Universidade de Trás-os-Montes e Alto Douro

Contributo para o estudo da aplicação de probióticos comerciais em aquacultura.

Sua utilização no cultivo de espécies com interesse comercial

Tese de Doutoramento em

Ciência Animal

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Resumo

A administração de probióticos em aquacultura, tal como noutros setores da produção animal, é apontada como uma estratégia nutricional capaz de promover o estado de saúde do hospedeiro, aumentar a sua resistência perante situações causadoras de stress e doença e melhorar as taxas de crescimento e de eficiência alimentar dos animais. Embora o mercado internacional disponibilize algumas formulações probióticas específicas para a aquacultura, não existiam, à data do início deste trabalho experimental, trabalhos científicos relacionados com o assunto que pudessem comprovar os alegados benefícios da maioria dos produtos comerciais utilizados. Desta forma, este trabalho foi desenvolvido com o objetivo de aprofundar o conhecimento relacionado com a administração de probióticos comerciais a espécies de grande importância no panorama aquícola europeu e mundial, concretamente a truta arco-íris (*Oncorhynchus mykiss*) e a tilápia-do-Nilo (*Oreochromis niloticus*). Os estudos visaram sobretudo o acompanhamento e análise *in vivo* de parâmetros zootécnicos, imunológicos e da morfo-histologia intestinal após o fornecimento de dietas experimentais suplementadas com probióticos.

No capítulo 1 é feita uma revisão bibliográfica acerca da utilização de probióticos em aquacultura e dos diversos modos de acção dos microorganimos probióticos. Nos capítulos seguintes é desenvolvida a componente prática do trabalho que, de um ponto de vista experimental, foi desenvolvida em 3 etapas. Numa primeira fase começamos por estudar um probiótico composto por *Bacillus subtilis* e *Bacillus cereus*, em truta arco-íris e, em paralelo, em truta marisca (*Salmo trutta*), uma espécie de truta nativa. No capítulo 2 descrevem-se e discutem-se os resultados dessa suplementação. Não tendo sido observados efeitos benéficos em nenhum dos parâmetros avaliados e em nenhuma das doses testadas (0,3 $g.kg^{-1}$ e 0,6 $g.kg^{-1}$), numa segunda fase, estudaram-se dois novos produtos comerciais: um constituído por 4 estirpes bacterianas desconhecidas, conhecendo-se apenas o género (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., e *Lactobacillus* sp.) sendo que ao longo do trabalho foi designado por A ou multi-espécies e outro constituído apenas por uma só espécie, *Pediococcus acidilactici*, designado como B ou mono-espécie. Esses produtos foram testados em duas dosagens dentro do intervalo recomendado pelos respetivos fabricantes (A: 1,5 $g.kg^{-1}$ e 3 $g.kg^{-1}$; B: 0,1 $g.kg^{-1}$ e 0,2 $g.kg^{-1}$) numa experiência apresentada nos capítulos 3 e 4. No capítulo 3 são descritos e discutidos os resultados zootécnicos, imunológicos e histomorfológicos respeitantes a essa suplementação. Após oito semanas de administração, observou-se que a menor dosagem do

probiótico A promoveu o crescimento e a conversão alimentar dos animais, enquanto a maior dosagem do B, melhorou apenas a conversão alimentar. A imunomodulação foi pouco extensa e limitada apenas às dosagens com efeitos nas taxas zootécnicas. Não foi observada qualquer influência significativa na morfo-histologia intestinal. No capítulo 4 descreve-se o efeito da administração dos probióticos na microbiota intestinal, tendo sido comprovado o aumento da diversidade microbiana no intestino dos animais alimentados com as dieta contendo probióticos, em particular nos indivíduos que receberam a dose mais baixa do probiótico A. Essa observação veio reforçar aquelas do capítulo 3, indicando que a menor dose testada do probiótico A (1,5 $g.kg^{-1}$) originava superiores benefícios no hospedeiro.

Numa última experiência, propusemo-nos avaliar os eventuais efeitos benéficos do probiótico A, por se ter revelado mais promissor, numa outra espécie, a Tilápia-do-Nilo. No capítulo 5 apresentam-se a experiência e os resultados obtidos e faz-se a sua discussão. Após 8 semanas de suplementação, o probiótico A, incorporado em 3 $g.kg^{-1}$ e 6 $g.kg^{-1}$ de dieta, originou uma melhoria no crescimento das tilápias (na dosagem mais baixa) e aumentou a resposta imunitária inespecífica (via alternativa do sistema complemento) na dosagem mais elevada. A morfologia intestinal foi melhorada por ambas dosagens do probiótico, dado que o grupo controlo apresentou *villi* mais curtas e células caliciformes em menor número, responsáveis pela produção de muco, que constituem um importante mecanismo de proteção do epitélio intestinal. No capítulo 6 tecem-se as conclusões e considerações finais. O capítulo 7 reúne a bibliografia referenciada ao longo do trabalho.

Os resultados obtidos neste trabalho permitem-nos afirmar que, sob as condições experimentais testadas, as formulações microbianas comerciais A e B apresentam efeito probiótico se administradas durante 8 semanas, em particular a formulação A, actuando como promotor de crescimento nas doses de 1,5 $g.kg^{-1}$ em truta arco-íris e de 3 $g.kg^{-1}$ em Tilápia do Nilo. Numa nota final sugere-se a realização de ensaios do tipo desafio “challenge” com agentes patogénicos e/ou causadores de stress para confirmar a hipotética melhoria do estado de saúde dos animais.

Palavras-chave:

Probiótico; Aquacultura; Truta Arco-íris; Tilápia-do-Nilo; Crescimento; Morfologia intestinal

Abstract

The probiotic administration in aquaculture, similarly to terrestrial livestock production, is seen as a nutritional strategy to promote the health of the host, increase his resistance towards stressful and disease conditions and improve growth and feed efficiency rates. At the beginning of this experimental work, some specific probiotic formulations for aquaculture were available in the international market, nevertheless the scientific information to validate their putative probiotic action was very scarce. In this context, this work was conducted to provide additional information about the supplementation of diets with commercial probiotics, optimizing their administration to important European and world aquaculture species as rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*). The studies were based on *in vivo* evaluations of zootechnical parameters, selected immune responses and intestinal morphohistology after probiotic administration through experimental diets.

Chapter 1 reviews the probiotic methods of action and their utilization on aquaculture. Subsequently the practical work developed is presented. The experimental work was run in the 3 steps. In a first phase we began to study to a probiotic composed by *Bacillus subtilis* and *Bacillus cereus* in rainbow trout and comparatively in brown trout (*Salmo trutta*), a native trout species. In chapter 2 we describe and discuss the results of this supplementation. Since no beneficial effects were observed, in any of the evaluated parameters, at either the dosages tested (0.3 $g.kg^{-1}$ and 0.6 $g.kg^{-1}$), in a second stage, we investigated two new commercial products. One of the putative probiotics, was named A, or multi-species and consisted of 4 bacterial genera (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.); the other, was designed by B or mono-species and consisted in *Pediococcus acidilactici*. These products were tested at two dosages within the range recommended by the respective manufacturers (A: 1.5 $g.kg^{-1}$ and 3 $g.kg^{-1}$; B: 0.1 $g.kg^{-1}$ and 0.2 $g.kg^{-1}$) in an experiment presented in chapters 3 and 4. Chapter 3 describes and discuss the zootechnical, immunological and histological results of this supplementation. After eight weeks of administration, the lower dose of the probiotic A promoted growth and feed conversion, while the higher dose of B improved feed conversion. The immunomodulation was not extensive and limited to the dosages that influenced zootechnical performance. It was not observed any significant influence on intestine morphology. Chapter 4 describes the effect of probiotic administration in the intestinal microbiota. Overall, the specimens that received the probiotic diets had increased microbial diversity, but that effect was particularly noticed in the individuals that received the lowest dose

of the probiotic A. This observation reinforce the chapter 3 ones, indicating that the lowest dosage tested of the A probiotic (1.5 $g.kg^{-1}$) leads to higher benefits in the host.

In a last experiment, the probiotic A, that revealed previous superior benefits, was evaluated in another fish species, the Nile tilapia, at 3 $g.kg^{-1}$and $6g.kg^{-1}$ of diet. Chapter 5 presents the experience, the results and their discussion. After 8 weeks of supplementation, the lower dosage of probiotic promoted the growth of tilapia while the highest amount increased the alternative pathway of the complement system, an unspecific immune response. Intestinal morphology was improved by both probiotic dosages, since the control group revealed shorter *villi* and less goblet cells responsible for producing mucus, an important protection mechanism of the intestinal epithelium. Chapter 6 presents the final conclusions and remarks. Chapter 7 lists the bibliography referred along the thesis.

The results of this study suggests that, under the currents experimental conditions, the commercial microbial formulations A and B have a probiotic effect if administered for 8 weeks, in particular A formulation, acting as a growth promoter in doses of 1.5 $g.kg^{-1}$ for rainbow trout and 3 $g.kg^{-1}$ in Nile Tilapia. As a final note we suggest the realization of challenge tests with pathogens and/or other stress agents to confirm the hypothetical improvement of the health status of the animals.

Keywords:

Probiotic; Aquaculture; Rainbow trout; Nile Tilapia; Growth performance; Intestinal morphology

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List of abbreviations and acronyms

16S rDNA	16S ribosomal DNA (prokaryotic DNA gene that codes for 16S ribosomal subunit)
ABW	Average body weight
ACH50	Alternative haemolytic complement pathway
ADC	Apparent digestibility coefficients
Adm.	Administration
AFN	*Autoridade Florestal Nacional* (National Conservation Center)
ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
AOAC	Association of Official Agricultural Chemists
ap.	Apparent
BEAA	Bile Aesculin Azide Agar
BHT	Butylated hydroxytoluene
BS EN	British Standard European Norm
BSA	Bovine serum albumin
BW	Body weight
CAT	Catalases
CECAV	*Centro de Ciência Animal e Veterinária* (Animal and Veterinary research Centre)
CFU	Colony forming unit
CIIMAR	Centro Interdisciplinar de Investigação Marinha e Ambiental
COM	Commission of the European Communities
CP	Crude protein
CTAB	Cetyltrimethylammonium bromide; hexadecyltrimethylammonium bromide
DDGS	Dried Distillers Grains with Solubles
DGGE	Denaturing gradient gel electrophoresis
DGI	Daily growth index
DGV	*Direcção-Geral de Veterinária* (Portuguese Veterinary Authority)
DM	Dry matter
DNA	Deoxyribonucleic acid
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
EATiP	European aquaculture technology and innovation platform
EC	European Commission
EC	Enzyme Commission (number)
EFSA	European Food Safety Authority
ESAC	*Escola Superior Agrária de Coimbra*
EU	European Union
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FBW	Final body weight
FCR	Feed conversion ratio
FDA	Food and Drug Administration
FI	Feed intake
G/C	Guanine-cytosine
GALT	Gut-associated lymphoid tissue
GE	Gross energy
GI	Gastrointestinal
GIALT	Gill-associated lymphoid tissue
GIT	Gastro-intestinal tract
GnRH	Gonadotropin releasing hormone
GPx	Glutathione peroxidases
GR	Glutathione reductases
GRAS	Generally Recognized as Safe
GSH	Glutathione
GSSG	Glutathione disulphide
GST	Glutathione S-transferases
H´	Shannon diversity index
HE	Haematoxylin and eosin
HIS	Hepatosomatic index

HK	Head-kidney
i.m.	Intramuscular
i.p.	Intraperitoneal
IBW	Initial body weight
ICBAS	*Instituto de Ciências Biomedicas Abel Salazar*
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
ISAPP	International Scientific Association for Probiotics and Prebiotics
ISI	Intestine somatic index
ISO	International Organization for Standardization
IU	International unit
K	Condition factor
LAB	Lactic acid bacteria
LP	Lamina propria
LPO	Lipid peroxidation
LPS	Lipopolysaccharides
MALT	Mucosa-associated lymphoid tissues
MAMPs	Microbe-associated molecular patterns,
MRS	Man, Rogosa, Sharpe
MUC	Mucin
MYP	Mannitol egg yolk polymyxin
n.a.	Not available
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NALT	Nasopharynx-associated lymphoid tissue
NOD	Nucleotide oligomerization domain
NRC	Nation Research Council
OD	Optical densities
OIE	*Office International des Epizooties* (World Organization for Animal Health)
OS	Oxidative stress
OTUs	Operational taxonomic units
PAMPs	Pathogen-associated molecular patterns
PAS	Periodic acid-Schiff reagent
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PER	Protein efficiency ratio
PRB	Probiotic
PRRs	Pathogen pattern recognition receptors
QPS	Qualified Presumption of Safety
R	Richness (of OTUs)
RaRBC	Rabbit red blood cells
RLP	Relative level of protection
RNA	Ribonucleic acid
RNaseA	Ribonuclease A
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SALT	Skin-associated lymphoid tissue
Sb	Submucosa
Sc	*Stractum compactum*
SCFA	Short-chain fatty acids
SD	Standard deviation
SGR	Specific growth rate
SOD	Superoxide dismutases
SSI	Spleen somatic index
ssp.	Subspecies
T	Temperature
TAE	Tris-acetate-EDTA (Ethylenediamine tetraacetic acid)
TBARS	Thiobarbituric acid reactive substances

TG	Total glutathione
TGFβ	Transforming growth factor beta
TLR	Toll-like receptors
TNFα	Tumour necrosis factor alpha
TSA	Tryptone Soy Agar
U	Units
UP	*Universidade do Porto*
UPGMA	Unweight Pair Group Method with Arithmetic Mean
USA	United States of America
UTAD	*Universidade de Trás-os-Montes e Alto-Douro*
VFI	Voluntary feed intake
VSI	Viscerosomatic index
WHO	World Health Organization

Bacteria genera abbreviations

A.	*Aeromonas*
B.	*Bacillus*
E.	*Enterococcus*
Ed.	*Edwardsiella*
L.	*Lactobacillus*
Lacto.	*Lactococcus*
M.	*Micrococcus*
P.	*Pediococcus*
S.	*Saccharomyces*
Strept.	*Streptococcus*
V.	*Vibrio*
Y.	*Yersinia*

1 *General introduction*

1.1 INTRODUCTION TO THE STUDY

The latest available data about the state of the world fisheries and aquaculture sectors annually provided by FAO, states that we have never consumed so much fish, or this sectors have impacted so much in the wellbeing of population, as these days (FAO, 2014). The same source highlights the role played by the sectors Fisheries and Aquaculture in the health promotion and on the mitigation of poverty and malnourishment. The worldwide fish consumption *per capita* increased from 9.9 kg in 1960, to 19.2 kg in 2012 (FAO, 2014). According to the latest statistics, aquaculture continues to expand more rapidly than any other food production sector (FAO, 2015). Fish capture is stagnant since 1980s (FAO, 2009), whereas aquaculture contribution to the total consumed fish has increased from 6% in 1970, to 50% in 2013, with an estimate contribution of 62% by 2030 (FAO, 2014).

However, the overall analysis of the global aquaculture growth rate in recent years indicates a slowdown in the growth worldwide, apart from specific accelerations for certain species and regions. It seems certain that this trend will continue in the near future. But it is also true that aquaculture will continue to grow in response to increasing demand of fish for human consumption. It is expected that the increase of population, higher incomes, urban lifestyle and dietary diversification, will increase the demand and shift the food consumption habits towards higher consumption of animal protein in developing countries. However, in industrialized countries, and particularly in Europe, given the (slow) reduction of the population, it is expected that the demand for food will increase only moderately and that issues as food safety, nutritional quality of products, environmental concerns and the animal welfare will become increasingly more important to consumers (FAO, 2009). In fact, in the recent years, the collective consciousness on the agricultural practices and their environmental consequences, along with food safety issues, has increased. In 2009, the Commission of the European Communities (COM 162, 2009), promoting the development of a sustainable European aquaculture, communicated that "*Environmental sustainability is a necessity and consumers want to be assured that aquaculture products are produced and transported taking account of high standard environmental requirements. Applying high standards will eventually also improve the image of the aquaculture industry and facilitate its access to the markets. The Community shall ensure that the EU aquaculture industry develops in a way which is compatible with a high level of protection of the natural environment. On the same lines, aquatic food products that are manufactured in or imported to the EU shall comply with*

high protection standards of consumer health and safety. The Community should also pursue its objectives for a high level of protection of health and welfare in farmed aquatic animals." More recently, the European Commission, recognizing the contribution of aquaculture for the food basket, economy and employment and facing the stagnation of its aquaculture production in the last decade, reinforced the need to develop and diverse the aquaculture sector, while maintaining high safety and environmental standards (COM 229, 2013; Lane *et al.*, 2014).

