 = Alternative complement pathway activity. Values represent mean ± SE, n = 12 for 36-days growth; n = 6 for 2 and 15 days p.c.

Table 4 - Intestinal morphology of Senegalese sole juveniles after the 36-day growth trial and after 2 and 15 days p.c. with *Photobacterium damselae* sp. p*iscicida.*

	Dietary treatments								
	CTRL		PB1		PB2		p value		
	NON-INFECTED	***INFECTED***	***NON-INFECTED***	***INFECTED***	***NON-INFECTED***	***INFECTED***	**D**	**I**	**D*I**
36-day growth trial									
ISA (mm²)	2.7 ± 0.3	-	2.9 ± 0.1	-	2.5 ± 0.3	-		0.57	
MLT (µm)	107.7 ± 6.6^{ab}	-	115.3 ± 5.1^{a}	-	88.8 ± 5.2^{b}	-		0.01	
VL (µm)	535.0 ± 37.6	-	553.0 ± 12.5	-	494.5 ± 22.4	-		0.32	
VW (µm)	107.6 ± 3.8	-	106.5 ± 2.2	-	99.6 ± 4.4	-		0.26	
GC (nº/ section)	117.6 ± 25.8^{b}	-	240.2 ± 35.1^{a}	-	110.6 ± 26.1^{b}	-		0.01	
15 days post-challenge									
ISA (mm²)	3.3 ± 0.7	3.2 ± 0.3	1.9 ± 0.6	2.6 ± 0.2	3.1 ± 0.2	3.1 ± 0.3	0.06	0.48	0.60
MLT (µm)	84.1 ± 15.6	106.8 ± 7.2	63.3 ± 15.3	108.5 ± 9.0	98.2 ± 9.1	94.0 ± 10.9	0.67	0.11	0.33
VL (µm)	609.3 ± 13.2^{a}	587.2 ± 29.1^{ab}	381.5 ± 88.1^{b}	488.1 ± 22.4^{b}	513.8 ± 31.9^{ab}	552.2 ± 4.2^{ab}	0.00	0.94	0.01
VW (µm)	94.1 ± 14.6	103.4 ± 5.2	71.8 ± 15.8	104.0 ± 2.2	99.8 ± 5.5	99.5 ± 3.3	0.52	0.31	0.31
GC (number/ section)	221.6 ± 45.8	279.6 ± 36.4	256.9 ± 11.6	257.5 ± 45.3	289.5 ± 67.7	250.4 ± 50.6	0.91	0.86	0.57

In each line, different superscript letters (a, b) indicate significant differences (P<0.01) among dietary treatments. Dietary treatments are abbreviated as CTRL, PB1 and PB2 for diets without supplementation, supplemented with *Enterococcus raffinosus* and supplemented with *Pseudomonas protegens*, respectively. Abbreviations are: D = Diet factor; I = Infection factor; ISA = Intestine section area; MLT = Muscular layer thickness; VL = Villus length; VW = Villus Width; GC = Goblet cells. Values represent mean ± SE, n = 8 for 36-days growth; n = 6 for 2 and 15 days p.c.



A)

		PROXIMAL INTESTINE							
		CTRL				PB1			
		F1	F2	F3	F4	F1	F2	F3	F4
PB1	F1	33.4	**66.3**	**61.4**	48.4				
	F2	25.7	**70.2**	39.7	**58.7**				
	F3	22.2	38.8	41.9	25.0				
	F4	31.3	**79.7**	**58.1**	**68.8**				
PB2	F1	26.6	**81.8**	48.0	**64.1**	**71.6**	**62.9**	**53.3**	**79.8**
	F2	31.0	**73.2**	**57.6**	**68.1**	**79.1**	**58.2**	**64.8**	**94.4**
	F3	26.9	**65.2**	49.1	**54.0**	**72.3**	**76.5**	**60.9**	**72.8**
	F4	25.7	**53.3**	33.8	26.5	**57.7**	**62.9**	48.4	39.9

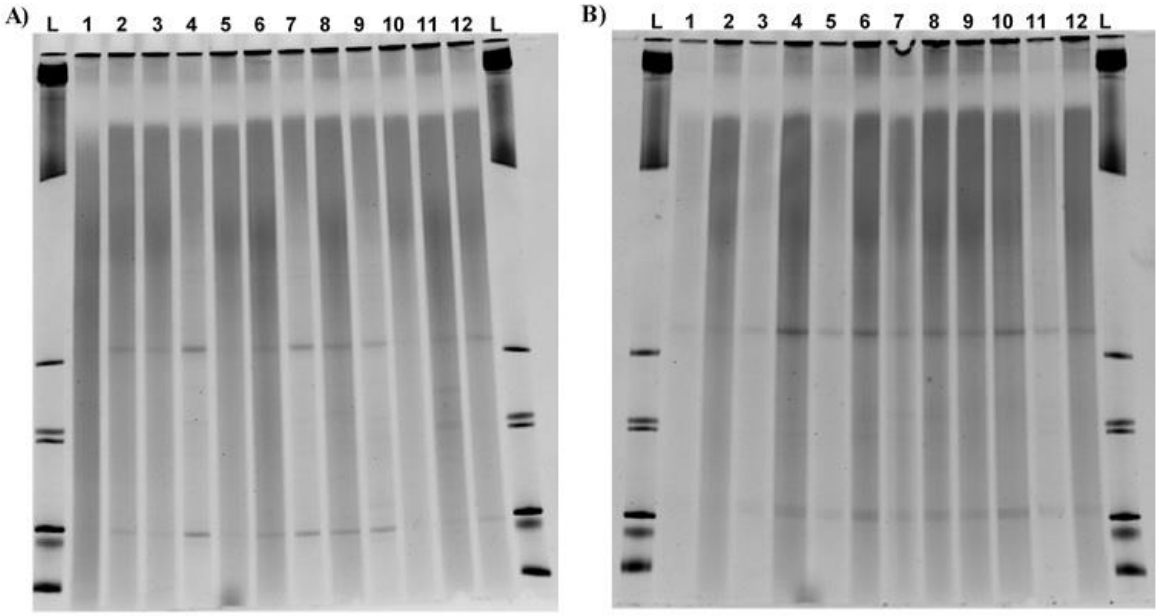
MEAN ± STANDARD DEVIATION
CTRL *vs* PB1 – 48.1 ± 18.3[b]
CTRL *vs* PB2 – 49.1 ± 18.7[b]
PB1 *vs* PB2 – 66.0 ± 13.5[a]