Worldwide, the intensification of the animal production in the recent decades allowed the increase of the production while decreased the costs. Such positive aspects were associated, however, with the pollution of natural resources and the spread of antibiotic resistance due to the indiscriminate use of antibiotics. The selective breeding of high productive animals and their farming at high densities contributed to the observed efficiency. However, also contributed to less robust animals with increasing susceptibility to diseases. Antibiotics were used extensively to treat and/or prevent bacterial disease outbreaks. In recent years, the presence of antibiotic residues in food, faeces and water and the antibiotic resistant bacteria, forced to rethink the use of antibiotics in animal production and to their prudent utilization. The prophylactic use of antibiotic agents was banned in EU countries since 1 January 2006 (Regulation (EC) 1831/2003) and the rules are strict concerning the use of therapeutic agents for food safety safeguard (Council Directive 96/23/EC; Council Directive 2006/88/EC).

Within fish farms and surroundings, a high bacterial resistance to antibiotics has been observed, mainly in the effluent samples, and in periods not corretaled with antibiotic therapy, indicating fish as reservoirs of antibiotic resistance bacteria (Alcaide *et al.*, 2005; Miranda & Zemelman, 2002). The evidences concerning the transference of resistant bacteria and antibiotic resistance factors from aquatic to terrestrial environments are increasing (Cabello, 2006; Rhodes *et al.*, 2000), including pathogenic bacteria for animals and man (Rhodes *et al.*, 2000). According to Cabello (2006), current data suggests that the indiscriminate use of antibiotics in aquaculture, has the potential to affect the Human and animal health on a global scale.

The frequent use of antibiotics is, therefore, a practice to avoid. However, if infectious diseases cause significant economic impact in all livestock sectors, in aquaculture the problem is even more severe. The water supports an important pathogen density and their effortless dispersion (Verschuere *et al.*, 2000). Intensive aquaculture fish experience situations of confinement, handling, transport and high densities, which are known stress factors (Arends

et al., 1999; Barton & Iwama, 1991; Wuertz *et al.*, 2006). The surrounding aquatic environment (*e.g.* temperature, salinity, dissolved oxygen, pH, pollutants) can cause environmental stress *per se* (Peters *et al.*, 1991; Reiser *et al.*, 2010; Reiser *et al.*, 2011). Stress increases fish susceptibility to infectious disease (Barton & Iwama, 1991; Crumlish *et al.*, 2003) since it affects immune responses (Costas *et al.*, 2011; Mock & Peters, 1990; Peters *et al.*, 1991), integrity (Olsen *et al.*, 2002) and intestinal functions (Olsen *et al.*, 2005). Those animals become more susceptible to opportunistic microbial infections (Sweetman *et al.*, 2010; Verschuere *et al.*, 2000), that may cause reduction of performance, quality of fish food and increase mortality rates. Thus, the application of prophylactic strategies is of best interest to producers in order to increase fish resistance to diseases.

More than a decade ago, the recommendations of FAO concerning the areas to research and developed for disease control in aquaculture included *i)* good nutrition to promote health, *ii)* affordable and effective vaccines, *iii)* immunostimulants and nonspecific immune enhancers, *iv)* probiotics (Subasinghe, 1997). The vaccination against some specific pathogens has contributed to reduce the use of antibiotics, mainly in salmon culture (Markestad & Grave, 1997). Nevertheless, vaccines are restricted to finfish and to a limit number of diseases and aquatic species (Subasinghe, 2009). At 2012, the European aquaculture technology and innovation platform (EATiP) considered the necessity to increase the use of alternative remedies and methods of biological control, such as probiotics (EATiP, 2012).

The use of probiotics, “Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2001; Hill *et al.*, 2014)” has been identified as an interesting solution to reduce the use of antimicrobials in all sectors of animal production and to promote livestock health. Aquaculture is one of the production sectors where the research on prophylactic practices has grown rapidly. Several review articles on the use of probiotics in aquaculture have been published recently (Akhter *et al.*, 2015; Balcázar *et al.*, 2006a; Bidhan *et al.*, 2014; Hai, 2015; Kesarcodi-Watson *et al.*, 2008; Merrifield *et al.*, 2010c; Merrifield & Ringø, 2014; Nayak, 2010; Newaj-Fyzul *et al.*, 2014; Wang *et al.*, 2008b) revealing the interest, dynamism and potential of this field. The evidences of probiotics application for immune and microbiota modulation, disease control and zootechnical benefits are accumulating.

1.2 THESIS OBJECTIVES

At the beginning of the current study, the international market provide some formulations of microbial additives with alleged probiotic action for aquaculture. The products were not very specific regarding fish hosts, dosage levels or the supplementation period. In the EU only one probiotic was authorized for aquaculture - *Pediococcus acidilactici* MA 18/5M - for salmonids and shrimps, increasing respectively, the number of animals with proper conformation (prevention of Vertebral Compression Syndrome) and improving survival rate, growth and resistance against infections caused by *Vibrio* sp. (Regulation (EC) 911/2009). Later on, this additive, marketed under the name Bactocell®, would obtain EU authorization to be use in all fish species (Regulation (EU) 95/2013).

Although we assumed that manufacturers have tried and tested their microbial additives, the scientific information on the outcomes of these products was limited or unavailable. Most studies published in scientific journals testing commercial probiotics in fish farming referred to products developed for terrestrial animals such as Bioplus2B® (*Bacillus subtilis* + *B. licheniformis*), Biomate SF-20® (*Enterococcus faecium*), ALL-LAC® *(Lactobacillus acidophilus* + *Streptococcus faecium*), Mycolactor® (*Saccharomyces cerevisiae*, *E. faecium*, *L. acidophilus*, *L. casei*, *L. plantarum*, *L. brevis*) and Bactocell®. Even concerning this last product, the one authorized in EU aquaculture, the available scientific information was scarce.

In this context, the current PhD study was designed to carry out *in vivo* feeding trials in rainbow trout (*Oncorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*), testing three commercial probiotic products. The commercial probiotics were: a *Bacillus* mixture (PAS TR®), originally developed for terrestrial livestock species, a bacterial mixture (Aquastar®), consisting in four distinct genera, and a monospecies probiotic (Bactocell®), the only probiotic product authorized in the EU to fish species. Trout and tilapia were chosen given their importance in European and World aquaculture, respectively.

The selected products were supplemented to the experimental diets at concentrations within the range recommended by the manufacturers.

The general thesis objective was to contribute to the application of commercial probiotics in aquaculture, in order to promote prophylactic nutritional strategies, towards a safer and sustainable aquaculture with improved fish welfare. The specific objectives were to evaluate the effects of the type of probiotics and their dosage level on the growth performance, dietary nutrient utilization, innate immune responses and intestinal morphology in trout and tilapia. To do so, the following parameters were determined:

• Zootechnical parameters as mortality, growth rates, feed conversion, ingestion and retention of nutrients, apparent digestibility of diets; organosomatic indexes;

• Carcass composition (moisture, ash, protein and lipid contents);

• Immune indicators in plasma (lysozyme, alternative complement pathway and peroxidase) and head kidney respiratory burst;

• Intestine morphometric influence;

• Intestinal microbiota modulation, mainly by molecular means.

1.3 BRIEF INTRODUCTION TO SELECTED FISH SPECIES CULTURE

In the experimental work leading to this thesis, three fish species were used: rainbow trout (*Oncorhynchus mykiss*), brown trout (*Salmo trutta*) and Nile tilapia (*Oreochromis niloticus*). Table 1-1 present their taxonomic classification and common designation in several languages.

Table 1-1 Taxonomic classification and designation of the model fish species.

Common name (English, Portuguese, French and Spanish)	**Rainbow trout** Truta arco-íris Truite arc-en ciel Trucha arco iris	**Brown trout** Truta comum, marisca ou fário Truite (fario) Trucha comun	**Nile Tilapia** Tilápia do Nilo Tilapia du Nil Tilapia del Nilo
Kingdom	Animalia		
Phylum	Chordata		
Subphylum	Vertebrata		
Class	Osteichthyes		
Subclass	Actinopterygii		
Order	Salmoniformes		Perciformes
Family	Salmonidae		Cichlidae
Genus	*Oncorhynchus*	*Salmo*	*Oreochromis*
Species	***Oncorhynchus mykiss*** (Walbaum, 1792)	***Salmo trutta*** (Linnaeus, 1758)	***Oreochromis niloticus*** (Linnaeus, 1758)

Source: (Fernandes, 1998; Gonçalves, 1998).

These species were chosen because of their importance to Portuguese, European and world aquaculture. Rainbow trout is a salmonid native from the Pacific coast of North America; by the end of 19th century was introduced to regions of all over the world, due to the properties of fast growth rates and tolerance to wide range of temperatures (Gonçalves, 1998). Survives at near freezing temperatures to 27ºC. For spawning and growth, trout require a narrower temperature range, between 9-18°C, whereas 21ºC is considered as the upper limit (FAO, 2005-2016). Gonçalves (1998) recommend temperatures of 9-12ºC on incubation and fry stages, and 13 to 18ºC for the juvenile and grow-out stages. Rainbow trout is the main trout farmed worldwide with total global production of about 0.8 million tonnes and Portuguese production of 751 tonnes in 2014 (FAO, 2011-2016). Brown trout or European trout, is a Eurasian and North African indigenous species (MacCrimmon & Marshall, 1968) that can adapt to diverse environments with a worldwide distribution (Klemetsen *et al*., 2003). Brown trout is not especially important for food fish, being generally used for recreational fisheries (Merrifield *et al.*, 2010c). The Portuguese and global production in 2014 was about 1.1 and 4388 tonnes, respectively (FAO, 2011-2016). Brown trout withstands temperatures ranging from 3ºC to 26ºC, with optimum range between 13-18ºC (Klemetsen et al., 2003).

Both salmonids are believed to be essentially freshwater species, but they can be also reared in seawater, when properly adapted. Some wild populations of rainbow and brown trout are found permanently in freshwater environment, while other have a migratory (anadromous) behaviour (Klemetsen *et al.*, 2003). *Salmo trutta trutta* is the subspecies with anadromous behaviour, also named as sea trout and *Salmo trutta fario* is the non-migratory subspecies.

Tilapia is a common designation of species from three genera of the Cichlidae family: *Oreochromis*, *Sarotherodon*, and *Tilapia*. The most important aquaculture species belong to genus *Oreochromis* and include the Nile tilapia, *O. niloticus*, the Mozambique tilapia, *O. mossambicus*, the blue tilapia, *O. aureus*, and *O. urolepis hornorum* (Watanabe et al., 2002). Nile tilapia is the most produced (El-Sayed, 2006), with a total production exceeding 3.6 million tonnes in 2014 (FAO, 2011-2016). Tilapias are tropical fishes native to Africa and the Middle East, but are currently globally distributed (FAO, 2012; Watanabe *et al.*, 2002). Their success lies on their resistance to diseases, adaptability to large aquatic conditions, production systems and diet formulations (El-Sayed, 2006; Fitzsimmons *et al.*, 2011; Watanabe *et al.*, 2002). Tilapias are freshwater species, but they can be also cope with increased salinities (El-Sayed, 2006; Fernandes, 1998). They tolerate temperatures of 8 to 42ºC (Fernandes, 1998), but the optimum temperature range between 24-32ºC (El-Sayed & Kawanna, 2008).

Rainbow trout sexually mature between 15-18 months old for males and after two years old for females (FAO, 2005-2016). Brown trout mature between 1-2 years old for males and 2-3 years of age old for females (FAO, 2012-2016). Tilapia sexually mature significantly earlier than trout, between 3 to 6 months old (El-Sayed, 2006). To avoid sexual maturation, inconvenient for grow-out fish, monosex populations are frequently used in commercial production; female gender in the trout species and male in tilapia (El-Sayed, 2006). Frequently female triploid trout are also used (FAO, 2012-2016; Gonçalves, 1998).

Feeding habits and nutrient requirements

Rainbow and brown trout have predominantly carnivorous feeding habits while tilapia are generally omnivorous. Wild adult trout feed on aquatic and terrestrial insects, molluscs, crustaceans, fish eggs, small fishes, and freshwater shrimp (FAO, 2005-2016; Klemetsen *et al.*, 2003). Tilapias feed on algae, aquatic plants, small invertebrates, detritus and associated bacterial films and diverse feeds of animal origin (Fitzsimmons *et al.*, 2011; Watanabe *et al.*,

2002). The relative position in trophic hierarchy and protein requirements is higher for salmonids and lower for tilapias (Huntington & Hasan, 2009).

Tables 1-2 and 1-3 presents the nutrient requirements for rainbow trout, tilapias (*Oreochromis* spp.) and Atlantic salmon (NRC, 2011). No data was assembled for brown trout so data from Atlantic salmon (*Salmo salar*), closest species to brown trout, are presented.

Table 1-2 Nutrient requirements of freshwater fish species (dry-matter basis).

	Rainbow trout	Atlantic salmon	Tilapia
Typical energy and protein concentrations [a]			
Digestible energy (kcal/kg diet)	4200	4400	3400
Digestible protein (%)	38	36	29
Amino acids (%)			
Arginine	1.5	1.8	1.2
Histidine	0.8	0.8 [b]	1
Isoleucine	1.1	1.1	1
Leucine	1.5	1.5	1.9
Lysine	2.4	2.4	1.6
Methionine	0.7	0.7	0.7
Methionine + cysteine	1.1	1.1	1
Phenylalanine	0.9	0.9	1.1
Phenylalanine + tyrosine	1.8	1.8	1.6
Threonine	1.1	1.1	1.1
Tryptophan	0.3	0.3	0.3
Valine	1.2	1.2	1.5
Taurine [c]	NR [c]	NR	NT
Fatty acids (%)			
18:3 *n*-3	0.7-1	1	NT
n-3 LC-PUFA [d]	0.4-0.5	0.5-1	R
18:2 *n*-6	1	NT	0.5-1
Cholesterol (%)	NT	NT	NT
Phospholipids (%)	NT (4-14) [e]	NT (4-6) [e]	NT

Source: NRC (2011). According to the authors the values represent near 100% bioavailability, due the determination of requirements with purified ingredients with highly digestible nutrients. R, required in diet but quantity not determined; NR, not required under practical conditions (diets containing ingredients from marine and terrestrial animal proteins and fish oil and water of at least medium hardness); and NT, not tested

a – Usual digestible energy and digestible crude protein levels (digestible N x 6.25) in commercial diets.
b – Young Atlantic salmon undergoing rapid growth after transfer to seawater appear to require up to 1.4% dietary histidine to prevent ocular pathology (bilateral cataracts).
c – Taurine (an amino-sulfonic acid that is a derivative of cysteine) was included. One study reported taurine requirement by rainbow-trout fry fed all-vegetable diets.
d – 20:5 *n*-3 and/or 22:6 *n*-3
e – Values in parentheses represent requirements reported for larval/early juvenile stages.

As mentioned by the authors, the values represent minimum requirements for maximum performance under optimal experimental conditions and with ingredients with high bioavailability. Practical diets may contain a safety margin, to compensate processing or storage losses, variation in composition or digestibility of ingredients and higher requirements due to environmental effects (NRC, 2011).

Table 1-3 Mineral and vitamins requirements (dry-matter basis).

NUTRIENT REQUIREMENTS	Rainbow trout (*Oncorhynchus mykiss*)	Atlantic salmon (*Salmo salar*)	Tilapia *Oreochromis* spp.
Macrominerals (%)			
Calcium	NR	NR	R/0,7[f]
Chlorine	NT	NT	0.15
Magnesium	0.05	0.04	0.06
Phosphorus	0.7	0.8	0.4
Potassium	NT	NT	0.2-0.3
Sodium	NR	NR	0.15
Microminerals (mg/kg)			
Copper	3	5	5
Iodine	1.1	R	NT
Iron	NT	30-60	85
Manganese	12	10	7
Selenium	0.15	NT	NT
Zinc	15	37	20
Fat-soluble vitamins [g]			
A (mg/kg)	0.75	NT	1.8
D (µg/kg)	40	NT	9
E (mg/kg)	50	60	60
K (mg/kg)	R	<10	NT
Water-soluble vitamins (mg/kg)			
Thiamin	1	NT	NT
Riboflavin	4	NT	6
Vitamin B6	3	5	15
Pantothenic Acid	20	NT	10
Niacin	10	NT	26
Biotin	0.15	NT	0.06
Vitamin B12	R	NT	NR
Folacin	1	NT	1
Choline [h]	800	NT	1000
Myoinositol [h]	300	NT	400
Vitamin C [i]	20	20	20

Source: NRC (2011). According to the authors the values represent near 100% bioavailability, due the determination of requirements with purified ingredients with highly digestible nutrients. R, required in diet but quantity not determined; NR, not required under practical conditions (diets containing ingredients from marine and terrestrial animal proteins and fish oil and water of at least medium hardness); and NT, not tested

f – Dietary requirement in the absence of waterborne calcium.

g – Conversion factors: 10000 IU ≈ 3000 µg vitamin A (retinol), 1 IU=0.025 µg vitaminD (cholecalciferol).

h- Diet without phospholipids.

i- As L-ascorbyl-2-monophosphate or L-ascorbyl-2-polyphosfate.

According to Lovell (2002) the diets for rainbow trout in North America usually have 36% of crude protein (CP) and 4 Kcal per g, while in Europe is frequently used more dense diets with 50% crude protein (CP) and 5 to 6 Kcal per g, and higher lipid contents (20 to 30%). El-Sayed (2006) recommends 30 to 40% of CP for young tilapias and 20-30% of CP for adult fish; accordingly to the author the lipid levels for maximum growth should be between 10-15% but are frequently lower (6-8%).

The feeding frequency (number of meals per day) and feeding level are inversely related to fish size. At early life stage, fish need to be fed several times a day (table 1-4) and then feeding frequency gradually decrease with increase in fish size (Lovell, 2002). Feeding level is usually expressed as a percentage of body weight (may vary from 5 to 15% and to 0.8 to 2.3 % for fry and juvenile, respectively), decreasing as the fish grows. Moreover, the feeding levels is related to the water temperature (table 1-5); quoting Tacon and Cowey (1985) "an increase in water temperature (up to an optimum level) is accompanied by an increased feed intake (Brett *et al.*, 1969; Choubert *et al.* , 1982), increased growth rate and metabolic rate (Jobling, 1983b) and a faster gastro-intestinal transit time (Fauconneau *et al.*, 1983)".

Table 1-4 Feeding rates and frequencies for growing tilapia at 28ºC.

Tilapia size (g)	Daily feeding (% fish weight)	Meals per day
2 days old to 1 g	30-10	8
1-5g	10-6	6
5-20g	6-4	4
20-100g	4-3	3 or 4
>100 g	3	3

Source: Lovell (2002).

Table 1-5 Recommended amount of feed per day to rainbow trout raised at different temperatures.

Rainbow trout size (cm)	Daily feeding (% fish weight) 5ºC	10ºC	15ºC
< 2.5 cm	6.6	9.9	14.5
2,5-5	5.5	8.1	12
5-7.5	4.4	6.5	9.7
7.5-10	3.3	4.9	7.2
10-12.5	2.5	3.7	5.4
12.5-15	2.0	2.9	4.4
15-17.5	1.7	2.5	3.6
17.5-20	1.4	2.1	3.1
20-22.5	1.2	1.9	2.7
22.5-25	1.1	1.6	2.4
>25	1.0	1.5	2.2

Adapted from Halver and Hardy (2002).

1.4 USE OF PROBIOTICS IN AQUACULTURE

1.4.1 Definition and concept evolution

The term *probiotic* currently is used for beneficial microorganism for men and animals. There is not a unique and universal definition and over time the concept has evolved. Lilly and Stillwell, in 1965, used the term to name *substances produced by one microorganism that stimulate the growth of another*, in opposition to antibiotic. That authors are frequently proposed as the first that used the term (FAO/WHO, 2001; Salminen *et al.*, 1999), but not according to Lauzon *et al*. (2014) in a detailed historic review of the concept. Parker, in 1974, considered probiotics as the *organisms and substances which contribute to the intestinal microbial balance* (Lauzon *et al.*, 2014). Latter, Fuller (1989) propose the following definition: "*live microbial feed supplement which beneficially affects the host animal by improving its microbial balance"*.

If initially the benefits where attributed to gastrointestinal tract (Madsen *et al.*, 2001; Marteau *et al.*, 2002), latter the actions were realized to be extended far beyond it (Lenoir-Wijnkoop *et al.*, 2007). There have been reports of benefits in skin and fur, in atopic dermatitis (Erdman & Poutahidis, 2014; Michail *et al.*, 2008), in reproduction (Erdman & Poutahidis, 2014), on urogenital (Reid, 2001) and rheumatic disorders (Sheil *et al.*, 2004; Yeoh *et al.*, 2013) and in the diminishing of cardiovascular disease risk (De Smet *et al.*, 1998). More recently the implications of gut microbiota and probiotics at neurological and behaviour level have been revealed (Bourassa *et al.*, 2016; Cryan & Dinan, 2012; Jiang *et al.*, 2015).

In this context, the term was redefined by Guarner and Schaafsma (1998, quoted in FAO/WHO, 2001) as *live microorganisms which when consumed in adequate amounts confer a health benefit on the host.* Based on that definition, a joint specialist panel from FAO/WHO redefined as: "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*, changing deliberately the term *consumed* to *administered* (FAO/WHO, 2001). That is one of the most consensual definitions and probably the most referred on literature. In 2013, the International Scientific Association for Probiotics and Prebiotics (ISAPP) retain that definition (small grammatical change[1]) considering it "inclusive

[1] "Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"

of a wide range of microbes and applications, while comprising the core features of probiotics: microbial, viable and beneficial to health" (Hill *et al.*, 2014).

The aquatic environment is more favourable to microbial growth compared to air (Gomez *et al.*, 2013; Hansen & Olafsen, 1999). The digestive tract of aquatic animals is an open system in continuous contact with the surrounding aquatic environment (Lazado & Caipang, 2014). Comparatively to the aquatic environment, the intestine is richer in nutrients, favouring microbial growth (Cahill, 1990; Wang *et al.*, 2008b). In this way, the surrounding microorganisms can exert more influence in the health of aquatic species than in terrestrial counterparts (Balcázar *et al.*, 2006a; Verschuere *et al.*, 2000). Moriaty (1998) proposed that the term probiotic in aquaculture should be extended to water microbial additives. Verschuere and co-authors (2000), accordingly, proposed the following definition: "*a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment*". Merrifield and co-authors (2010c) extended even more the concept "*any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer*", allowing the inclusion of viable or not viable cells in the definition.

Although conceptually is more usual the characterization of probiotics as viable (live) cells, some studies indicate that dead (not viable) microorganisms have probiotics effects, so giving scientific justification for expanding the concept (Caselli *et al.*, 2011; Lahtinen, 2012). In that context, Salminen and co-authors (1999) defined probiotics as "*microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host"*. In fact, cell wall components or DNA sequences of dead microorganisms exert benefits through immune stimulation, toxin sequester or connection to mucosal epithelia. Rachmilewitz *et al*., (2004) observed similar results, using live or inactivated cells, concluding that microbial DNA was the responsible for the Tool-like receptors stimulation. Diaz-Rosales and co-authors (2006) observed enhanced immune responses after heat-inactivated probiotic administration to gilthead sea bream. Abbass *et al*, (2010) reported higher survival of rainbow trout infected with Yersiniosis, after intraperitoneal or intramuscular injection of several subcellular components of the probiotics *Aeromonas sobri*a GC2 and *Bacillus subtilis* JB-1. These studies are valuable contributors for the understanding of probiotic modes of action, and open interesting possibilities for the industry of immunestimulant additives (Adams, 2010). Nevertheless, it remains more suitable

the association of probiotic with live microorganism. Shenderov (2013) used the term *metabiotics* (or pharmacobiotics or postbiotics) to name the "structural components of probiotic microorganisms and/or their metabolites and/or signalling molecules with a determined (known) chemical structure that can optimize host-specific physiological functions, regulator, metabolic and/or behaviour reactions". Thus, the probiotic definition and concept is in constant turnover, prone to change with the new insights and research findings.

1.4.2 **Mechanisms of action**

Probiotics act in complex and multifactorial ways, not completely elucidated but that depend on the strain and the host (Lazado & Caipang, 2014). In most cases the mechanisms of action (Figure 1-1) result on the microbiota modulation and their consequent influence on mucosal immunity (Balcázar *et al.*, 2007a; Merrifield & Carnevali, 2014b).

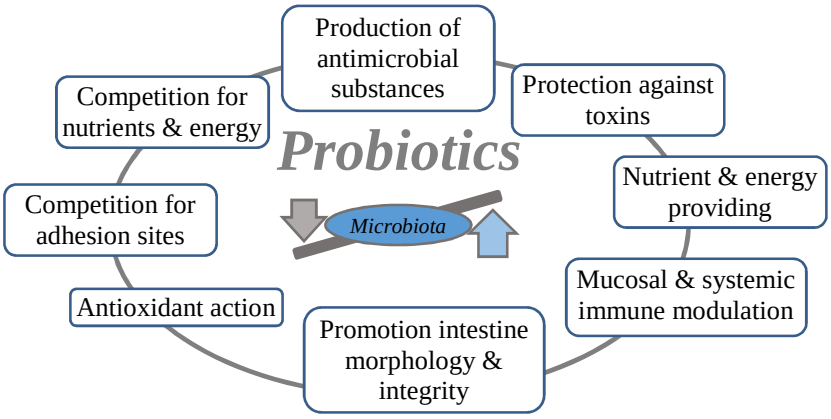

Figure 1-1 Probiotics major mechanisms of action.

The displayed actions can be triggered by the probiotic microorganisms or indirectly by the promotion of the growth of microorganisms exhibiting those properties. Nevertheless, the result will be the favouring of a healthier microbiota, in which the microorganisms with positive actions are dominant relative to the potential pathogenic ones, situation defined as *eubiosis* or *normobiosis* (Figure 1-2) while the opposite is named *dysbiosis* (Iebba *et al.*, 2016).

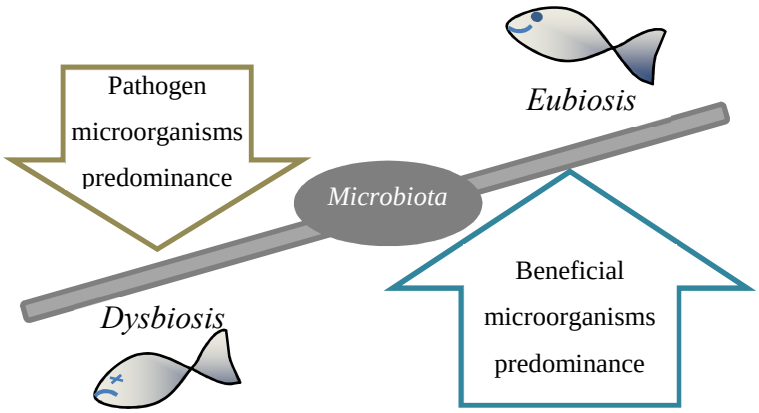

Figure 1-2 Microbiota balance positive (eubiosis) or negative to host (dysbiosis).

Although some probiotics may act at in several ways, it is very unlikely that a single microorganism display all the abilities described in Figure 1-1, justifying the use of a blend of bacteria and/or yeast for more effectiveness (Oelschlaeger, 2010; Salinas *et al.*, 2008; Verschuere *et al.*, 2000). The central mechanisms of probiotics action are described below.

1.4.2.1 Competitive exclusion

For the establishment in gastro-intestinal tract (GIT), the microorganismos must adhere to mucus or epithelial cells. To achieve that, they must compete for adhesion sites. Competition with pathogens for adhesion and colonization of the mucosal surfaces is one of the known probiotics modes of action (Balcázar *et al.*, 2006a; Chabrillón *et al.*, 2005a; Chabrillón *et al.*, 2005b; Gueimonde *et al.*, 2006; Vine *et al.*, 2004). Balcazar *et al.* (2007d) observed *in vitro*, low adhesion of important fish pathogens (*Aeromonas Salmonicida*, *Carnobacterium piscícola*, *Yersinia ruckeri* and *Lactococcus garvieae*) to rainbow trout gut mucus, subsequently to the adhesion of certain strains of *Lactococcus* and *Lactobacillus*.

The authors observed distinct competitive exclusion abilities among the different bacteria strains from same species.

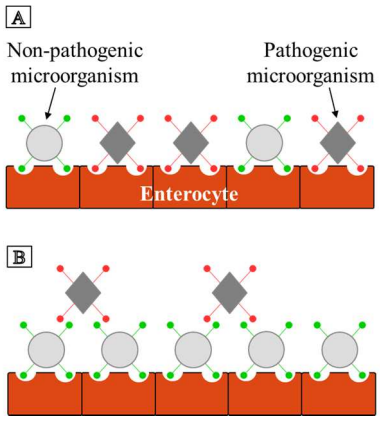

Figure 1-3 Simple representation of competitive exclusion by probiotics.
Situation A) represents a diverse population, including pathogenic microorganisms. Situation B) represents competitive exclusion of pathogenic microorganismos resulting from adhesion of non-pathogenic ones. Adapted from Ewing (2008).

1.4.2.2 Competition for nutrients

The microorganisms compete with each other for nutrients and energy substrates, diminishing their abundance on the surrounding environment. In that way, probiotic bacteria may affect the proliferation of other microorganisms, including putative pathogenic bacteria (Brown, 2011).

The competition for iron is well-known, given the importance of this mineral for bacterial growth. Some microorganism can produce siderophores, low molecular weight iron-binding compounds, that are produced extracellularly and facilitate iron solubility and capture (Messenger & Barclay, 1983). Gatesoupe (1997) studying the possibility of inhibition of a pathogenic vibrio by a probiotic strain, observed higher survival to infection of turbot larvae fed probiotic enriched rotifers, with no significant impact on larval growth. However, in a second trial, in a condition of iron deficiency, the probiotic supplementation improved significantly the larvae survival and growth. The author related those results to the ability of the probiotic strain to produce a siderophore while the pathogen were unable. Likewise, Sugita *et al*. (1998) identified as a siderophore, a substance with large antibacterial spectrum produced by *Bacillus* and Brunt *et al*. (2007) observed that siderophore produced by probiotics (*Bacillus* sp. and *Aeromonas sobria*) protected rainbow trout against several important pathogens.

1.4.2.3 Production of inhibitory compounds

The microorganisms, including probiotics, may produce substances with antimicrobial action, as antibiotics, bacteriocins, nitric oxide, hydroxide peroxide, lytic enzymes, organic acids and siderophores (Dobson *et al.*, 2012; Nair *et al.*, 1985; Sugita *et al.*, 1998; Williams & Vickers, 1986). Those antagonist compounds affect the surrounding microbial community, and may destroy or limit the proliferation of putative pathogenic or opportunistic agents (Balcázar *et al.*, 2007d).

Bacteriocins, produced by most bacteria and archaea, are antibacterial peptides, heterogenic on size, structure, antimicrobial potency, as cellular target receptors (Dobson *et al.*, 2012; Ghanbari *et al.*, 2013; Gillor *et al.*, 2008). *Lactobacillus* (Ghanbari *et al.*, 2013) and *vibrio* (Sugita *et al.*, 1997) isolated from fish intestine showed the ability to produce antagonistic bacteriocins against Human and aquatic pathogens. According to Dobson (2012)

there are three main mechanisms through which bacteriocin production might contribute to probiotic functionality. Bacteriocin may function as: *i)* colonizing peptides, allowing a better colonization of probiotics within the resident microbiota; *ii)* killing peptides, directly destroying pathogens and *iii)* signalling peptides to the host immune system or other bacteria. The latter may be involved in bacteria communication (*quorum sensing*) through extracellular diffusion of signalling molecules that facilitates group synchronization and coordination (Dobson *et al.*, 2012). According to Brown (2011) some probiotics may interfere in the *quorum sensing* amongst pathogenic bacteria, limiting their growth and/or their negative influence on the host.

Probiotics may produce deconjugated bile acids, which are derivatives of bile salts with stronger antimicrobial activity, comparatively to bile salts synthesised by the host (Oelschlaeger, 2010). That property has also consequences in the diminishing of the absorption of cholesterol, influencing the cardiovascular disease risk (De Smet *et al.*, 1998).

Some bacteria produce organic acids as lactic acid and short-chain fatty acids (SCFA), mainly acetic, propionic and butyric acids (Clements *et al.*, 1994; Mountfort *et al.*, 2002). Those metabolites decrease the intestine pH, which inhibit the proliferation of pathogenic bacteria, by perturbance of the optimum pH for their proliferation (Topping & Clifton, 2001). The organic acids production and the consequent low pH of the gut lumen are also involved in other probiotic form of actions, as described later.

1.4.2.4 Host protection against pathogen toxins

Probiotics may protect the host against bacteria, cyanobacteria and fungi toxins. In some cases, probiotic bacteria may have a protective effect by inhibiting pathogenic bacteria from producing the toxins. Such effect is mainly related to the high concentrations of organic acids like acetic, lactic or butyric acid in the medium (Ogawa *et al.*, 2001). In other cases, the protective effect may occur by the binding of probiotic cells to toxins. This physical mechanism, limits toxin uptake by the host and increases their faecal excretion and can occur even using dead probiotic cells (Oelschlaeger, 2010). Gratz *et al*. (2006) and Nybom *et al*. (2008) showed that *Lactobacillus rhamnosus* strains had the ability to bind several distinct mycotoxins and cyanobacteria toxins. Unviable *L. rhamnosus* showed superior binding ability of aflatoxins comparatively to live bacteria (Haskard *et al.*, 2000). The toxin safeguard

functionality may take place by enzymatic damage: *Sacchromyces boulardii* produced a protease that inhibited *Clostridium difficile* toxin A in the rat ileum (Castagliuolo *et al.*, 1996).