B)

		DISTAL INTESTINE							
		CTRL				PB1			
		F1	F2	F3	F4	F1	F2	F3	F4
PB1	F1	40.6	**75.1**	1.4	**62.6**				
	F2	**51.1**	**83.9**	7.3	**74.4**				
	F3	**50.3**	**70.2**	2.5	**70.4**				
	F4	43.6	**83.8**	2.0	**69.6**				
PB2	F1	40.4	41.6	1.7	**51.3**	34.5	39.6	45.3	36.7
	F2	38.6	**69.5**	1.3	**58.0**	**92.4**	**64.6**	**52.5**	**83.8**
	F3	**54.7**	**82.5**	2.7	**74.8**	**63.5**	**74.2**	**76.7**	**70.0**
	F4	**61.4**	**72.4**	5.3	**75.3**	**58.8**	**68.2**	**71.4**	**64.2**

MEAN ± STANDARD DEVIATION
CTRL *vs* PB1 – 49.3 ± 30.3
CTRL *vs* PB2 – 45.7 ± 28.7
PB1 *vs* PB2 – 62.3 ± 16.8

Figure 4 – DGGE images showing bands corresponding to the intestinal bacteria present in the proximal (A) and distal intestine wall (B) samples of Senegalese sole juveniles (n = 4) fed for 36-days the dietary treatments. Dietary treatments are abbreviated as CTRL, PB1 and PB2 for diets without supplementation, supplemented with *Enterococcus raffinosus* and supplemented with *Pseudomonas protegens*, respectively. Lanes: L – molecular weight marker 1 Kb Plus Ladder; 1 to 4 – Fish fed CTRL diet; 5 to 8 – Fish fed PB1 diet; 9 to 12 – Fish fed PB2 diet. Tables present percentage of similarity computed using Quantity One® program, for the DGGE profiles. If the lanes are identical to each other, the percentage of similarity is 100. Values higher than 50% are in bold. Different superscript letters (a, b) indicate significant differences (P<0.05).



A)

		NON-INFECTED							
		CTRL				PB1			
		F1	F2	F3	F4	F1	F2	F3	F4
PB1	F1	0.0	**68.4**	41.9	47.1				
	F2	25.8	48.5	47.6	**52.8**				
	F3	**54.9**	**73.3**	**73.3**	**93.4**				
	F4	32.3	**62.1**	49.0	**69.4**				
PB2	F1	27.5	**64.1**	48.4	**76.8**	**52.0**	**53.5**	**76.4**	**68.3**
	F2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	F3	27.8	**51.5**	44.0	47.9	33.0	32.9	43.2	39.3
	F4	37.7	**71.9**	**57.1**	**79.2**	**51.2**	**59.8**	**78.9**	**86.8**

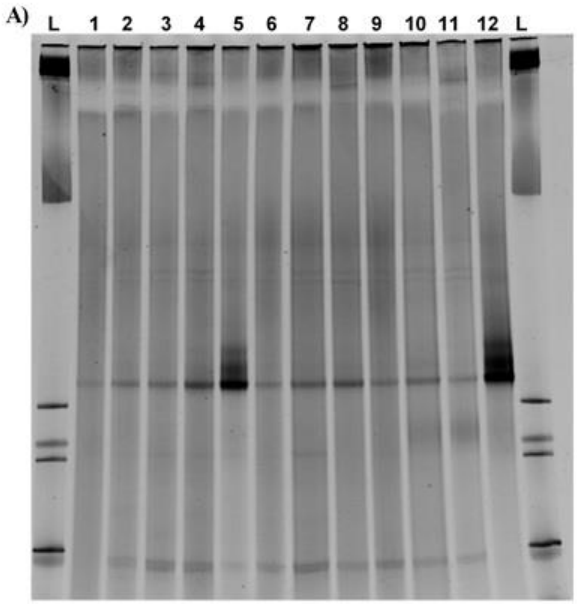
MEAN ± STANDARD DEVIATION
CTRL *vs* PB1 – 52.5 ± 22.0
CTRL *vs* PB2 – 39.6 ± 28.0
PB1 *vs* PB2 – 42.2 ± 29.6

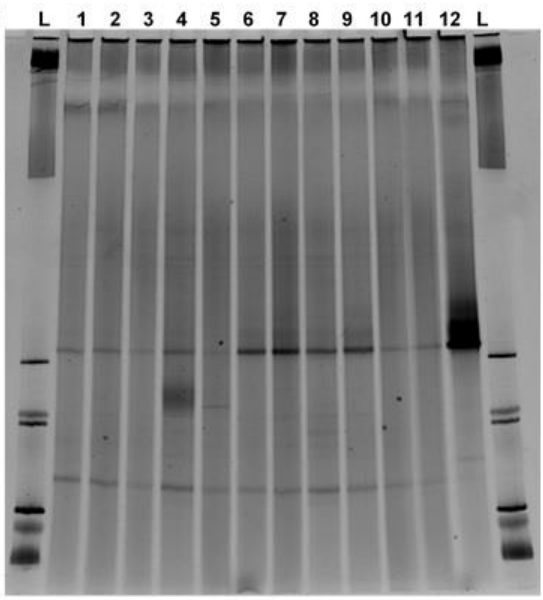
B)

		INFECTED							
		CTRL				PB1			
		F1	F2	F3	F4	F1	F2	F3	F4
PB1	F1	**74.0**	**65.2**	**90.0**	**61.9**				
	F2	46.9	**50.9**	**55.2**	**86.1**				
	F3	**80.2**	**59.5**	**84.5**	**54.7**				
	F4	33.5	**59.5**	**52.2**	47.9				
PB2	F1	36.5	**64.1**	**56.8**	**59.5**	**60.6**	**65.4**	**51.7**	**84.6**
	F2	**68.4**	**76.1**	**90.0**	**55.8**	**89.3**	**57.0**	**75.9**	**61.6**
	F3	**72.0**	**78.7**	**93.9**	**51.5**	**84.8**	**52.9**	**79.2**	**57.8**
	F4	**60.9**	**87.3**	**84.1**	45.4	**75.6**	47.1	**69.6**	**64.9**

MEAN ± STANDARD DEVIATION
CTRL *vs* PB1 – 62.6 ± 16.2
CTRL *vs* PB2 – 67.6 ± 16.7
PB1 *vs* PB2 – 67.4 ± 13.0

Figure 5 – DGGE images showing bands corresponding to the intestinal bacteria present in the proximal intestine wall samples of non-infected (A) and infected (B) of Senegalese sole juveniles (n = 4), 15-days post-challenge. Dietary treatments are abbreviated as CTRL, PB1 and PB2 for diets without supplementation, supplemented with *Enterococcus raffinosus* and supplemented with *Pseudomonas protegens*, respectively. Lanes: L – molecular weight marker 1 Kb Plus Ladder; 1 to 4 – Fish fed CTRL diet; 5 to 8 – Fish fed PB1 diet; 9 to 12 – Fish fed PB2 diet. Tables present percentage of similarity computed using Quantity One® program, for the DGGE profiles. If the lanes are identical to each other, the percentage of similarity is 100. Values higher than 50% are in bold.





A)

		NON-INFECTED							
		CTRL				PB1			
		F1	F2	F3	F4	F1	F2	F3	F4
PB1	F1	32.0	28.2	37.3	**56.9**				
	F2	**67.3**	**70.0**	**72.2**	**50.1**				
	F3	45.2	**50.1**	**63.9**	**63.1**				
	F4	44.6	**52.9**	**63.1**	**84.9**				
PB2	F1	**60.7**	**76.2**	**74.9**	47.1	24.8	**80.3**	41.1	**57.9**
	F2	**58.5**	**68.5**	**78.1**	**66.8**	**54.7**	**74.6**	**57.0**	**72.1**
	F3	22.0	41.4	40.9	**54.3**	22.1	40.1	37.7	**55.4**
	F4	25.1	25.4	33.7	49.8	**77.8**	34.2	42.0	49.4

MEAN ± STANDARD DEVIATION
CTRL *vs* PB1 – 55.1 ± 15.5
CTRL *vs* PB2 – 51.5 ± 18.9
PB1 *vs* PB2 – 51.3 ± 18.2

B)

		INFECTED							
		CTRL				PB1			
		F1	F2	F3	F4	F1	F2	F3	F4
PB1	F1	40.0	45.2	**56.6**	41.1				
	F2	30.5	36.1	26.8	38.6				
	F3	36.9	42.6	34.6	43.1				
	F4	**56.3**	**56.7**	**50.6**	**57.9**				
PB2	F1	42.4	**50.0**	40.1	**50.5**	39.0	**80.8**	**85.6**	**78.5**
	F2	47.7	**52.9**	**70.2**	46.0	**67.9**	35.5	37.9	48.0
	F3	44.8	44.5	35.6	41.7	**50.4**	**51.0**	48.4	**51.9**
	F4	16.0	22.2	22.3	25.5	21.5	**55.0**	**59.6**	42.6

MEAN ± STANDARD DEVIATION
CTRL *vs* PB1 – 43.4 ± 9.8^{ab}
CTRL *vs* PB2 – 40.8 ± 13.8^{b}
PB1 *vs* PB2 – 53.4 ± 17.6^{a}

Figure 6 – DGGE images showing bands corresponding to the intestinal bacteria present in the distal intestine wall samples of non-infected (A) and infected (B) of Senegalese sole juveniles (n = 4), 15-days post-challenge. Dietary treatments are abbreviated as CTRL, PB1 and PB2 for diets without supplementation, supplemented with *Enterococcus raffinosus* and supplemented with *Pseudomonas protegens*, respectively. Lanes: L – molecular weight marker 1 Kb Plus Ladder; 1 to 4 – Fish fed CTRL diet; 5 to 8 – Fish fed PB1 diet; 9 to 12 – Fish fed PB2 diet. Tables present percentage of similarity computed using Quantity One® program, for the DGGE profiles. If the lanes are identical to each other, the percentage of similarity is 100. Values higher than 50% are in bold. Different superscript letters (a, b) indicate significant differences (P<0.05).

5.4. Discussion

In the current study we have depicted in an integrative way the effects of dietary supplementation with two promising probiotics in Senegalese sole. In previous studies with probiotics, with the same fish species, the effects of probiotics on growth performance were contradictory, as a significant increase in final body weight and growth rate was obtained with a monospecies probiotic after 2 months administration period (García de la Banda et al., 2012; García de La Banda et al., 2010; Sáenz de Rodrigáñez et al., 2009). However previous studies did not confirm such positive effect after 72 days of feeding (Barroso et al., 2014) or after 1-month feeding (Batista et al., 2014), as in our study. This may be due to differences in the feeding times. In our case, a 36 days growth trial could not had been enough to demonstrate/ evidence the potential effect of probiotic in growth. In addition, we cannot rule out the effect of intrinsic internal factors such as genotype or the interaction among genotype and environment on the growth performance (Bagley et al., 1994). Senegalese sole has a slow growth rate and despite growth performance was not a main objective of our work, it is noteworthy that only with 36 days growth trial the fish were able to achieve good DGI results (> 1.5), values in accordance with previous growth studies with Senegalese sole (Guerreiro et al., 2012; Rema et al., 2008; Silva et al., 2009).

The probiotics used in the supplemented diets, *E. raffinosus* and *Ps. protegens* were detected in both PI and DI wall. *E. raffinosus* was detected in almost all fish intestine analysed, regardless of the dietary treatment, indicating their normal presence in Senegalese sole intestine microbiota. *Enterococcus* species are not considered as belonging to aquatic environments, but they have been found in freshwater fish and their surrounding environment (González et al., 2000) as well as in marine fish (Sahnouni et al., 2012). *Ps. protegens* was detected in 100% of fish fed PB2 diet, but not in all fish fed CTRL or PB1 diets. This finding indicates that dietary supplementation of *Ps. protegens* was effective in modulating the intestine microbiota, leading to increased *Ps. protegens* colonization in the intestinal wall. Tapia-Paniagua et al. (2010), Tapia-Paniagua et al. (2015) and Martin-Antonio et al. (2007) have previously detected *Pseudomonas* sp. as part of the intestinal microbiota of sole. *Vibrio* and *Pseudomonas* are the most common genera reported in marine fish intestine (Toranzo et al., 1993).