1.4.2.5 Production of nutrients

Probiotics produce metabolites with nutritive and energetic value: vitamins (Laiño *et al.*, 2013; LeBlanc *et al.*, 2011; Sugita *et al.*, 1991), amino acids and short-chain fatty acids (SCFA, Balcázar *et al.*, 2006a; Iehata *et al.*, 2009; Mountfort *et al.*, 2002). The probiotic cell components such as nucleotides, amino acids (Metges, 2000) or fatty acids (Yano *et al.*, 1997) have nutritional value. Those elements may constitute energetic or nutritive substrates for the host or other microorganisms, modulating the surrounding microbiota (O'Toole & Cooney, 2008).

In mammals, SCFA are known stimulators of colonic blood flow and fluid and electrolyte uptake (Topping & Clifton, 2001). Particularly, butyrate is an important energetic source, since is preferentially oxidised on colonocytes, and promotes a normal phenotype in these cells (Topping & Clifton, 2001). In addition, butyrate has a proliferative action on normal colon cells and anti-proliferative action on neoplasic colonocytes (O'Toole & Cooney, 2008). In mammals, butyrate is also involved in several functions besides the intestine, as reviewed by Guilloteau *et al*. (2010). In fish, the information about SCFA roles is limited, nevertheless the role of acetate, produced by microbial fermentation, as energetic substrate for herbivorous fish is known (Clements *et al.*, 1994; Mountfort *et al.*, 2002). Moreover, butyrate plays a role on the energetic budget, providing energy and having a protein-sparing effect, and on the enterocyte metabolism (Robles *et al.*, 2013), intestinal morphology and immunology (Liu *et al.*, 2014).

The reduction of intestinal pH, as a result of microbial fermentation, has additional positive effects on the availability of other nutrients as calcium or sodium (Lutz & Scharrer, 1991). Several studies in mammals associated the probiotic and prebiotic administration with the increase of bone mass or mineral bone content (Scholz-Ahrens *et al.*, 2007). In fish, that research area has been scarcely studied, possibly due to the ability of fish gills to retain minerals directly from the water (Bakke *et al.*, 2011), making more difficult the experimental design. Avella *et al.* (2012) observed an increase in bone calcification in zebrafish treated for

ten weeks with *Lactobacillus rhamnosus* 501®, whereas Abd El-Rhman *et al.* (2009) observed in increase in body ash content in Nile tilapia fed with diets containing *Pseudomonas*.

Some probiotics may have a hypocholesterolaemic action, particularly interesting for Human nutrition. Lactic acid bacteria (LAB) can diminished the cholesterol available for absorption in the intestine, by bonding cholesterol or bile acids to bacteria surface, by their assimilation or alteration (Dilna *et al.*, 2015; Matur & Eraslan, 2012). Moreover, there are indications of the role of *L. acidophilus* on the cholesterol absorption, through the down regulation of a key protein (Huang *et al.*, 2010).

1.4.2.6 Production and stimulation of digestive enzymes

Fish gastrointestinal microbiota produce diverse digestive enzymes: amylases, cellulases, lipases, proteases, chitinases and phytases (Askarian *et al.*, 2013; Brunt *et al.*, 2007; Ray *et al.*, 2010; St.John *et al.*, 2006), as comprehensively reviewed by Ray and co-authors (2012). In this way, the intestinal microorganisms may help to decompose nutrients, particularly those indigestible for host, such as non-starch polysaccharides. Additionally, the microbiota may stimulate the production of digestive enzymes (Bairagi *et al.*, 2002; Balcázar *et al.*, 2006a; De Schrijver & Ollevier, 2000; Murillo & Villamil, 2011; Wang *et al.*, 2008b), helping the digestion and absorption of dietary nutrients. Bairagi *et al*. (2002) observed on the intestine of nine teleost fish species, the production of digestive enzymes by bacteria relevant to the digestive process. The administration of photosynthetic bacteria, *Bacillus* or combination of both, resulted in increased activity of amylase, lipase and proteases in common carp (*Cyprinus carpio*) juveniles (Wang & Xu, 2006) and cellulase in *Penaeus vannamei* shrimp (Wang, 2007). The supplementation of *B. subtillis*, *L. plantarum* or both resulted in an increased amylase, lipase and protease activities in juvenile Nile tilapia, and an increase in amylase activity when fed diets supplemented with *S. cerevisae* yeast (Essa *et al.*, 2010). The probiotic strain Pdp13 (*Shewanella* genus) increased the alkaline phosphatase activity on distal intestine in juvenile Senegalese sole (Rodrigáñez *et al.*, 2009). Sharifuzzaman *et al*. (2014) observed in rainbow trout, a probiotic strain-specific increase in some glycosidases. Lara-Flores *et al*. (2010) observed higher disaccharidases activity in tilapia intestine treated with probiotics.

The probiotic influence on digestive enzymes seems to be more significant in fish starting the exogenous feeding. The administration of yeasts, *Saccharomyces cerevisiae boulardii* (Waché *et al.*, 2006) and *Debaryomyces hansenii* HF1 (Tovar *et al.*, 2002) and the lactic acid bacteria, *Lactobacillus* spp. (Suzer et al., 2008), *Lactobacillus curvatus* and *Leuconostoc mesenteroides* (Askarian *et al.*, 2011) resulted on an early maturation of digestive system in freshwater and marine fish species, with the increase of pancreatic and intestine digestive enzymes activity. Tovar *et al*. (2002) observed that polyamines (putrescine, but mainly spermidine and spermine) produced by *D. hansenii* had an effect on the early maturation of amylase and intestine brush border enzymes (aminopeptidase, maltase and alkaline phosphatase) in sea bass larvae. Additionally, fish treated with *D. hansenii* had higher survival and the lower incidence of spinal malformation. Polyamines are responsible for initiation and control of several functions on intestinal cells, by direct control of gene expression (Iacomino *et al.*, 2012; Löser *et al.*, 1999).

The effects on digestive system maturation by probiotic polyamines is one of the possible ways that dietary probiotics supplementation may improve growth performance and feed conversion. Nevertheless, Tovar *et al*. (2002) did not observed an increase in growth rate in sea bass larvae treated with *D. hansenii*.

Some bacteria isolated from fish intestine produce phytases. Askarian *et al*. (2013) observed in Atlantic cod microbiota, two promising probiotic strains (*Brochothrix* sp. and *Carnobacterium* sp.) with inhibitory action against important fish pathogens that showed high protease activity and some (although limited) phytase activity. Das & Ghosh (2013) isolated several phytase producing bacterial strains from carps intestine, including *Bacillus subtilis*. Those enzymes may contribute to the increase in availability of mineral elements, as observed in broiler chickens fed with *Mitsuokella jalaludinii*, a ruminal phytase producing bacteria (Lan *et al.*, 2002). This may be of particular interest in fish fed diets rich on plant ingredients.

1.4.2.7 Production of metabolites with antioxidant action

The role of oxidative stress (OS, Figure 1-4) at the initiation or development of several cardiovascular, neurodegenerative and tumoral diseases (Dichi *et al.*, 2014; Kullisaar *et al.*, 2012), explains why several products with antioxidant properties are being intensively studied and applied in Human and animal nutrition (Esteban *et al.*, 2014; Lushchak, 2012). The

participation of some probiotic microorganisms on the host antioxidant defence (Figure 1-5) has been observed (Mishra *et al.*, 2015).

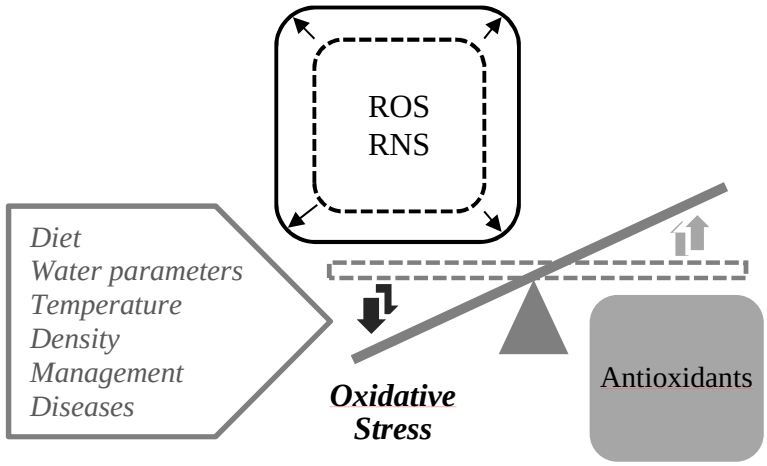

Figure 1-4 Unbalance between reactive species and antioxidant defences leads to a state of oxidative stress.

Oxidative stress (OS) occurs when "the concentration of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is transient or chronically enhanced, disturbing cellular metabolism and damaging cellular constituents" (Lushchak, 2011). Under several management and environmental stress situations (as handling, poor water quality, exposure to chemicals, parasites and pathogens) the rate of generation of ROS can exceed that of their removal, ending in OS (Morales *et al.*, 2004).

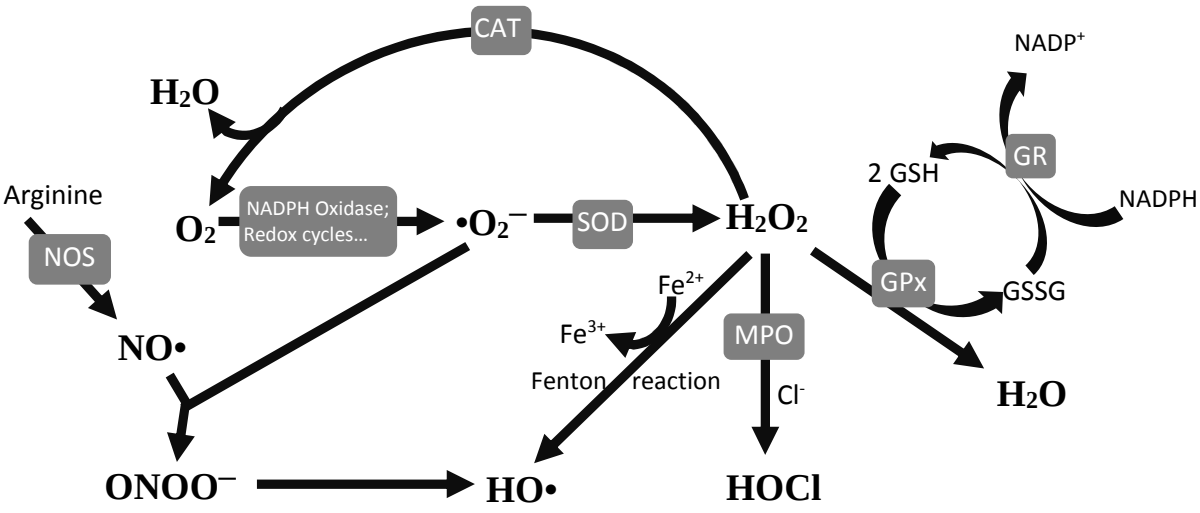

Figure 1-5 Routes of production of main reactive species and antioxidant enzymes.

ROS like molecular oxygen (O_2), superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and hipochlorous acid (HOCl) and RNS as nitric oxide radical ($NO^{\bullet}$) and peroxynitrite ($ONOO^-$), are generated continuously in the cell metabolism, as intermediate or side products or being produced on purpose, during respiratory burst by phagocytes (Babior, 2000; Lushchak, 2011; Martínez-Álvarez *et al.*, 2005). Those highly reactive species may damage cell components, mainly lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrates and nucleic acids (Blokhina *et al.*, 2003). To counteract it, the cell possess enzymatic and non-enzymatic antioxidant systems (Davies, 1995). Enzymes such as superoxide dismutases (SOD; EC 1.15.1.1), catalases (CAT, EC 1.11.1.6), glutathione peroxidases (GPx; EC 1.11.1.9), glutathione reductases (GR, EC 1.6.4.2) and glutathione S-transferases (GST, EC 2.5.1.18) are part of the enzymatic antioxidant defence system, while vitamin C (ascorbic acid), E (α-tocopherol) and glutathione (GSH) are examples of non-enzymatic antioxidants (Davies, 1995; Lushchak, 2011). Their role is to maintain low cellular levels of ROS, avoiding oxidative damage to macromolecules and influencing the response to pathogens.

Some probiotic strains, particularly LAB and their cellular extracts have shown antioxidant activity, very variable and strain-dependent (Amaretti *et al.*, 2013; Lin & Yen, 1999). Although not entirely understood, the mechanisms of action may involve the scavenging and the prevention of oxidants production in the intestine (Azcárate-Peril *et al.*, 2011). Some bacteria, such as *Lactococcus lactis*, produce protective enzymes (SOD, catalases and peroxidases) against reactive oxygen species (ROS, Azcárate-Peril *et al.*, 2011). Others (e.g. *Lactobacillus plantarum*), devoid of SOD, are rich in manganese (II) that play a role in the scavenging mechanism for the elimination of superoxide radical anion (Archibald & Fridovich, 1981). *L. plantarum* LP 1, *Streptococcus thermophilus* Z 57, *Bifidobacterium lactis* B 933 (Zanoni *et al.*, 2008) and *Lactobacillus fermentum* (Mikelsaar & Zilmer, 2009; Peran *et al.*, 2006) are known to produce glutathione, that play an important role on non-enzymatic antioxidant defence system. Additionally, probiotic bacteria may enhance the host glutathione biosynthesis, as observed in rats (Lutgendorff *et al.*, 2009; Lutgendorff *et al.*, 2008). It is thought that butyrate, produced directly by the probiotics or indirectly by the modulated microbiota, may be involved. Butyrate can induce host gene expression of proteins involved on oxidative stress response, particularly glutathione-S-transferase (Scharlau *et al.*, 2009). According to Kullisaar *et al*. (2012) glutathione is involved in the mucosa integrity maintenance. Moreover, probiotics possess metal ions chelating ability (Lin & Yen, 1999); metallic ions are known oxidative stress inducers (Lushchak, 2011). Some microbial exopolysaccharides have been reported to have antioxidant activity and may constitute a way to the removal of reactive oxygen and nitrogen species by probiotics (Abdhul *et al.*, 2014; Dilna *et al.*, 2015; Li *et al.*, 2014).

The administration of *Lactobacillus fermentum*, a glutathione-producing bacteria, showed to facilitate the recovery from inflammatory injuries in colitic rat (Peran *et al.*, 2006). Several LAB strains were able to reduce oxidative stress in rats subject to experimental oxidant (Amaretti *et al.*, 2013).

In aquaculture, the antioxidant potential of probiotics has been already reported, and likewise, particularly after a stressful situation as pathogenic infections. Castex *et al.* (2009) observed lower oxidative stress on shrimp digestive gland receiving *P. acidilactici*, after *Vibrio nigripulchritudo* infection; before infection the probiotic impact was less obvious. The authors assign the oxidative stress modulation as (part of) the reason for the higher survival observed. The same was observed on leopard grouper (*Mycteroperca rosacea*) fed with the yeast *Debaryomyces hansenii* before or after *Aeromonas hydrophila* infection (Reyes-Becerril

et al., 2011) or the *Amyloodinium ocellatum* parasite (Reyes-Becerril *et al.*, 2008). Rainbow trout receiving a multi-species probiotic in the diet presented higher activity of some antioxidant enzymes and lower lipid peroxidation (Ozório *et al.*, 2016). In tilapia, the probiotic addition to the water resulted in higher serum SOD and catalase activity, even if the total antioxidant capacity has not changed significantly (Zhou *et al.*, 2010). Giannenas *et al.* (2015) studied a multibacteria probiotic influence on the quality of fish muscle, reporting a lower lipid oxidation in rainbow trout muscle, immediately after death or after five days of storage.

1.4.2.8 Influence on intestine structure integrity, morphology, functionality

The microbiota and probiotics can influence positively the integrity, structure, morphology, motility and intestine functionality (Awad *et al.*, 2008; Caipang & Lazado, 2015; Dimitroglou *et al.*, 2009; Matur & Eraslan, 2012; Sweetman *et al.*, 2010). In Humans, there are increasing evidences of the involvement of some microorganisms in the maintenance of intestine barrier homeostasis (Madsen *et al.*, 2001) and their efficacy in the recovery of gastroenteritis (Marteau *et al.*, 2002) and other disorders (Aragon *et al.*, 2010). The probiotic usage for the prevention or treatments of such disorders is due to the vast research on animal models and cellular mammal lines, revealing the ability of certain microorganisms to alter the intestine structure minimizing diarrhoea (Taras *et al.*, 2005), inflammation, oedema (Fitzpatrick *et al.*, 2012) and apoptosis and to promote the epithelial cells proliferation (Aliakbarpour *et al.*, 2012; Im *et al.*, 2009). Matur and Eraslan (2012) reviewed comprehensively the subject.