Usually, the innate immune system of the fish is the main target in profiling the immunomodulatory properties of candidate probiotics (Lazado and Caipang, 2014b). The effects of probiotic on different microorganisms in the immune system has been studied in several fish species (Aly et al., 2008; Cerezuela et al., 2012b; Irianto and Austin, 2002b). In the present study, dietary supplementation of *E. raffinosus* and *Ps. protegens* did not change the humoral innate immune response of sole after 36 days of growth trial. However, at 15-days p.c. the bacterial pathogen was associated with a significant decrease in the peroxidase activity in all the groups, despite at 2-days p.c. that decrease was not so evident. The exposure to an inflammatory agent triggers an immune response with the subsequent release of antimicrobial substances found in neutrophil granules. The observed decrease could be due to the long-term consumption of the peroxidases released by granulocytes to combat the bacteria, or to a decrease in the number of circulating granulocytes. At shorter time p.c. (24h), an increase in peroxidase activity was observed in Senegalese after an LD_{50} intraperitoneal injection of Phdp (Costas et al., 2013).

Yishuai et al. (2015) investigated several enzyme activities of a number of innate immune parameters in the serum, skin mucus and skin of Atlantic salmon, after challenge with a pathogenic strain of *Aeromonas salmonicida*. Superoxide dismutase, peroxidase and catalase activities significantly decreased at days 4 and 6 p.c., in mucus and skin. According to Yishuai et al. (2015), the decreased antioxidant capacity of the infected fish could possibly contribute to the mortality of the challenged groups.

The effect of probiotics in the intestinal structure and gut microbiota has been reported in fish (Lazado and Caipang, 2014b; Tapia-Paniagua et al., 2012; Tapia-Paniagua et al., 2015). Batista et al. (2014) observed that in sole juveniles fed multispecies probiotics, had a thicker muscular layer (MLT), but no effects on the villus length and number of GC were detected. In the current study, fish fed PB1 diet also had a thicker muscular layer than PB2 fed fish at the end of the growth trial. Moreover, PB1 group had more GCs than the other treatments. A higher number of GC in the intestinal epithelium is associated to an increase in the mucus production, leading to a thicker protective mucus layer. The epithelial surfaces of the body are the first defences against infection. Infections occur only when the pathogen can cross through these external protective barriers and colonize the fish tissues

(Janeway et al., 2001). The main constituents of the mucus are mucins, and its composition and thickness is key for the equilibrium between commensal and pathogenic microorganisms dwelling in this mucus layer (Janeway et al., 2001). Thus, the increase in MLT and in the number of GC may indicate an enhancement in the protection against some pathogens. This could explain, at least in part, the lower susceptibility tendency of fish fed PB1 compared to control or PB2 groups. In terms of protection against photobacteriosis, despite the cumulative mortality was not significantly different among the dietary treatments, fish fed PB1 diet seem to be less susceptible to the bacteria than those fed PB2 and control diets.

The fact that fish fed PB1 diet (infected or non-infected) had shorter villus than non-infected fish fed the CTRL diet, could suggest a reduction of the surface area for absorption, despite no differences were detected in growth performance.

The ability to modulate intestinal microbiota has been reported in fish treated with different probiotics, such as lactic acid bacteria (Carnevali et al., 2006; Carnevali et al., 2004; Planas et al., 2006), *Bacillus* sp and *Enterococcus faecium* (Avella et al., 2010a; Avella et al., 2010b; Avella et al., 2011). DGGE results in the current study suggested that the addition of probiotics deeply influenced the intestinal microbiota of Senegalese sole after 36 days of the growth trial. Fish fed the PB1 and PB2 diets had similarity values higher than 60%, whereas fish fed CTRL diet had low similarity values (<50%) with fish fed PB1 or PB2 diets. This indicate that dietary supplementation of *E. raffinosus* and *Ps. protegens* bacteria in the basal diet modulates the microbiota population, once that animals fed the PB1 and PB2 diets have different profile from the ones fed CTRL diet.

The ability of probiotic bacteria to modulate Senegalese sole intestinal microbiota has been already reported for *Shewanella putrefaciens* (Pdp11) and *Sh. baltica* (Pdp13). García de La Banda et al. (2010) observed that intestinal microbiota was differently influenced depending on the strain assayed, since only fish fed the Pdp13 diet showed a compact clustering, with a higher similarity index (80%). Tapia-Paniagua et al. (2010) observed that Pdp11 caused an increase in the dominance of *Vibrio* species in the intestinal microbiota. However, Tapia-Paniagua et al. (2014) showed that both Pdp11 and Pdp13 caused an increase on the predominance of *Shewanella* spp., whereas Pdp13 caused a decrease in *Vibrio* spp. Similarly, Tapia-Paniagua et al. (2015), tested the use of Pdp11 associated to oxytetracycline (OTC), and observed that a decreased intensity of the DGGE bands related to *Vibrio* genus

and the presence of DGGE bands related to *Lactobacillus* and *Shewanella* genera in the specimens that received OTC and Pdp11 jointly. Moreover, Pdp11 played a role in preventing the negative effect of OTC in decreasing the richness and diversity of the intestinal microbiota.

At day 15 p.c., intestinal microbiota from PI of infected fish showed higher similarity index, with mean values superior to 60%. This fact reveals a very similar microbiota among fish subjected to the Phdp infection, suggesting that Phdp was able to successfully colonize the intestine and compete with autochthonous microbiota. Cipriano (2011) demonstrated reduced bacterial diversity with dominance shifting to opportunistic pathogens on the skin and mucus after *Aeromonas salmonicida* infection in Atlantic salmon. Similarly, bacterial diversity decreased dramatically with community dominance shifting to *Pseudomonas* and *Vibrio* during disease caused by a variety of organisms in turbot *Scophthalmus maximus* (Toranzo et al., 1993). As the infection proceeds, the pathogens grows, replicates and displaces other species of the normal microbiota until becoming the dominant one. This situation apparently enabled the pathogen to overwhelm the host defence mechanisms and initiate a systemic infection, which resulted in clinical disease and mortality (Cipriano, 2011).

In the DI, the opposite pattern occurred. Non-infected fish had higher similarity values among fish, when compared to infected fish. However, this similarity was less pronounced (> 50%) than in the PI of infected fish (> 60%). Kristiansen et al. (2011) evaluated the bacterial community of the PI and DI segments of Atlantic salmon fed control or prebiotic supplemented diets. They also observed that the indigenous microbiota of the gastro-intestinal tract was modulated by the feeding regime and varied between PI and DI segments, with a less clear prebiotic effect in the DI. Indeed, the gastro-intestinal tract has a complex set of microbial communities that differ between the various segments (Sartor, 2008), that can explain the presently observed results.

5.5. Conclusion

In the present study, sole fed diets supplemented with two potential probiotic bacteria *(E. raffinosus* and *Ps. protegens*) for 36 days, did not show significant differences on growth performance or innate immune parameters. Fish fed *E. raffinosus* probiotic had increased muscular layer thickness and higher number of

GC, indicating a possible enhancement in the protection against some pathogens. PB1 groups had lower cumulative mortality after at the end of the challenge (albeit not significant), indicating that fish fed diet with *E. raffinosus* had a slight edge over the other groups in terms of protection against photobacteriosis.

Dietary supplementation of *E. raffinosus* and *Ps. protegens* bacteria in the basal diet have been able to modulate the microbiota, having the animals fed the PB1 and PB2 diets different profile from the ones fed CTRL diet. Fish challenged with Phdp had higher similarity in their intestinal microbiota, especially in the proximal area, perhaps due to dominance of Phdp during disease. In addition, a decrease in the peroxidase activity was observed in infected fish, revealing a lower antioxidant capacity than their non-infected counterparts.

E. raffinosus seems to be the best candidate to be administered to sole before stressing periods, enhancing the protection capacity of the fish leading to less mortalities during photobacteriosis. However additional studies with challenges would be needed to confirm this result.

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CHAPTER 6

General discussion, highlighted conclusions and future perspectives

6.1. General discussion

Probiotics confer beneficial actions to the host or to their environment through different modes of action. Their application in aquaculture has been regarded as a sustainable and promising strategy not only in the context of disease control but also in nutrition, growth and immunity (Lazado and Caipang, 2014a)

Plant protein (PP) ingredients are becoming increasingly used in aquafeeds as an affordable replacement for fishmeal. Nevertheless, the application of PP ingredients at relatively high levels in the diets may cause nutritional imbalances in carnivorous fish species and influence the immune response. These ingredients often contain a wide range of anti-nutritional factors that have a negative impact on fish health as well as feed utilization (Hardy, 2010). The current study evaluated the use of probiotic supplementation in Senegalese sole diet at different biotic and abiotic conditions, including intercropping their use with high levels of plant ingredients to mitigate the anti-nutritional effects of the latter.

In the **chapter 2** fish fed probiotic supplemented diets did not show changes on growth performance. Nevertheless, fish fed high content of multispecies probiotic (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.) presented higher growth homogeneity. This finding was also observed in previous studies (García de la Banda et al., 2012; Sáenz de Rodrigáñez et al., 2009; Varela et al., 2010; Wang et al., 2008b). The beneficial effect of probiotics on the growth performance and dietary nutrient utilization has been commonly reported for several fish species (Carnevali et al., 2006; El-Haroun et al., 2006; Taoka et al., 2006a; Wang et al., 2008b) including Senegalese sole (Díaz-Rosales et al., 2009; García de la Banda et al., 2012; Sáenz de Rodrigáñez et al., 2009). However, in **chapters 3** and **4**, final body weight and feed conversion ratio were negatively affected by the use of the same multispecies bacteria tested in **chapter 2**. In **chapter 5**, the two autochthonous bacteria used as probiotic did not affect growth performance. In fact, the effects of probiotics and prebiotics on the growth performance are not consistent. Positive effects on feed efficiency and growth performance was observed in Senegalese sole (García de la Banda et al., 2012), Japanese flounder (Taoka et al., 2006a) and in Atlantic cod (Lauzon et al., 2010). Conversely, no changes in growth (Ferguson et al., 2010) or growth retardation (Gunther and Jimenez-Montealegre, 2004) were observed in Nile tilapia. The trial duration may play a role in the outcome of the dietary probiotic treatment. In our case, a 36 days

growth trial could not had been enough to demonstrate/ evidence the potential effect of probiotic in growth. In addition, we cannot rule out the effect of intrinsic internal factors such as genotype or the interaction among genotype and environment on the growth performance (Bagley et al., 1994). Senegalese sole has a slow growth rate and despite growth performance was not a main objective of our work, it is noteworthy that only with 36 days growth trial the fish were able to achieve good daily growth index results (> 1.5), values in accordance with previous growth studies in Senegalese sole (Guerreiro et al., 2012; Rema et al., 2008; Silva et al., 2009).

In **chapters 3** and **4** we demonstrated that growth performance of Senegalese sole was not negatively affected by high content of plant ingredients. Similarly, previous reports (Cabral et al., 2011; Cabral et al., 2013; Silva et al., 2009) proved the capacity of Senegalese sole to cope with diets rich in plant ingredients as replacement for the marine-derived ingredients.

Serum peroxidase, lysozyme and ACH50 activities, are commonly used as indicators of non-specific immune status in fish. In **chapters 2, 3, 4** and **5** the dietary probiotic supplementation, did not affected the innate immune parameters measured. Nevertheless, in **chapter 2** fish fed dietary probiotic supplementation showed a tendency to have increased lysozyme and ACH50 activities. This trend was also observed by Díaz-Rosales et al. (2009) working with Senegalese sole where probiotics induced a slightly increase in some of the studied immune parameters. Some probiotics are known to be effective in enhancing the natural complement activity in fish (Choi and Yoon, 2008; Panigrahi et al., 2007; Salinas et al., 2008). Certain nutrients and immunostimulants can be supplemented in the diet to modulate serum lysozyme activity in fish (Kim and Austin, 2006a). These effects on the immune system seem to be temporary (reversible) and dependent on the inclusion level and trial duration (Ortuño et al., 2002). Conversely, Balcázar et al. (2007a), Merrifield et al. (2010b) and Merrifield et al. (2010b) did not find significant effects of probiotics on serum lysozyme activity. In **chapter 3**, we addressed the molecular basis of the innate immune response in fish fed PP diets supplemented with a commercial multispecies probiotic and an immunostimulant (autolyzed yeast). Dietary probiotic supplementation altered the immune and stress responses in sole. In the distal intestine, rectum and head-kidney, the transcript level of the genes *c3* paralogues, *lyzc* and *lyzg* did not show treatment-related differences, which is in agreement with the results of the humoral innate immune indicators. In fish, the

induction of heat shock proteins (*hsp*) is a component of the cellular stress response against a diversity of stressors, such as osmotic stress, heat shock or infections (Basu et al., 2002). Senegalese sole fed yeast supplemented diets had higher *hsp90b* expression in the distal intestine, while fish fed multispecies probiotic displayed higher *hsp90b1* and *hsp90a* transcript levels in distal intestine and rectum, respectively. In addition, *hsp90b* in distal intestine and *hsp90b1* mRNA levels in head-kidney were significantly affected by diets, with lower expression in fish fed the diet with the highest PP content.