In fish, similarly to terrestrial counterparts, there are observations of probiotic influence on intestine morphology, promoting an elongation of mucosae folds or *villi*[2] (Gisbert *et al.*, 2013; Pirarat *et al.*, 2011) and an increase on microvilli density (Rodrigáñez *et al.*, 2009;

[2] To enhance the intestine absorption surface the fish mucosa is folded and sometimes the primary folds present new folds (second and tertiary folds), projecting towards the lumen, similarly to villi presented on the higher vertebrate intestine. But, oppositely to them, fish "villi" do not present the central lacteal lymphatic vessel, neither present Lieberkuhn crypts between them (Wilson & Castro, 2010); in this context, numerous authors consider that fish do not possess true villi, naming those projections as mucosa folds (Gargiulo *et al.*, 1998; Jutfelt, 2006; Løkka *et al.*, 2013; Sealey *et al.*, 2009; Wilson & Castro, 2010). Nevertheless, the term villi or the plural villus is also observed in the literature (Dimitroglou *et al.*, 2009; Gisbert *et al.*, 2013; Heikkinen *et al.*, 2006; Pirarat *et al.*, 2011; Sweetman *et al.*, 2010).

Standen *et al.*, 2015) and length (Merrifield *et al.*, 2010d). The intestine wall thickness may also suffer influence (Barroso *et al.*, 2014), particularly in the muscular layer (Batista *et al.*, 2015). Standen *et al*. (2016) observed an increase in the relative perimeter (an indicator of absorptive area) in mid-intestine and an increase in goblet cells counts in Nile tilapia fed diets supplemented with mixed-species probiotic (*Lactobacillus* spp., *Pediococcus* spp., *Bacillus* spp. *and Enterococcus* spp.), with a clear dose-dependent effect. The intestinal section affected by probiotic treatment may differ with the fish species. In rainbow trout, microvilli length was increased in the proximal but not distal section (Merrifield *et al.*, 2010d), whereas in Nile tilapia, the probiotic effect was observed in the proximal and distal section, but not in the mid-section (Pirarat *et al.*, 2011).

Probiotic administration often results in a higher presence of goblet cells, responsible for the production of enteric mucus (Gisbert *et al.*, 2013; Pirarat *et al.*, 2011; Standen *et al.*, 2016; Standen *et al.*, 2013). Conversely, a decrease of goblet cell counts (Cerezuela *et al.*, 2013) or no significantly effect (Batista *et al.*, 2015; Harper *et al.*, 2011; Standen *et al.*, 2015) were also observed. Another indication of immune-stimulation by probiotic treatment is the increase in the intraepithelial leucocytes infiltration in the gut mucosae (Gisbert *et al.*, 2013; Harper *et al.*, 2011; Pirarat *et al.*, 2011; Standen *et al.*, 2016; Standen *et al.*, 2015). There are also observations of probiotic influence on enterocyte activity (Merrifield *et al.*, 2010d). In Senegalese sole probiotics promoted the enterocyte functionally by interfering on the excessive lipid accumulation (García de La Banda *et al.*, 2010; Rodrigáñez *et al.*, 2009).

Nevertheless, the influence of dietary treatment are not always clear (Ferguson *et al.*, 2010; Kristiansen *et al.*, 2011; Merrifield *et al.*, 2010d), with the administration dosage (Standen *et al.*, 2016) and/or the probiotic species (Merrifield *et al.*, 2010d) playing a role on the beneficial outcome. There are less reported cases in which probiotic treatment had negative impact. The administration of *B. subtilis* damaged the intestine mucosae (oedema and inflammation) in gilthead sea bream, particularly after co-administration with inulin (Cerezuela *et al.*, 2013).

The effects of probiotics on the epithelial proliferation have been attributed to the increase of short chain fatty acids (SCFA) production in the intestine. Ballester-Lozano *et al.* (2013) demonstrated in gilthead seabream the participation of butyrate on several cellular functions, promoting the integrity (regulation of several tight junctions elements), proliferation and health of intestine, especially on proximal section.

The microbiota has an influence in the motility and transit of gastro-intestinal tract (GIT). Usually the observations consist in the acceleration of gastric emptying and intestine transit (Miller & Ouwehand, 2013). Yet, on disorders characterized by passage rates higher than normal, the microbiota act in a way of normalization of the gastro-intestinal transit (Matur & Eraslan, 2012). The putative influence may be distinct on elderly or young healthy population, or woman and men (Matur & Eraslan, 2012; Meance *et al.*, 2011). According to Matur e Eraslan (2012), how intestinal microbiota may influence motility is not clear. Some findings may indicate that microbiota influence on inflammation mediators and SCFA content, with well-known influence on the GIT motility (Cherbut, 1995). To our best knowledge, no study has been carried out on the effects of probiotic supplementation on intestine motility in fish.

1.4.2.9 Imunnomodulatory action

The skin and mucosal surfaces establish a physical barrier separating the organism from the external environment (Foey & Picchietti, 2014; Snoeck *et al.*, 2005), protecting against invasion by microorganisms and harmful foreign substances. Mucosa, with the associated lymphoid tissue, plays a vital role on the immune resistance and constitute the largest defence organ (Lazado & Caipang, 2014). Generically is named as MALT (mucosa-associated lymphoid tissues), or specifically as GALT (gut-associated lymphoid tissue), GIALT (gill-associated lymphoid tissue), SALT (skin-associated lymphoid tissue) and the recently discovered NALT (nasopharynx-associated lymphoid tissue) (Salinas, 2015; Tacchi *et al.*, 2014).

The microbiota and digestive secretions make the very first line of defence against the invasion by microorganisms and external substances. The digestive function creates a hostile environment to pathogens by the acidic pH, digestive enzymes (specially pepsin and pancreatin; Mayer & Walker, 2005) and bile (Dalmo et al., 1997, cited by Mulder *et al.*, 2007), acting as a chemical barrier. The microbiota is a biological barrier against the adhesion and proliferation of pathogens. A healthy and balanced microbiota may limit the pathogen contact with the host by antimicrobial substances production, pH lowering and competition for substrates or adhesion sites, as described previously. Additionally, microorganisms are active players in the regulation of host immune system. The modulation can be systemic or local

(Lazado & Caipang, 2014). Nayak (2010) and Lazado & Caipang (2014) reviewed extensively the influence of probiotics on fish systemic and mucosal immunity, respectively. Probiotics influence/communicate with host immune system through their metabolites, side-products, cell wall components or DNA (Oelschlaeger, 2010).

The innate immune response is a dynamic reaction involving humoral elements present on the blood or tissues, cellular elements and inflammation, responsible for the attraction of immune cells to the scene. All those elements may be influenced by probiotic administration.

The GIT is exposed to a huge panoply of antigen from feed or resident or external microorganism (Mayer & Walker, 2005). The host must have mechanisms to recognize potential aggressors and distinguish them from non-pathogenic agents (Gomez *et al.*, 2013). The innate immune response in vertebrates is based on the recognition of *pathogen-associated molecular patterns* (PAMPs) also called *microbe-associated molecular patterns*, MAMPs, (Lazado & Caipang, 2014) by receptors called *pathogen pattern recognition receptors* (PRRs). PAMPs are well conserved molecular structure of pathogens, including bacteria, virus, fungi, protozoa (Kawai & Akira, 2005) and parasites (Alvarez-Pellitero, 2008), as lipopolysaccharides (LPS), peptidoglycan; ß-1,3 glucan, mannan-oligosaccharide (Magnadóttir, 2006; Whyte, 2007), flagellin, zymosan and microbial nucleic acids (Barton & Medzhitov, 2003; Lazado & Caipang, 2014). Additionally to pathogen patters, also molecules released during lesions, infection, inflammation or apoptosis, that usually are not expressed on cell surface, constitute danger signals, initiating an innate immune response (Magnadóttir, 2006). Several PRRs were already identified in teleosts, classified on four main types: toll-like receptors (TLR); NOD-like receptors; C-type lectin receptors; and peptidoglycan recognition proteins (Lazado & Caipang, 2014). The activation of PPRs activates intracellular signalling pathways, releasing specific cytokines and transmitting signals to surrounding cells to exert pro or anti-inflammatory effects (Lazado & Caipang, 2014). Also leads to alterations in the gene expression of mucus mucins and antimicrobials, promoting the release of antimicrobial substances and mucus and altering mucus composition (McGuckin *et al.*, 2011).

Several probiotic strains were able to modulate pro-inflammatory cytokines (as TNFα and IL-1ß) and anti-inflammatory cytokines (as IL-10 and TGFß) in tilapia intestine (Standen *et al.*, 2016) and (HK) head-kidney (HK, Pirarat *et al.*, 2011) and rainbow trout HK and spleen (Panigrahi *et al.*, 2007; Panigrahi *et al.*, 2011). Thus, probiotics are recognized as non-pathogenic, playing active role on the intestinal immunology and morphology of the host towards a more tolerant and less inflammatory mucosa (Lazado & Caipang, 2014; Ringø *et*

al., 2007), ameliorating the epithelial barrier function (Madsen *et al.*, 2001). As example, *Pediococcus acidilactici* was able to contribute by alleviating the inflammation of the intestine of Atlantic salmon, subjected to induced gut inflammation (Vasanth *et al.*, 2015).

Probiotics may also ameliorate the antigen recognition, given the up regulation of TLR2 gene expression observed on Nile tilapia supplemented with a multispecies probiotic through feed (Standen *et al.*, 2016).

At local level (gut mucosa) the immune stimulation might be more notorious, but numerous studies reported the probiotic influence on nonspecific humoral and cellular elements at a systemic level. In that way, fish become more effective in protecting against pathogenic bacteria and fungi.

The increase of phagocytic activity was observed in rainbow trout supplemented with lactic acid bacteria (Balcázar *et al.*, 2007b; Kim & Austin, 2006b; Panigrahi *et al.*, 2004; 2005; 2007; Sharifuzzaman & Austin, 2009) or *Bacillus subtilis* (Newaj-Fyzul *et al.*, 2007), Senegalese sole treated with *Shewanella putrefaciens* and *Shewanella baltica* (Díaz-Rosales *et al.*, 2009), gilthead sea bream treated with two *Vibrionaceae* bacteria (Díaz-Rosales *et al.*, 2006), tilapia treated with *Bacillus amyloliquefaciens* (Selim & Reda, 2015) and grouper treated with *B. pumilus* and *B. clausii* (Sun *et al.*, 2010).

Similarly, the intensification of the respiratory burst has been observed by enhancement of superoxide anion and nitric oxide production and peroxidase activity. The increase of respiratory burst was observed in rainbow trout (Kim & Austin, 2006b; Newaj-Fyzul *et al.*, 2007; Nikoskelainen *et al.*, 2003; Panigrahi *et al.*, 2007; Sharifuzzaman & Austin, 2010a), gilthead sea bream (Cerezuela *et al.*, 2012) and tilapia (Selim & Reda, 2015). Nevertheless the probiotic effect is not always clear, as observed in several studies with rainbow trout (Balcázar *et al.*, 2007b; Panigrahi *et al.*, 2004; 2005; 2007) and Nile tilapia (Ferguson *et al.*, 2010). The enhancement of peroxidase activity by *Bacillus subtilis* was observed on rainbow trout (Newaj-Fyzul *et al.*, 2007) and gilthead sea bream (Salinas *et al.*, 2008). Still, the administration of *Bacillus* sp. or *Aeromonas sobria* in rainbow trout did not affect the serum peroxidase content (Brunt *et al.*, 2007). *Lactobacillus pentosus* and *Kocuria* SM1 induced an increase of peroxidase activity in eel (Lee *et al.*, 2013) and in rainbow trout (Sharifuzzaman & Austin, 2009), respectively.

Taoka *et al.* (2006b) observed higher serum bactericidal activity after administrating to Nile tilapia a multi species commercial probiotic, either as live (viable) or inactivated forms in the feed or added to the rearing water in viable form.

Lysozyme activities were increased by probiotic treatment in brown trout (Balcázar *et al.*, 2007a), rainbow trout (Brunt *et al.*, 2007; Newaj-Fyzul *et al.*, 2007; Panigrahi *et al.*, 2004; Sharifuzzaman & Austin, 2009), grouper (Sun *et al.*, 2010), Nile tilapia (Pirarat *et al.*, 2011; Selim & Reda, 2015; Taoka *et al.*, 2006b) or *Labeo rohita* (Giri *et al.*, 2013). Conversely, Balcazar *et al*. (2007b), Panigrahi *et al*. (2005), Shelby *et al*. (2007) and Merrifield *et al*. (2011a) did not observed a probiotic effect on lysozyme activities.

Complement activity, specially the alternative pathway, was increased after probiotic treatment on brown trout (Balcázar *et al.*, 2007a), rainbow trout (Balcázar *et al.*, 2007b; Nikoskelainen *et al.*, 2003; Panigrahi *et al.*, 2004; Panigrahi *et al.*, 2005), gilthead sea bream (Salinas *et al.*, 2008), grouper (Sun *et al.*, 2010), Nile tilapia (Pirarat *et al.*, 2011) and *Labeo rohita* (Giri *et al.*, 2013).

Probiotic administration can raise serum immunoglobulin (Ig) levels, as observed in rainbow trout (Nikoskelainen *et al.*, 2003), gilthead sea bream (Salinas *et al.*, 2008), grouper (Sun *et al.*, 2010) and eel (Lee *et al.*, 2013). The Ig gene expression was up-regulated on HK and spleen of tilapia receiving *L. rhamnosus* by the diet (Panigrahi *et al.*, 2011).

The modulation of those important innate responses were strain, time and dose-dependent (Balcázar *et al.*, 2007a; Nikoskelainen *et al.*, 2003).

1.4.3 **Possible results of probiotic administration**

As result of the several mechanisms described above, the probiotic administration may strengthen the general fish health, limiting inflammation and oxidative stress, providing resistance against pathogenic infections and ultimately improving zootechnical performances. In some cases, a reduction in larvae malformation incidence was observed. Those aspects will be further developed.

1.4.3.1 Improvement of zootechnical performances

Probiotics can act as growth promoters in numerous fish species, since their administration may result in better growth rates comparatively to non-supplemented group, such as rainbow trout (Bagheri *et al.*, 2008; Sealey *et al.*, 2009), Senegalese sole (Díaz-Rosales *et al.*, 2009), sea bass (Carnevali *et al.*, 2006), Nile tilapia (Lara-Flores *et al.*, 2003), carp (Wang & Xu, 2006), Atlantic cod (Lauzon *et al.*, 2010a), gilthead sea bream (Avella *et al.*,

2010), rohu (Giri *et al.*, 2013) and grouper (Son *et al.*, 2009) among others. However there are reports of no significant influence (He *et al.*, 2009; Hidalgo *et al.*, 2006) and even negative effects on growth (Batista *et al.*, 2016; Shelby *et al.*, 2006). Interestingly, the negative observations of decreased growth rates by probiotic treatment were counteracted by the observations of a lower mortality (Shelby *et al*., 2006) or the up-regulation of some antioxidant enzymes (Batista *et al*., 2016) on probiotic treated fish.

The feed conversion ratio (FCR) and protein efficiency ratio (PER) were improved by probiotic treatment in Senegalese sole (Díaz-Rosales *et al.*, 2009; Rodrigáñez *et al.*, 2009), tilapia (Lara-Flores *et al.*, 2003) and rainbow trout (Bagheri *et al.*, 2008). Conversely, Senegalese sole fed diets supplemented with a mixed-species formulation had a reduction in efficiency of dietary nutrient utilization (Batista *et al*., 2016).

The tables 1-6 and 1-7 present data on growth studies testing the effects of probiotic in rainbow trout and Nile tilapia, respectively. The tables present the results on survival, growth rate (SGR, specific growth rate), FCR, PER and feed intake (FI). The effects of probiotic on body composition is presented for some of the studies. To our best knowledge, no growth study on probiotic utilization was carried in brown trout. The outcomes of probiotic administration on zootechnical performance are diverse: null, positive or, in some circumstances, negative, as observed by Hidalgo *et al*. (2006). The authors observed in dentex supplemented with *Bacillus cereus*, a higher mortality without any apparent zootechnical benefit since the growth and feed efficiency rates were not affected.

The use of probiotics may increase the apparent digestibility of dietary protein and organic matter, as observed in Nile Tilapia (Lara-Flores *et al.*, 2003). The administration of *Vibrio proteolyticus* tended to increase protein digestibility in turbot juveniles (De Schrijver & Ollevier, 2000).