The main high density lipoprotein (Apoa1) has anti-inflammatory, antimicrobial and antioxidant properties (Barter et al., 2004) and is involved in regulation of fish complement (Magnadóttir, 2006). A*poa1* expression in rectum of fish fed low PP content (PP35) was up-regulated by the dietary yeast supplementation. However, the same gene *apoa1* was down-regulated in the distal intestine of fish fed high PP diet, suggesting that the use of different protein sources (marine-derived vs. plant protein) may modulate the *apoa1* expression.

The expression of the key effector caspase *casp3* in rectum was also significantly affected by PP content, suggesting that plant ingredients may reduce apoptotic activity. Nevertheless, van Breda et al. (2005) observed in mice that 7 genes involved in apoptosis were up-regulated by a consumption of 40% plant protein diet.

The biosynthesis of ferritin is repressed under conditions of iron deprivation (Torti and Torti, 2002). The dietary multispecies probiotic supplementation down-regulated ferritin m gene (*ftm)* in Senegalese sole rectum, compared to unsupplemented diets. This *ftm* down-regulation occurred maybe due to an indirectly mobilization of iron (Pantopoulos and Hentze, 1995) during an oxidative stress response in the rectum. Further the up-regulation of *gpx1* and *cat* transcript levels in the distal intestine of sole may also indicate an antioxidative effect of dietary probiotic supplementation. Catalases are a class of enzymes that facilitate the dismutation of hydrogen peroxide to oxygen and water (Nicholls, 2012) and *gpx1* is as an enzyme counteracting oxidative stress due to its hydroperoxide-reducing capacity (Brigelius-Flohe and Maiorino, 2013). Rueda-Jasso et al. (2004) suggested a relationship between *cat* activity and diet composition (lipid level and starch type). Nutritional imbalances and diet composition may play a role in oxidation processes and antioxidative defense mechanisms (Rueda-Jasso et al., 2004). In Senegalese sole, a high dietary plant protein content was associated with a decrease in *cat* and

gpx1 transcript levels in rectum, which indicates modulatory effects of plant ingredients on the antioxidative defense mechanisms. In **chapter 4**, the innate immune parameters were affected by the increase of dietary PP content. At 17 and 38 days of the feeding trial, fish fed PP72 diets had higher lysozyme and ACH50 activities than fish fed PP35 diets, suggesting a stimulation of the innate immune response by plant ingredients. Also Geay et al. (2011) observed an increase in the ACH50, while the lysozyme was lower, in fish fed a PP diet when compared to fish fed fishmeal based diet. On the other hand, Sitjà-Bobadilla et al. (2005) reported that high level of fishmeal replacement by plant protein sources had no significant effect on lysozyme activity. Jalili et al. (2013) showed that rainbow trout fed diets with high PP levels (70 and 100%) resulted in a decrease on the ACH50, as reported by Sitjà-Bobadilla et al. (2005) in gilthead seabream. Hepatocytes provide the major source of complement factors (Abelseth et al., 2003) and the progressive fat degeneration of liver with PP inclusion could decrease complement proteins synthesis (Sitjà-Bobadilla et al., 2005). According to our results, this effect could be improved by a long-term feeding diets with high plant ingredients content. Liver from fish fed PP72 diets showed a higher level of vacuolization comparing to fish fed PP35 diets. The use of high levels of plant ingredients is recognised to have several disadvantages, particularly related to the amino acid profiles and unsaturated fatty acid imbalances, but especially for their anti-nutritional factors levels (Geay et al., 2011). High PP level seems to result in an acute stimulation of lysozyme and ACH50, since fish fed PP72 diets for 2 and 17 days showed higher activities than fish fed PP35 diets. Nevertheless, at 73 days of feeding trial lysozyme activity returned to original values.

Intestinal microbiota changed with the diet and this effect is of particular interest considering that the effects of high fishmeal replacement by plant ingredients are still poorly understood, especially in flatfish. The intestinal tract of fish harbors a high density of nonculturable bacteria whose composition of which has not yet been reported, leading to differences between viable and total microbial counts (Shiina et al., 2006). Molecular techniques have facilitated culture-independent studies, becoming a valid support to traditional techniques (Ercolini, 2004).

In **chapters 3** and **4** it has been possible to demonstrate the ability of PP diets with probiotic or autolyzed *Saccharomyces cerevisiae* to modulate the intestinal microbiota of Senegalese sole. After 73 days of feeding, fish fed PP72 diets had a

higher number and diversity of bacteria in their gut compared to the PP35 diets. Microbial diversity was also affected by soybean meal in gilthead sea bream (*Sparus aurata*) (Dimitroglou et al., 2010) and Atlantic salmon (*Salmo salar*) (Bakke-McKellep et al., 2007) where fish fed the soybean meal diet had higher total number as well as a more diverse population composition of adherent bacteria in the distal intestine. In **chapter 3**, it was also observed that the distal intestine showed higher microbiota diversity, when compared to rectum segment, which may indicate variable immune properties across the different segments of the intestine (Inami et al., 2009). In addition to ascertaining microbial diversity by molecular methods, we used a conventional microbiological techniques in **chapter 4** to identify culturable bacteria in the gastrointestinal tract.

The predominant bacteria found in *Solea senegalensis* intestine content were *Vibrio* sp., which is in agreement with other studies carried out with the same flatfish cultured under extensive, semi-extensive or intensive production systems (Martin-Antonio et al., 2007). This trend was also detected in farmed Senegalese sole fed fresh or lyophilized *Shewanella putrefaciens* (Tapia-Paniagua et al., 2010; Tapia-Paniagua et al., 2015). The supplementation of diet with the multispecies probiotic reduced the intestinal bacteria related to *Vibrio* sp. Previous studies also observed that strains of probiotic *Bacillus* (Liu et al., 2015; Luis-Villasenor et al., 2015; Wu et al., 2014), *Enterococcus* (Lin et al., 2013) and *Pediococcus* and *Lactobacillus* (Munoz-Atienza et al., 2013) showed an antibacterial activity against fish pathogens, including *Vibrio* species. *V. harveyi* was detected in sole fed probiotic supplemented and especially in fish fed yeast supplemented diets, and not detected in fish fed non-supplemented diets. These *Vibrio* species are included in the Harveyi Clade and are well-known to cause pathogenic outbreaks in marine fish farms (Austin and Zhang, 2006; Cano-Gomez et al., 2011; Gomez-Gil et al., 2004), including *Solea senegalensis* in the case of *V. harveyi* (Arijo et al., 2005a; Zorrilla et al., 2003). However, the presence of virulent and non-virulent strains of *V. harveyi* (Rico et al., 2008; Zorrilla et al., 2003) is frequent, and for this reason more studies are necessary to evaluate the potential virulence of the strains of these species and how diet formulation may modulate the presence of these bacteria in the intestine. In addition, the modulation of intestinal microbiota has been demonstrated in fish treated with *S. cerevisiae* (yeast) fermentation products (He et al., 2011) and isolated glucans (Kuhlwein et al., 2013).

On the other hand, the dietary probiotic supplementation in PP35 diet induced the presence of *S. saprophyticus,* normally associated to microorganisms that play a critical role in fish fermentation and are known to be resistant to different antibiotics (Sergelidis et al., 2014; Zhang et al., 2015). Although there are no reports that *S. saprophyticus* may cause diseases in fish, Sun et al. (2011) speculate that may be potentially harmful as it is often implicated in human urinary tract infections (Kuroda et al., 2005). Its presence in the intestinal microbiota of fish must be considered as a potential route for the transmission of antibiotic resistance (Chajecka-Wierzchowska et al., 2015).

In **chapter 5**, both autochthonous bacteria tested (*Enterococcus raffinosus* and *Pseudomonas protegens*) deeply influenced the intestinal microbiota of Senegalese sole after 36 days of the growth trial after 36 days of the growth trial. Fish fed control diet had low similarity values (<50%) compared to fish fed diets supplemented with *E. raffinosus* (PB1) or *P. protegens* (PB2), indicating that dietary supplementation of both bacteria had significantly altered the microbial populations of fish intestine. At day 15 of post-infection, proximal intestinal microbiota of infected fish showed higher similarity index (> 60%), suggesting that Phdp was able to successfully colonize the intestine and compete with autochthonous microbiota. Cipriano (2011) demonstrated reduced bacterial diversity with dominance shifting to opportunistic pathogens on the skin and mucus after *Aeromonas salmonicida* infection in Atlantic salmon. Similarly, bacterial diversity decreased dramatically with community dominance shifting to *Pseudomonas* and *Vibrio* during disease caused by a variety of organisms in turbot, *Scophthalmus maximus* (Toranzo et al., 1993). Conversely, distal intestine of non-infected groups had higher similarity values between dietary treatments, when compared to distal intestine of infected fish. However, this similarity was less pronounced (> 50%) than the proximal intestine of infected fish (> 60%). The gastro-intestinal tract has a complex set of microbial communities that differ between the various segments (Sartor, 2008).

Intestinal microbiota is confined within highly specialized barrier defenses, composed by the stratified mucous layer, an epithelium and a lamina propria with several innate and adaptive immune cells (Maynard et al., 2012). Dietary probiotic supplementation may interfere with intestinal health, by altering its morphology (length, width, surface area of the villi and muscular layer thickness), improving the tract nutrient absorption capacity and enhancing the animal protection against

pathogen outbreak (Liu et al., 2007; Peinado et al., 2012). In chapters **2**, **4** and **5** the effects of experimental diets on intestinal morphology, were studied. In **chapter 2**, the muscular layer thickness showed a significant difference between fish fed mono- and multi-species probiotic diets, at lower probiotic dosage. Fish fed monospecies probiotic diet had the thinnest muscular layer among treatments, denoting a possible influence of the type of probiotic inclusion in the intestine morphology. Although the changes in the muscular thickness have already been mentioned for different species (broilers, rabbits and rats) the physiological/metabolic mechanisms behind is yet poorly understood.

In **chapter 4,** the same multispecies probiotic tested in chapter 2 did not influence the intestinal morphology of sole. However, fish fed unsupplemented PP72 diets showed larger intestine section area and longer villus than fish fed unsupplemented PP35 diets, after a very short-term (2 days) of feeding. At 17 days of feeding, fish fed unsupplemented PP72 diets showed more goblet cells (GC) than the other dietary groups, except the group fed PP35 diet supplemented with the yeast. At 73 days, however, villus length and width, and GC were significantly increased in fish PP35 diets compared to PP72 diets. The latter may indicate the immune defence and nutrient absorption are stimulated during short-term feeding with high levels of plant protein. In contrast, when animals are fed high plant protein levels for long-term periods the effects described above disappeared, probably due to habituation of fish and/or to a negative effect caused by chronic consumption of antinutritional compounds. The intestinal epithelium is covered by a layer of mucus continuously produced by GC enabling first line barrier against microbes (Maynard et al., 2012). It is plausible to infer that the reduction of GC after 73 days of feeding PP72 diets may have caused the reduction of mucous production, consequently reducing intestinal protection capacity of those fish.

Yeast cells have been reported to be a source of nucleotides, which contribute for the intestinal maintenance in aquatic animals by improving mucosal flora and mucosal surfaces with relative elongation of the intestinal tract (Li et al., 2007). After 38 days of feeding YEAST diets, villus length was significantly increased, when compared to control groups. Abu-Elala et al. (2013) observed that fish treated with *S. cerevisiae* showed yeast colonization in intestine, followed by an increase in the length and density of the intestinal villus. Such morphological alterations are often associated with an improvement in feed digestion and absorption. After 2 days

feeding the YEAST diets, fish had higher hepatocyte glycogen content comparing with fish fed probiotic diets. A stimulation of the glycogen liver storage can be interpreted as a defense mechanism since blood glucose concentration falls when fish is subjected to stress, such as disease outbreaks. *S. cerevisiae* is rich in bioactive ingredients and nutrients such as cell wall carbohydrates. According to Wilson (1994), fish fed diets rich in digestible carbohydrates, showed high hepatic glycogen reserves. However, when fish were fed YEAST for av longer duration (73 days), such trend in energy storage disappeared.