As described for the antioxidant action of probiotics, frequently more notorious in animals subject to sub-optimal conditions, a similar situation seems to occur on growth performances. Gonçalves *et al*. (2011) administrated *L. Rahmnosus* to juvenile tilapia, with no significant effect on growth performance when compared to fish fed control diets, when the fish density was adequate. However, in tilapia subjected to crowding stress, the positive impact of *L. Rahmnosus* supplementation was evident. The stressed tilapia treated with probiotic presented similar growth as non-stressed fish, whereas the non-supplemented stressed fish showed an inhibition of growth and efficiency in feed utilization (Gonçalves *et al*., 2011). A similar outcome was observed in seabream treated with PdP11 *Shewanella*

putrefaciens and subjected to high stocking density (Varela *et al.*, 2010). Those results indicate that probiotics may be an efficient way to counteract the growth performance impairment observed in fish reared at high densities, commonly applied in intensive aquaculture.

Some studies reported the enhancement of appetite by probiotics supplementation, especially at the early phase of the dietary treatment (Irianto & Austin, 2002; Robertson *et al.*, 2000). Conversely, Shelby *et al*. (2007) observed a negative influence on feed intake in one of the probiotic formulations in evaluation in catfish (*Enterococcus faecium* and *Pediococcus acidilactici*).

Recent studies with Senegalese sole (Batista *et al.*, 2015) and rainbow trout (Gisbert *et al.*, 2013) observed higher weight homogeneity within tanks in fish fed diet supplemented with probiotics. That is an important achievement of reduction of costs in management operations.

1.4.3.2 Carcass composition influence

The chemical composition of fish muscle or carcass is rarely assessed on probiotic experiments (tables 1-6 and 1-7). Yet, some studies observed an influence of probiotics in the carcass composition in fish fry. In rainbow trout, a commercial formulation supplemented with mix-species bacteria and yeast (*Saccharomyces cervisiae, Enterococcus faecium, Lactobacillus acidophilus, L. casei, L. plantarrum and L. brevis*) increased significantly the body protein content (Sealey *et al.*, 2009). In addition, a mixture of *Bacillus* (*B. subtilis* and B. *licheniformis*) was able to increase protein and decrease lipid content in trout carcass (Alizadeh *et al.*, 2011; Bagheri *et al.*, 2008). On the opposite, *Streptococcus faecium* plus *L. acidophilus* or *S. cerev*isiae supplementation enhanced the body lipid content in tilapia fry (Lara-Flores *et al.*, 2003). In older fish, the probiotic impact in carcass composition attenuates, since no significantly effect was observed in tilapia (Standen *et al.*, 2016; Standen *et al.*, 2013; Telli *et al.*, 2014) or rainbow trout (Gisbert *et al.*, 2013; Merrifield *et al.*, 2010a; Merrifield *et al.*, 2011a; Merrifield *et al.*, 2010b; Ramos *et al.*, 2015). Interestingly, in Senegalese sole, a *Shewanella* bacteria, while not affecting significantly the whole carcass composition, affected the muscle composition, by increasing the lipid content (Rodrigáñez *et al.*, 2009), and the protein and n-6 fatty acids levels (García de La Banda *et al.*, 2010).

Table 1-6 Summary of zootechnical results of previous probiotic supplementation trials in rainbow trout.

Probiotic Microorganisms	Origin	CFU/g diet	% additive diet	Administration route	Time	IBW (g)	T °C	Feeding % BW meals/day	Survival (%)	Growth (SGR)	Feed conversion (FCR)	Carcass composition	Source
L. rhamnosus ATCC53103	Valio product	3×10^9, 4×10^{12}		Feed coated with rapeseed oil with PRB	51 days	32	15↗ 18-19	n.a.	n.a.	=	n.a.	n.a.	(Nikoskelainen *et al.*, 2001)
		10^4, 10^6, 10^8, 10^{10}, 10^{11}		Feed coated with sunflower oil with PRB	2 weeks	83	13	0.7-1% n.a.	n.a.	=	n.a.	n.a.	(Nikoskelainen *et al.*, 2003)
L. plantarum CLFP 238 *L. mesenteroides CLFP 196*	salmonids	10^6 10^6		Feed coated with PRB suspension	30 days	27	14↗19	1.5% 2/day	n.a.	=	n.a.	n.a.	(Vendrell *et al.*, 2008)
B. subtilis & *B. licheniformis*	BioPlus 2B ®	4.8×10^8 1.2×10^9 2.0×10^9 3.8×10^9 6.1×10^9		Feed sprayed with PRB suspension	63 days	0.12	14	n.a.	↑ in higher doses	↑ in higher doses	↓ FCR & ↑ PER in higher doses	Protein ↑ lipid ↓	(Bagheri *et al.*, 2008)
		10^6-10^7	0.1, 0.2, 0.5, 0.75, 1	Feed sprayed with PRB suspension	36 days	0.4	14	n.a.	=	↑ in higher doses	↓ FCR & ↑ PER (**except** highest dose)	Protein↑ (except lower dose); lipid↓	(Alizadeh *et al.*, 2011)
B. subtilis & *B. licheniformis*	BioPlus 2B ®	10^8		Feed coated with fish oil containing PRB	10 weeks	70 45	15	2-2.5% 2/day	=	↑ after antibiotic treatment	↓ FCR & ↑ PER + after antibiotic treatment	=	(Merrifield *et al.*, 2010a; Merrifield *et al.*, 2010b)
E. faecium	Lactosan product	10^8								=	↓ FCR **except** after antibiotic treatment	=	
E. faecium + *B. subtilis* & *B. licheniformis*		10^8+10^8										=	
B. mojavensis & *Enterobacter cloacae*	Rainbow trout gut	10^2, 10^4,10^6, 10^8, 10^{10}		Feed coated with fish oil containing PRB	60 days	0.71	18	*ap.satiation* 3/day	n.a.	↑ ($1x10^8$)	n.a.	n.a.	(Capkin & Altinok, 2009)
Pediococcus acidilactici CNCM MA 18/5M	Bactocell®	1.5×10^6		Feed coated with fish oil containing PRB	5 months	larvae	11	n.a.	=	=	n.a.	n.a.	(Aubin *et al.*, 2005)
		10^8		Feed coated with fish oil with PRB or added prior to extrusion	10 weeks	9	15	2-2.5% n.a.	=	=	=	=	(Merrifield *et al.*, 2011a)

S. cervisiae & *E. faecium* & *L. acidophilus* & *L. casei* & *L. plantarum* & *L. brevis*	Mycolactor Dry Probiotic®	*1.5×10⁵*	0.1	PRB incorporated as feed additive before extrusion	8 weeks (I) 12 weeks (II)	0.13 (I) 6 (II)	15	*ap.satiation* n.a.	= (I) ↑ (II)	↑ (I) ↑ (II) ↑ soybean meal	= (I) = (II)	Protein ↑	(Sealey *et al.*, 2009)
B. cereus var. *toyoi* (NCIMB 40112)	Toyocerin®	2×10^4		Feed coated with fish oil containing PRB	93 days	2.8	13	3.3% 4/day	=	=	=	=	(Gisbert *et al.*, 2013)
B. subtilis & *E. faecium* & *P. acidilactici* & *L. reuteri*	Aquastar® hatchery	*3×10⁶* *1.5×10⁷*	0.1 0.5	Feed	8 weeks	113	23	*ap.satiation* 3/day	=	↑ in lower dose	↓ (more intense in lower dose)	n.a.	(Giannenas *et al.*, 2015)
	Aquastar® growout	2×10^6	0.2	Feed coated with fish oil containing PRB	9 weeks	87	14↘7	0.8-1.8% n.a.	n.a.	=	n.a.	n.a.	(Ozório *et al.*, 2016)

PRB (Probiotic); IBW (initial body weight); T. (temperature); n.a. (information not available); SGR (specific growth rate); FCR (feed conversion ratio); PER (protein efficiency ratio); *ap.satiation* (apparent satiation).
L. Lactobacillus.; *B. Bacillus*; *E. Enterococcus*; *P. Pediococcus*; S. *Saccharomyces.*
↑ significantly higher; ↓ significantly lower; = no statistical difference relative to control animals; ↗↘ temperature raising or decreasing through the experiment.
CFU values in grey italic: estimated CFU/g of diet using the information about CFU/g of product and the incorporation dosage.

Table 1-7 Summary of zootechnical results of previous supplementation trials in Nile tilapia.

Probiotic Microorganisms	Origin	CFU/g diet	% additive diet	Administration route	Time	IBW (g)	T °C	Feeding % BW meals/day	Survival (%)	Growth (SGR)	Feed conversion (FCR)	Carcass composition	Source
Strept. faecium & *L. acidophilus*	All-Lac™		0.1	Feed	9 weeks	152 mg	28	*ap.satiation* 4/day	↑	↑	↓	Lipid ↑ (at some tested densities)	(Lara-Flores *et al.*, 2003)
S. cerevisae	BioSaf™		0.1						↑	↑	↓		
B. licheniformis & *B. subtilis*	Biogen®		0.5, 1.5, 2.0, 2.5	PRB incorporated as feed additive before pelleting	120 days	23	27-28	3% 3/day	n.a.	↑	↓ ↑ PER = FI	CP= Lipid↓(0.5%) Ash↑ (2%) GE↓(0.5, 1.5%)	(El-Haroun *et al.*, 2006)
E. faecium	Biomate®	1×10^6		PRB suspension added to the feed	39 days	13 mg	27	Excess feeding 2/day	=	=	n.a.	n.a.	(Shelby *et al.*, 2006)
B. subtilis & *B. licheniformis*	Bioplus2B®	1×10^6							=	=			
B. subtilis & *B. licheniformis*	Bioplus2B®	1×10^8		PRB suspension added to the feed	56 days	29 mg	27	Excess feeding 2/day	↑	↓	n.a.	n.a.	(Shelby *et al.*, 2006)
P. acidilactici	Bactocell®	1×10^8							=	↓			
S. cerevisae	Levucell®	1×10^8							=	↓			
B. subtilis (ATCC 6633)	Sigma product	1×10^7		Feed	1 & 2 months	5	26	5% n.a.	=	↑	↓	n.a.	(Aly *et al.*, 2008a)
L. acidophilus		1×10^7							=	↑	↓		
B. subtilis & *L. acidophilus*		0.5×10^7 & 0.5×10^7							↑	=	↓		
B. pumilis		10^6 10^{12}		Feed	1, 2-8 months	6.5	n.a.	1%-3% 2/day	↑ (higher dosage)	↑ (lower dosage)	n.a.	n.a.	(Aly *et al.*, 2008b)
L. acidophillus & *B. subtilis* & *S.* & *Aspergillus oryzae*	Organic Green™	*1 × 10⁵* *2 × 10⁵*	0.1 0.2						↑ (higher dosage)	↑ (lower dosage)			
E. faecium Zj4	Piglet gut	PRB added to water every 4 days (1×10^7 cfu.ml^{-1})			40 days	6.8	24-26	3% 3/day	=	↑	n.a.	n.a.	(Wang *et al.*, 2008a)
M. luteus	tilapia gonads and intestine	10^7		Feed coated with PRB suspension	90 days	2.4	26-28	3% 2/day	=	=	↓	Lipid↓	(Abd El-Rhman *et al.*, 2009)
Pseudomonas		10^7							↓	↓	=; ↓ PER	Lipid↓ CP↓, ash↑	
M. luteus & *Pseudomonas*		10^7							=	=	=	Lipid↓; ash↑	
LAB	Tilapia gut	5×10^4		Sprayed on the feed	134 days	0.14	20-25	*ap.satiation* n.a.	=	↑	n.a.	n.a.	(Apun-Molina *et al.*, 2009)
Bacilli	Tilapia pond	*Bacilli* added to water every 15 days (1×10^3 CFU mL^{-1})							=	↑			
Bacilli & LAB									=	↑			

B. subtilis NIOFSD017	Tilapia gut	10^7		PRB suspension incorporated as feed additive before pelleting	60 days	25	28	3% 3/day	=	↑	↓ ↑ PER ↑ FI	CP ash GE↑	(Essa *et al.*, 2010)
L. plantarum NIOFSD018		10^7										CP ash↑	
B. subtilis & *L. plantarum*		10^7										Lipid GE↑	
S. cerevisiae NIOFSD019		10^4										=	
B. subtilis B10	Carp pond	PRB added to aquaria water at a final concentration 1×10^7 cfu.ml^{-1} every 2 days			40 days	7	25	3% 2/day	=	=	n.a	n.a.	(Zhou *et al.*, 2010)
B. coagulans B16									=	↑			
Rhodopseudomonas palustris G06									=	↑			
L. plantarum	Tilapia gut	1×10^8		PRB suspension sprayed on the feed	12 weeks	15.4	24-30	5-3.5% 4/day	=	↑	↓	n.a.	(Jatoba *et al.*, 2011)
L. rhamnosus GG (ATCC 53103)		10^{10}		Feed	3 weeks	12.3 12.7	26	3 % n.a.	=	↑in stressed fish	=	n.a.	(Gonçalves *et al.*, 2011)
		10^{10}		Feed	30 days	22	29	1.5 % 1/day	n.a.	= (+but not significant)	= (+ but not significant)	n.a.	(Pirarat *et al.*, 2011)
P. acidilactici MA 18/5 M	Bactocell®	10^7		Feed coated with PRB suspension	32 days	175	24.5	1.5 % 3/day	↑	= (+but not significant)	= (+ but not significant)	n.a.	(Ferguson *et al.*, 2010)
		2.8×10^6		Feed coated with oil with PRB before extrusion	6 weeks	9	27	4% 3/day	=	=	=	=	(Standen *et al.*, 2013)
L. brevis JCM 1170		$10^5, 10^7, 10^9$		Feed	5 weeks	1		*ap.satiation* 2/day	=	=	=	n.a.	(Liu *et al.*, 2013)
L. acidophilus JCM 1132		$10^5, 10^7, 10^9$											
B. subtilis C-3102	Casporin®	7.8×10^6		Feed sprayed with soybean oil with PRB	84 days	33	n.a.	*ap.satiation* 3/day	=	=	= FI =	=	(Telli *et al.*, 2014)
B. subtilis & *Aspergillus oryzae* & *S. cerevisiae*	Biogenic product	1.5×10^9 & 1×10^9 & 2×10^9	0.5	Feed coated with soybean oil containing PRB	4 weeks	25	27	2% 2/day		=	=	n.a.	(Iwashita *et al.*, 2015)
		3×10^9 & 2×10^9 & 4×10^9	1							=	↓		
B. subtilis & *E. faecium* & *P. acidilactici* & *L. reuteri*	Aquastar® growout	*5×10^6*	0.5	PRB incorporated as feed additive before extrusion	8 weeks	55	28	1-3% 4/day	=	=	n.a.	n.a.	(Standen *et al.*, 2015)
		1.5×10^6 3×10^6	0.15 0.3		6 weeks	29	28	1-5% 4/day	n.a.	↑ in higher dose	= PER =	=	(Standen *et al.*, 2016)

PRB (Probiotic); IBW (initial body weight); T. (temperature); n.a. (information not available); SGR (specific growth rate); FCR (feed conversion ratio); PER (protein efficiency ratio); FI (feed intake); *ap.satiation* (apparent satiation). CP (crude protein); GE (gross energy); + positive impact although not statistically significant *Strept. Streptococcus*; *L. Lactobacillus*.; *B. Bacillus*; *E. Enterococcus*; *P. Pediococcus*; S. *Saccharomyces; M. Micrococcus;* LAB (Lactic acid bacteria).
↑ significantly higher; ↓ significantly lower; = no statistical difference relative to control animals; CFU values in grey italic: estimated CFU/g of diet using the information about CFU/g of product and the incorporation dosage.

1.4.3.3 Reduction of malformation incidence

The administration of some probiotics resulted on a beneficial influence on larvae conformation, with a reduction of animals with spinal malformation. That effect was observed in seabass larvae, treated with *Pediococcus acidilactici* MA185M (Lamari *et al.*, 2013) or *Debaryomyces hansenii* HF1 (Tovar *et al.*, 2002), and in rainbow trout larvae fed *Pediococcus acidilactici* MA185M (Aubin *et al.*, 2005).

1.4.3.4 Increased resistance towards disease or stress agents

Probiotic administration can be a nutritional strategy to improve fish healthiness (Dimitroglou *et al.*, 2011; Kiron, 2012). As result, fish become more resilient to infectious diseases (Aly *et al.*, 2008a; Iwashita *et al.*, 2015; Reyes-Becerril *et al.*, 2008; Selim & Reda, 2015), high density stress (Gonçalves *et al.*, 2011; Lara-Flores *et al.*, 2003; Telli *et al.*, 2014), to heat-shock (Taoka *et al.*, 2006a) or subnutritional conditions (Lara-Flores *et al.*, 2003; Sealey *et al.*, 2009).

The findings on enhanced survival against important pathogenic agents after probiotic supplementation are accumulating for several fish species. Tables 1-8 and 1-9 summarizes the outcome of the pathogenic challenge studies in fish species used as animal model in the current thesis: rainbow trout, brown trout and Nile tilapia. The probiotic administration reduced the severity of the infections in the majority of the trials.