In the growth trial of **chapter 5**, fish fed a diet supplemented with *E. raffinosus* (PB1) had a thicker intestinal muscular layer (MLT), when compared to fish fed *P. protegens* (PB2) at the end of the growth trial. Moreover, PB1 group had more GC than the other treatments. A higher number of GC in the intestinal epithelium is associated to an increase in the mucus production, leading to a thicker protective mucus layer. The epithelial surfaces of the body are the first defences against infection. Infections occur only when the pathogen can cross through these external protective barriers and colonize the fish tissues (Janeway et al., 2001). The main constituents of the mucus are mucins, and its composition and thickness is key for the equilibrium between commensal and pathogenic microorganisms dwelling in this mucus layer (Janeway et al., 2001). The changes in MLT and number of GC may indicate an enhancement in the protection against pathogens. This could explain, at least in part, the lower susceptibility tendency of fish fed PB1 compared to control or PB2 groups. In terms of protection against photobacteriosis, even if the cumulative mortality was not significantly different among dietary treatments, fish fed PB1 diet seem to be less susceptible to this bacterial pathogen than those fed PB2 and control diets.

The fact that fish fed PB1 diet (infected or non-infected) had shorter villus than non-infected fish fed the CTRL diet, could suggest a reduction of the surface area for absorption, despite no differences were detected in growth performance.

The effects of dietary probiotic supplementation, following an infection with *Photobacterium damselae* subsp. *piscicida* (Phdp) were tested in **chapter 5**. At 15-days post-infection the pathogenic bacteria caused a decrease in the peroxidase activity in fish fed control and probiotic diets. Exposure to an inflammatory agent triggers an immune response with the subsequent release of antimicrobial substances found in neutrophil granules. The observed decrease could be due to

the long-term consumption of the peroxidases released by granulocytes to combat the bacteria, or to a decrease in the number of circulating granulocytes. At shorter time post-chalenge (24h), an increase in peroxidase activity was observed in Senegalese sole after an LD_{50} intraperitoneal injection of Phdp (Costas et al., 2013). Yishuai et al. (2015) investigated several enzyme activities corresponding to a number of innate immune parameters in the serum, skin mucus and skin of Atlantic salmon, after challenge with a pathogenic strain of *Aeromonas salmonicida*. Superoxide dismutase, peroxidase and catalase activities significantly decreased at days 4 and 6 p.c., in mucus and skin. According to Yishuai et al. (2015), the decreased antioxidant capacity of the infected fish could possibly contribute to the mortality of the challenged groups.

6.2. Highlighted conclusions

Growth performance

- Senegalese sole was able to cope with diets in which the marine-derived protein was replaced by plant protein.
- Dietary probiotic supplementation may decrease costs associated to size grading, being however strain- and dose-dependent.

Immune and stress response

- The use of probiotics and plant ingredients in sole diet was associated with changes in immune- and stress-related gene expression. Overall, fish fed diets with high plant protein content showed lower transcript levels of those genes, when compared with their counterparts fed diets with low PP content.
- Multispecies bacteria supplementation may have triggered the activation of the antioxidative stress response, with an up-regulation of *gpx* and *cat* transcript levels in distal intestine, concomitantly with the down-regulation of *ftm* mRNA in rectum.
- Fish subjected to the Phdp infection had a decrease in the peroxidase activity, suggesting a lowest antioxidant capacity.

Gut morphology

- Probiotics and the yeast supplementation in sole diets were able to change the intestinal morphology, influencing some parameters like the muscular layer thickness, number of goblet cells and *villus* length. The autochthonous *Enterococcus raffinosus* bacteria has demonstrated some kind of enhancement in the protection against pathogen disease, by increasing the muscular layer thickness and the number of goblet cells in fish fed diets with that bacteria.
- Short-term feeding with high PP content may improve the intestinal surface area, which would be beneficial in increasing intestinal absorptive capacity in fish. However, it seems that this was a temporary (acute) effect that was reversed in long-term feeding high PP diets, leading to decreased immune competence and energy storage in the liver.

Intestinal microbiota

- The predominant bacteria present in sole intestine were *Vibrio sp.* Probiotics and the inactivated yeast supplementation in sole diets had an effect on the intestinal microbiota, with predominant species varying with the dietary formulation.
- Distal intestine of Senegalese sole showed higher microbiota variability than rectum and the inclusion of plant ingredients was associated with more diverse microbiota profile.
- Fish subjected to Phdp infection had higher similarity of intestinal microbiota, especially in the proximal intestinal samples, showing the dominance of Phdp during disease.

Disease resistence

- Fish fed diets with the autochthonous *Enterococcus raffinosus* bacteria had lower cumulative mortality after 17 days post infection, indicating that fish fed diet with *E. raffinosus* had a slight edge over the other groups against photobacteriosis.

6.3. Future perspectives

The beneficial effects of dietary probiotics and immunostimulants in Senegalese sole remain to be further elucidated, especially regarding the supplementation doses, duration, alternative candidate bacteria to be used, as well as which abiotic and biotic conditions the probiotic treatment is beneficial to fish. In addition, the modulatory effects of probiotic on intestinal microbiota needs to be better evaluated using additional technologies, such as next-generation sequencing. Metagenomics is a timely approach in the field of microbial ecology and is used in the same manner as 16S rRNA gene fingerprinting to describe global microbial community profiles. However, even metagenomics cannot reveal the actual activities at a specific time and place, or how those activities change in response to environmental forces or biotic interactions. On the other hand, metatranscriptomics is a cutting edge method that can help us to understand how communities respond to changes in their environment and also could be a very important tool to study the interactions between intestinal probiotic bacteria and the fish host.

Furthermore, the analysis of the probiotic effects on the immune system would be improved using genome-wide transcriptomic and epigenetic approaches, targeting a high number of genes, covering both the adaptive and the innate branches of the immune system. In fish, virtually nothing is known about how probiotics influence DNA methylation, histone modifications and long non-coding RNAs in the host gut. Knowledge about these epigenetic effects of probiotics will help us to better understand the complex interplay between the gut microbiome, the host immune system and the environment, which will improve the application of probiotics in aquaculture.

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