1.4.3.5 Reproduction influence

The probiotics may influence fish reproduction, as previously observed in zebrafish (*Danio rerio*), an import vertebrate model species for biomedical research (Gioacchini *et al.*, 2011) and on four ornamental fish species (Ghosh *et al.*, 2007). The *Lactobacillus rhamnosus* 501®, after 10 days of supplementation in water (10^6 CFU.ml^{-1}), showed to improve zebrafish fecundity, revealed by higher number of ovulated eggs *in vivo* starting from the second day of administration. The probiotic administration also promoted oocyte maturation, higher hatching rate and acceleration of embryo development. The probiotic treatment influenced the gene expression of neuropeptide hormones and metabolic signalization compounds (Gioacchini *et al.*, 2010). Other experiments revealed that the same probiotic (administered

lyophilized in the diet; 10^6 CFU.g^{-1}) induced the responsiveness of incompetent follicles (stage IIIa) towards maturation (Gioacchini *et al.*, 2012; Giorgini *et al.*, 2010). The same probiotic influenced gonadal differentiation through effects on the GnRH and IGF system (Avella *et al.*, 2012). The authors observed a large anticipation of the gonad differentiation and a large influence on sex ratio (93% females, 7% males in control *vs*. 55% females, 45% males in the treated group). A *Bacillus subtilis* strain, isolated from a carp species, was able to increase significantly the female gonadosomatic index, fecundity, fry production and survival in four species of ornamental fish, while decreased the number of deformed fry fish (Ghosh *et al.*, 2007).

Table 1-8 Summary of challenge trials after probiotic supplementation in rainbow trout.
Roberton *et al*. (2000) studied Atlantic salmon as well. Balcazar *et al.* (2009) studied brown trout. All probiotics supplemented in the feed, except work of Abbass *et al*. (2010).

Probiotic microorganisms	Origin	CFU/g diet	Time adm. previous challenge	IBW (g)	Pathogenic agent CFU ad. per fish	Infection method	Survival (%)	Source
Atlantic Salmon								
Carnobacterium sp. strain K1	Atlantic salmon	5×10^7	7 or 14 days	20	*A. salmonicida* (Hooke)	cohabitation with i.p. injected fish (5×10^6 CFU)	= (7 days); + (14 days)	(Robertson *et al.*, 2000)
					V. ordalii (V 453)		= (7 days); + (14 days)	
					Y. ruckeri (PR110)		=	
					V. anguillarum (V72)		= (7 days); + (14 days)	
Carnobacterium sp. strain K1	Atlantic salmon	5×10^7	14 or 28 days	20	*A. salmonicida* (Hooke)	cohabitation with i.p. injected fish (5×10^6 CFU)	+ (14 >28 days)	(Robertson *et al.*, 2000)
			14 days		*Y. ruckeri* (PR110)		+	
Carnobacterium maltaromaticum B26 *Carnobacterium divergens B33*	Rainbow trout	10^7	14 days	25 g	*A. salmonicida* Hooke *Yersinia ruckeri* T1	i.p. (2.4×10^6 CFU) i.p. (1.6×10^6 CFU)	+	(Kim & Austin, 2006b)
Lactobacillus Rhamnosus ATCC53103	Valio	2.8×10^9 3.9×10^{12}	15 days	32	*A. salmonicida* ssp. *salmonicida*,	cohabitation with i.p. injected fish (1.8×10^6 CFU)	+ (10^9 >10^{12})	(Nikoskelainen *et al.*, 2001)
B. subtilis & *B. licheniformis*	Bioplus2B®	4×10^4	42 days	1.5	*Yersinia ruckeri* O1	i.p. (1.8×10^6 CFU)	+	(Raida *et al.*, 2003)
Lactococcus lactis ssp. Lactis CLFP 100 *Leuconostoc mesenteroides* CLFP 196 *Lactobacillus sakei* CLFP 202	Salmonids	10^6	2 weeks	40	*A. salmonicida* ssp. *salmonicida* CLFP 501	cohabitation with i.p. injected fish (1.7×10^5 CFU)	+	(Balcázar *et al.*, 2007b)
Brown trout								
Lactococcus lactis CLFP 100 *Leuconostoc mesenteroides* CLFP 196	Rainbow trout gut mucus	10^6	30 days	70	*A. salmonicida*	Temperature rising 14ºC to 16ºC	+	(Balcázar *et al.*, 2009)
Bacillus subtilis AB1 Viable	Rainbow trout	10^4, 10^5,10^6, 10^7, 10^8, 10^9	2 weeks	30	*Aeromonas* sp. ABE1	i.p. (2.3×10^6 CFU)	+ (100% **10^7**>10^6>10^5>10^8>10^9, 10^4)	(Newaj-Fyzul *et al.*, 2007)
formalized cells		10^7					+ (100%)	
sonicated cells		10^7					+ (100%)	
cell-free supernatant		10^7					+ (100%)	
Lactobacillus plantarum *L. mesenteroides*	Salmonids	10^6 10^6	30 days	27	*Lactococcus garvieae* CLFP LG1	cohabitation with i.p. injected fish (3.4×10^2 CFU)	+	(Vendrell *et al.*, 2008)
B. mojavensis & *Enterobacter cloacae*	Rainbow trout gut	10^2, 10^4, 10^6, 10^8, 10^{10}	20 days	0.71	*Yersinia ruckeri* (GA97016)	Added to water 6×10^7 CFU/ml	+ (**10^8**>10^6>10^{10}>10^4>10^2)	(Capkin & Altinok, 2009)
S. cervisiae & *E. faecium* & *L. acidophilus* & *L. casei* & *L. plantarum* & *L. brevis*	Mycolactor Dry Probiotic®	*1.5×10^5*	8 weeks	0.13	infectious hematopoietic necrosis virus (IHNV) strain 220-90	Added to water 10^5 plaque forming units/ml	=	(Sealey *et al.*, 2009)

Kocuria SM1	Rainbow trout gut	10^8	1, 2, 3 or 4 weeks	10	*Vibrio anguillarum*	i.p. (3×10^4 CFU)	+ (**2** > 4 > 3> 1 week)	(Sharifuzzaman & Austin, 2009)
		10^8	2 weeks PRB Challenge after 1, 2, 3, 4 or 5 weeks of PRB withdrawal	10	*Vibrio anguillarum*	i.p. (3×10^4 CFU)	+ (**1** > 2,3,4 weeks); = 5 weeks	(Sharifuzzaman & Austin, 2010a)
		10^8	2 weeks	10	*Vibrio anguillarum* *Vibrio ordalii*	i.p. (3×10^4 CFU) i.p. (5×10^3 CFU)	+	(Sharifuzzaman & Austin, 2010b)
Aeromonas sobria GC2 viable	Rainbow trout & Carp	10^3, 10^6, 10^7, 10^8, 10^{10}	14 days	20	*Lactococcus garvieae* 29-99 *Streptococcus iniae* 00-318	i.p. (2×10^6 CFU)	+ (**10^7**>10^8>10^{10}>10^3>10^6)	(Brunt & Austin, 2005)
formalized cells		10^7					=	
sonicated cells		10^7					+ (< than viable)	
supernatant		10^7					+ (< than viable)	
Bacillus JB-1	Rainbow trout & Carp	10^8	14 days	12	*A. salmonicida* *Lacto. garvieae* *Strept. iniae* *V. anguillarum* *V. ordalii* *Y. ruckeri*	i.p. (3×10^6 CFU) i.p. (2×10^6 CFU) i.p. (2×10^6 CFU) i.p. (3×10^6 CFU) i.p. (3×10^6 CFU) i.p. (3×10^6 CFU)	+	(Brunt *et al.*, 2007)
Aeromonas sobria GC2		10^7						
Aeromonas sobria GC2 or Bacillus subtilis JB-1:	Rainbow trout & Carp	*i.p. or i.m. injection*	7 days	12	*Yersinia ruckeri* EX5	i.p. (2.5×10^5 cells)		(Abbass *et al.*, 2010)
Live cells		*10^7cells*					+ (80-90%)	
GC2 or JB-1 formalized cells		*10^7cells*					=	
GC2 or JB-1 whole cell proteins		*0.2 mg*					+ (100%)	
JB-1 cell wall proteins		*0.2 mg*					+ (100%)	
GC2 outer membrane proteins		*0.2 mg*					+ (100%)	
GC2 lipopolysaccharides		*0.1 mg*					+ (100%)	
GC2 Extracellular products		*0.2 mg*					=	

PRB (probiotic). Adm. (administration); IBW (initial body weight); i.p. (intraperitoneal); i.m. (intramuscular)

Strept. Streptococcus; *L. Lactobacillus*.; *B. Bacillus*; *E. Enterococcus*; *P. Pediococcus*; S. *Saccharomyces; M. Micrococcus; A. Aeromonas*. *Lacto. Lactococcus. V. Vibrio; Y. Yersinia*

+ statistical improvement; = no statistical difference relative to control animals;

CFU values in grey italic: estimated CFU/g of diet using the incorporation dosage information.

Table 1-9 Summary of challenge trials after probiotic supplementation in Nile tilapia.
All probiotics supplemented in the feed, except Taoka *et al*. (2006) that administered via the diet or the water.

Probiotic microorganisms	Origin	CFU/g diet	Time adm. previous challenge	IBW (g)	Pathogenic agent CFU ad. per fish	Infection method	Survival %	Source
E. faecium	Biomate®	1×10^6	65 days	13 mg	*Streptococcus iniae*	i.p. (1×10^6 CFU)	=	(Shelby *et al.*, 2006)
B. subtilis & B. licheniformis	Bioplus2B®	1×10^6						
B. subtilis & B. licheniformis	Bioplus2B®	1×10^8	65 days	29 mg	*Streptococcus iniae*	i.p. (1×10^6 CFU)	=	(Shelby *et al.*, 2006)
P. acidilactici	Bactocell®	1×10^8						
S. cerevisae	Levucell®	1×10^8						
Lactobacillus rhamnosus (ATCC 53103)		1×10^8	2 weeks	60	*Edwardsiella tarda*	i.p. (1×10^9 CFU)	= (+ but not significantly)	(Pirarat *et al.*, 2006)
		1×10^{10}					+	
B. subtilis & L. acidophilus & Clostridium butyricum & S. cerevisiae	Commercial product	10^7 & 10^8 & 10^7 & 10^7	30 days	111	*Edwardsiella tarda*	i.p. (5×10^6 CFU)	+ (diet live cells > diet dead cells > water live cells)	(Taoka *et al.*, 2006b)
B. subtilis (ATCC 6633)	Sigma	1×10^7	1 or 2 months	5	*Aeromonas hydrophila, Pseudomonas fluorescens Streptococcus iniae*	i.p. (5×10^7 CFU)	+ (2 > 1 month; > in bacterial mix)	(Aly *et al.*, 2008a)
L. acidophilus		1×10^7						
B. subtilis & L. acidophilus		0.5×10^7 & 0.5×10^7						
B. pumilis		10^6	1 or 2 months prob. feeding. Then basal diet until 8 months	6.5	*Aeromonas hydrophila*	i.p. (5×10^7 CFU)	+ (> higher dosages; 2 > 1 month). 8 months post-treatment, the prob. still provided some protection, specially fish fed prob. for 2 months)	(Aly *et al.*, 2008b)
		10^{12}						
L. acidophillus & B. subtilis & S. & Aspergillus oryzae	Organic Green™	*1 × 10⁵* *2 × 10⁵*						
Micrococcus luteus	tilapia gonads and intestine	10^7	90 days	2.4	*Aeromonas hydrophila*	i.p. (3×10^6 CFU)	+	(Abd El-Rhman *et al.*, 2009)
Pseudomonas		10^7					=	
M. luteus & Pseudomonas		10^7					=	
L. brevis JCM 1170		$10^5, 10^7, 10^9$	5 weeks	1	*A. hydrophila* NJ-1	Bath immersion (10^8 cells/ml)	= (10^5,10^7); + (10^9)	(Liu *et al.*, 2013)
L. acidophilus JCM 1132		$10^5, 10^7, 10^9$					=	
Bacillus subtilis & Aspergillus oryzae & Saccharomyces cerevisiae	Biogenic product	1.5×10^9 & 1×10^9 & 2×10^9	4 weeks	25	*A. hydrophila* *Streptococcus iniae*	i.p. (2×10^5 CFU) i.p. (1×10^3 CFU)	+	(Iwashita *et al.*, 2015)
		3×10^9 & 2×10^9 & 4×10^9						
Bacillus amyloliquefaciens CECT-5940	Ecobiol Aqua® fish isolated	1×10^4	30 days	28	*Yersinia ruckeri Clostridium perfringens*	i.p. (1.5×10^7 CFU)	=	(Selim & Reda, 2015)
		1×10^6					+ (> *C. perfringens*)	

+ statistical improvement; = no statistical difference relative to control animals; *B. Bacillus*; *E. Enterococcus*; *P. Pediococcus*; *S. Saccharomyces*; *L. Lactobacillus*. *Strept. Streptococcus*. *M. Micrococcus; A. Aeromonas*. Prob. Probiotic.
CFU values in grey italic: estimated CFU/g of diet using the incorporation dosage information. i.p. intraperitoneal injection. RLP (relative level of protection).

1.4.4 **Influencing factors**

The extensive experimentation already performed makes evident the interest of probiotics for aquaculture. Nevertheless, the large variation between the trial's variables makes comparisons challenging, and is still impossible to predict the outcomes with exactitude. Several factors affect the results of probiotic administration (figure 1-6).

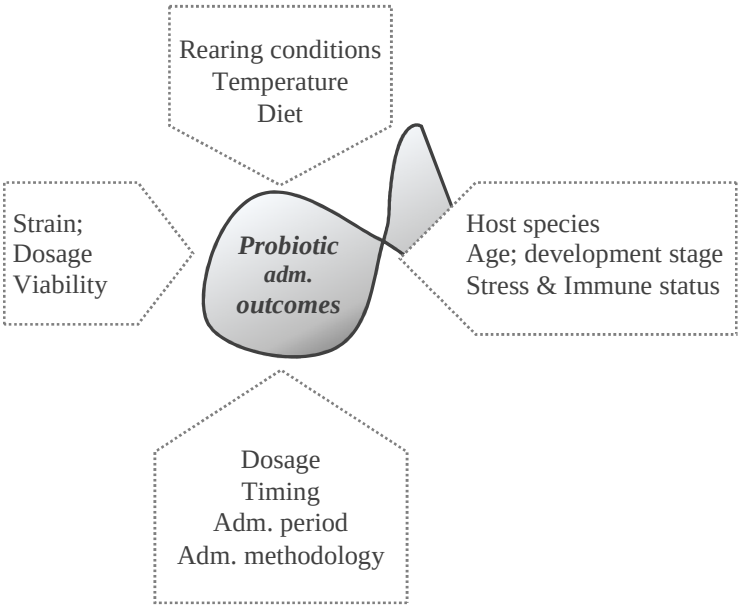

Figure 1-6 Factors that may influence the outcome of probiotic administration to aquaculture reared fish.
Adm. (Administration).

The probiotic **strain** is of major influence (D'Arienzo *et al.*, 2011; Ibrahim *et al.*, 2011; Itoi *et al.*, 2009). The individual properties of each strain determines their specific probiotic action (Oelschlaeger, 2010). The microbial **viability** as already approached, is a matter of debate. Although viable probiotics usually provide higher immune stimulation (Munoz-Atienza *et al.*, 2015; Panigrahi *et al.*, 2005), dead microorganisms are also able of immune modulation (Díaz-Rosales *et al.*, 2006; Salinas *et al.*, 2006), and heat-killed *Lactobacillus* may have higher toxin binding capacities (Gratz *et al.*, 2006). The viability form (vegetative or lyophilised), have shown to influence the results: while the freeze-dried form performed better than live-sprayed for Panigrahi *et al*. (2005), Merrifield *et al*. (2011a) observed the opposite. The **administration** form, through the diet or added to rearing water, may affect the result as well (Taoka *et al.*, 2006b).

The **dosage** is a determining factor. If low concentrations may be insufficient to exert observable benefits, high doses can be too disruptive. Giannenas *et al*. (2015)

observed growth improvement at the lower, but not at the highest dose tested in rainbow trout juveniles. In the same way, in pathogenic challenge studies, frequently the higher dosages do not translate into the higher survival rates (Capkin & Altinok, 2009; Nikoskelainen *et al.*, 2001).

The **extension** of probiotic administration is very important in his effectiveness, as Sharifuzzaman and Austin (2009) observed in rainbow trout, more resistant to *Vibrio anguillarum* challenge after two weeks of the probiotic *Kocuria* SM1 administration, comparing to 1, 3 or 4 weeks. **Temperature** is an important variable affecting the results, as would be expected in ectothermic animals (Ibrahim *et al.*, 2011; Verschuere *et al.*, 2000), as well as the remaining water parameters and rearing conditions, affecting the background microbiota. The composition of the **diet** used concomitantly to probiotic administration, can have positive or detrimental effects, since interactions among dietary ingredients or elements and the microbiota have been observed (Corcoran *et al.*, 2005; Ingerslev *et al.*, 2014b). Likewise, association or **interaction** effects among probiotic microorganisms have been observed (Makridis *et al.*, 2000; Merrifield *et al.*, 2010b; Wang & Xu, 2006). As example, the administration to gilthead seabream of *L. delbrueckii* ssp. *lactis* with *B. subtilis* was able to increase significantly the serum ACH50 activity and IgM levels, while the individual administration did not (Salinas *et al.*, 2008). Conversely, the diet supplementation with *B. subtilis* + *B. licheniformis* elevated rainbow trout lysozyme activity, but not after their combination with *Enteroccoccus faecium* (Merrifield *et al.*, 2010b).

The **host**, with his specific gastro-intestinal tract (GIT), immunity and background microbiota influence enormously the probiotics survival, establishment and immunomodulatory features (Lazado & Caipang, 2014). In that context, the **timing** of probiotic administration is very important. The probiotic administration in early moments of lifespan, is very promising since might impact on the establishment of the animal gut microbiota. In effect, the microbial GIT colonization occurs at larvae stage; the number of intestinal bacteria increase exponentially in the first days of larval development (Makridis *et al.*, 2000). Experiments with several fish species reveal the potential of probiotic application to larviculture (Avella *et al.*, 2010; Gatesoupe, 1994; Lauzon *et al.*, 2010b; Makridis *et al.*, 2000; Makridis *et al.*, 2008). On the same way, after antibiotic treatment, which imbalances the gut microbiota, an increased impact of the probiotic

treatment is observed (Merrifield *et al.*, 2010a; Merrifield *et al.*, 2010b). Similarly, at suboptimal environmental conditions or **stressful situations**, the probiotic benefits are more likely to be observed, as described.

1.4.5 **Criteria for selection of probiotics and regulatory considerations**

Several authors list the attributes of candidate probiotics (Balcázar *et al.*, 2006a; Brown, 2011; Lauzon *et al.*, 2014; Salminen *et al.*, 2005):

- Safe, without side effects, no pathogenic or toxic.
- Resistant to digestive process: acid, bile and digestive enzymes resistance
- Able to adhere to mucosal mucus and persist in gastro-intestinal tract
- Stable and resistant to commercial manufacturing and storage process, maintaining their viability under normal conditions
- Suitable for lyophilisation processing
- Beneficial to host
- Able to reduce pathogens
- Promoter of a balanced microbiota

The safety concerns should include the putative host, but other aquatic species, the consumer (Lauzon *et al.*, 2014) and the handler of the product (Castex *et al.*, 2014). Also the probiotic microorganisms should be free of plasmid-encoded antibiotic resistance genes (FAO/WHO, 2002; Lauzon *et al.*, 2014). Moreover FAO/WHO (2002) experts recommend the identification of the microorganisms genus, species and strain.

Except the safety concerns, mandatory for probiotics, not all the above characteristics must be reunited to achieve positive outcomes from a probiotic treatment. For instance, Sheil *et al*. (2004) observed that the subcutaneous injection of *Lactobacillus salivarius* 118 had anti-inflammatory effects, reducing murine colitis and arthritis. The intraperitoneal or intramuscular injection of probiotics protected rainbow trout against *Yersinia ruckeri* infection (Abbass *et al.*, 2010). Some studies report beneficial effects after the addition of probionts to the rearing water (Wang *et al.*, 2008a; Zhou *et al.*, 2010), and the probiotic preventing of cutaneous mucus adhesion by opportunistic agents was observed *in vitro* (Chabrillón *et al.*, 2005a; Chabrillón *et al.*, 2005b). Those observations suggest that, in some particular cases, the survival to digestive process or to industrial

feed processing may not be required. A similar situation occurs on probiotics for Human dermatological utilisation.

Numerous microorganisms belonging to distinct genus have been screened for his putative beneficial action, as extensively review by Newaj-Fyzul *et al*. (2014), mainly at laboratory or pilot scale. Their utilization at farm scale require safety considerations and is facilitated if the microorganisms have a long history of use, or figures on the lists of microorganisms *Generally Recognized as Safe* (GRAS) or with *Qualified Presumption of Safety* (QPS), assessed respectively, by USA Food and Drug Administration (FDA) and EU European Food Safety Authority (EFSA). That fact may explain why so many aquaculture trials use microorganism most associated to terrestrial livestock or Human microbiota.

In EU, probiotics are classified as *feed additives* in the category *zootechnical additives*. Their commercialization and utilization is subject of authorization, regulated by the Regulation (EC) 1831/2003. According to that regulation, only additives that have been through an authorization procedure may be placed on the market. Authorisations are granted for specific animal species, specific conditions of use and for ten years periods. Then, a renewal for the authorization must be requested. To obtain approval, a manufacturer is required to submit a technical dossier, containing data and studies demonstrating the safety and efficacy of the product for animals, consumers and the environment (Balcázar *et al.*, 2006a; Castex *et al.*, 2014). The Regulation (EC) 429/2008 describes with detail the procedures for the preparation and the presentation of applications and technical dossiers.

2 *Commercial Bacillus probiotic supplementation of rainbow trout (Oncorhynchus mykiss) and brown trout (Salmo trutta): growth, immune responses and intestinal morphology*

Commercial *Bacillus* probiotic supplementation of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*): growth, immune responses and intestinal morphology.

Key words: Probiotics, *Bacillus*, Rainbow trout, Brown trout, Innate immune responses, Intestinal morphology

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2.1 ABSTRACT

Probiotic administration is associated with enhancement of host resistance to environmental and nutritional stressors, improving survival and growth rates.

This study was carried out to evaluate the effects of dietary supplementation of *Bacillus subtilis* and *Bacillus cereus toyoi* on growth performance, innate immune responses and gut morphology of two trout species feeding a commercial diet high in soybean meal. A commercial probiotic (4.2×10^9 CFU.g^{-1} of additive) was supplemented to the experimental diets at 0% (control), 0.03% (P_1; 6×10^3 CFU.g^{-1} of diet) or 0.06% (P_2; 1.5×10^6 CFU.g^{-1} of diet) and fed to brown trout (*Salmo trutta)* and rainbow trout (*Oncorhynchus mykiss*) for 9 and 20 weeks, respectively. Rainbow trout showed significantly better growth performance than brown trout, regardless of the dietary treatment. No effect of dietary probiotic supplementation was detected on growth performance, body composition, or innate immune parameters (plasma lysozyme, alternative complement and peroxidase activities).

In both species, after 9 weeks, intestinal *lamina propri*a and submucosa were widened, with increased presence of inflammatory cells, significantly higher in groups fed probiotics. That inflammatory process, with *villi* and enterocytes noticeably damaged compared to the control group, was more pronounced in brown trout. Under the current trial conditions, the *Bacillus subtilis* + *Bacillus cereus toyoi* had no positive impact in either trout species, on the contrary a harmful effect was observed.

2.2 INTRODUCTION

The growing global concern about the necessity to reduce antibiotic use due to development and/or transmission of antimicrobial resistance within microorganisms (FAO/OIE/WHO, 2006) and the actual European prohibition of antibiotic use as growth promoters (Regulation (EC) 1831/2003) resulted in the development of new feeding and management strategies promoting animal's health and growth. One of them is the supplementation of feeds with probiotics, commonly defined as "living organisms, which when administered in adequate amounts confer a health benefit to the host" (FAO/WHO, 2002).

Spore-forming bacteria are being used as probiotic supplements in animal feeds and as human supplements due to their heat stability and ability to survive the gastric barrier (Cutting,

2011). *Bacillus* spores germinate in the gastrointestinal tract of mice (Casula & Cutting, 2002), chickens (Cartman *et al.*, 2008) and pigs (Leser *et al.*, 2008). Furthermore *Bacillus* spp. have been isolated from fish (Merrifield *et al.*, 2010b; Newaj-Fyzul *et al.*, 2007), crustaceans, bivalves and shrimps (Gatesoupe, 1999).

The use of *Bacillus* spp. has been verified in some aquaculture species with success, improving disease resistance and growth performance in rainbow trout (Bagheri *et al.*, 2008; Merrifield *et al.*, 2010a; Raida *et al.*, 2003) and Nile tilapia (Aly *et al.*, 2008a). Some studies reveal modulation of gut histomorphology by microbial feed additives, with an increase of *villi* absorptive surface (Gisbert *et al.*, 2013) and an improvement of the epithelial barrier (Vasanth *et al.*, 2015). Their use is already a common practice to treat Human enteritis (Marteau *et al.*, 2002) and microbiota imbalance, and there are accumulating evidence of their involvement in the homeostasis of gut mucosa barrier (Madsen *et al.*, 2001). That aspect could be particularly interesting to aquaculture of carnivorous fish, whose diets have increasing participation of vegetable ingredients, attending a more sustainable activity (Gatlin *et al.*, 2007). Some of those feed ingredients can impact on gut health given the usual presence of some anti-nutrients (Francis *et al.*, 2001; Krogdahl *et al.*, 2010). The soybean induced subacute enteritis is particularly well known in salmonids, and ideally their feed incorporation should not exceed 20% (Halver & Hardy, 2002) but in the field, in order to achieve cheaper and environmental friendly rations, the incorporation can be somewhat superior. Probiotics could play a role in the ability to utilize high levels of soybean in rainbow trout, as already observed by Sealey *et al*. (2009) and Dawood *et al*. (2015). Nevertheless the data on the impact of probiotics on gut morphology is limited, and the impact of *Bacillus* administration to brown trout has never been reported, to our knowledge.

Therefore, this study was conducted to evaluate the effects of dietary supplementation with a commercial probiotic blend *Bacillus subtilis* and *Bacillus cereus toyoi* (1:1) on growth performance, innate immune responses and gut morphology of rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta)* juveniles fed a commercial diet with soybean meal. These salmonids are significant contributors to fish production worldwide. Rainbow trout is the main trout farmed internationally (total global production exceeded 0.7 million tonnes in 2010, FAO, 2012) and native species of brown trout (*Salmo trutta*) are especially important in terms of their contribution to recreational fisheries (Merrifield *et al.*, 2010c).

2.3 MATERIAL 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), and according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

2.3.1 Experimental design and sampling

Three experimental diets were prepared, incorporating the commercial PAS-TR® (IMEVE S.A.) probiotic mixture of *Bacillus subtilis* and *Bacillus cereus toyoi* (4.2×10^9 CFU.g^{-1} dry powder) into a milled commercial trout diet (Alpis S.A., Póvoa do Varzim, Portugal; Table 2-1) at 0% (Control, C), 0.03% (P_1; 6×10^3 CFU.g^{-1} of diet) or 0.06% (P_2; 1.5×10^6 CFU.g^{-1} of diet), followed by cold pelleting (2.4 mm diameter) and drying (40ºC for 48 h). Each experimental diet was fed to six groups of rainbow trout (28 animals per tank; average weight 15.6 g) and to six groups of brown trout (23 animals per tank; average weight 15.3 g). Fish were transferred from AFN – National Conservation Center (Posto Aquícola do Torno, Marão, Portugal) to the Aquatic engineering laboratory facilities at ICBAS-UP. Fish were reared in fibreglass tanks (65 L) in a controlled semi-closed recirculating freshwater system (16.9 ± 0.5 ºC), and hand-fed until visual satiation twice a day (9:00 and 16:00 h), 6 days per week. Every 2 or 3 days tanks were cleaned, with approximately 30% of the recirculating water renewed each time.

The probiotic feeding lasted 9 weeks for both species, then continued only for rainbow trout until 20 weeks to study the impact of a longer probiotic administration on immune parameters. After 3 and 9 weeks of experimental feeding, animals from both species were sacrificed to determine selected humoral immune parameters (12 fish/treatment at 3 weeks and 6 fish/treatment at 9 weeks) and to study intestinal morphology (6 fish/treatment, 1/tank). Throughout the first 7 weeks, growth and feed conversion were monitored and carcass composition was assessed (9 animals/treatment pooled in 3 samples). The sacrificed animals were previously anesthetised by water immersion with ethylenoglycol monophenyl ether (1mL L^{-1}).

Table 2-1 Ingredients and proximate composition of the experimental diets

Ingredients (g kg^{-1}):[1]	Diet		
	Control (C)	**Probiotic 1 (P_1)**	**Probiotic 2 (P_2)**
Fish meal	475.0	475.0	475.0
Soybean meal 44	285.0	285.0	285.0
Wheat	120.0	120.0	120.0
Fish oil	80.0	80.0	80.0
Algae meal	30.0	30.0	30.0
Vitamin[2] and Mineral[3] Premix	10.0	10.0	10.0
Proximate composition:			
Dry matter (*g kg^{-1}*)	958.1	965.3	962.2
Ash (*g kg^{-1}* DM)	163.1	161.5	163.4
Crude protein (*g kg^{-1}* DM)	475.2	477.7	476.6
Crude lipid (*g kg^{-1}* DM)	126.1	127.2	122.1
Crude fiber (*g kg^{-1}* DM)	28.7	29.0	33.8
Gross energy (MJ Kg^{-1} DM)	19.2	19.8	19.6

[1]Supplier data.
[2] Vitamins (mg or IU kg^{-1} diet): retinyl acetate (12000 IU); cholecalciferol (2400 IU); alpha tocopheryl acetate (200 mg); menadione sodium bisulfite (4.2 mg), ascorbic acid (100 mg), thiamine hydrochloride (20 mg), riboflavin (30 mg), calcium pantothenate (50 mg), nicotinic acid (200 mg), pyridoxine hydrochloride (15 mg), folic acid (4 mg), cyanocobalamin (0.05 mg), biotin (0.4 mg); inositol (300 mg).
[3]Minerals (mg or g kg^{-1} diet): Cu (6 mg), Mg (500 mg), Fe (50mg), Mn (12 mg), F (1 mg), I (5.6 mg), Zn (40 mg), Se (0.3 mg),.Ca (1.6 g), Cl (0.69 g), K (0.54 g), Na (0.16 g).

2.3.2 **Innate immune parameters**

Respiratory burst and nitric oxide (NO) were determined in head-kidney (HK) leucocytes. Alternative haemolytic complement pathway (ACH50), lysozyme and peroxidase activities were determined in plasma. Respiratory burst activity was carried out according to Secombes (1990). Optical densities (OD) were converted to nmol of O_2^- produced by multiplying by 15.87 (Pick, 1986). NO was assessed as described by Neumann *et al*. (1995) using the Griess reaction to quantify nitrite content of the leucocytes supernatant. ACH50 activity was determined following the method described by Sunyer and Tort (1995). Results are presented as ACH50 units mL^{-1}. Lysozyme activity was determined according to Hutchinson and Manning (1996). The peroxidase activity (units.mL^{-1} plasma) was measured as described by Quade and Roth (1997). All analyses were conducted in triplicate.

2.3.3 **Diets and body composition analysis**

The proximate composition of diets (Table 2-1) and whole fish was carried out according to reference procedures (AOAC, 1997), in duplicate or triplicates: dry matter by drying at 105ºC for 24ºC (Memmert UL40, Schwabach Germany), total ash by combustion at 550ºC for 6h in a

muffle furnace (Thermolyne F6010, Thermo Scientific, Dubuque, USA), crude protein, by multiplying nitrogen content by 6.25 (N determined by Kjeldahl method, VELP DK20, Usmate, Italy), crude lipid by petroleum ether extraction (Soxhlet behr extractor, R 106S, Düsseldorf, Germany), gross energy by complete combustion in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany). The probiotic count of the diets was determined by counting the *Bacillus* sp. colony forming units (CFU) on mannitol egg yolk polymyxin (MYP) agar medium plates, using serial dilution, after 18 to 24 h incubation at 30ºC according to standard 7932/2004 of *International Organization for Standardization* (ISO) and Mossel et al. (1967).

2.3.4 **Growth performance**

Growth and feed conversion performance were assessed as follows: Weight gain, calculated as the difference between final and initial weight, Specific growth rate (SGR), calculated as $100 \times [(\ln \text{Final weight}) - (\ln \text{Initial weight}) / \text{number of days}]$ and Daily growth index (DGI), calculated as $100 \times [(\text{Final body weight})^{1/3} - (\text{Initial body weight})^{1/3}] / \text{days}$. Feed conversion ratio (FCR) was determined as *dry feed intake / weight gain*. Voluntary feed intake (VFI) was calculated as $(\text{dry feed intake} / \text{average weight} / \text{days}) \times 100$, where average weight is $(\text{final weight} + \text{initial weight})/2$. Protein efficiency ratio (PER) was determined as *weight gain/protein intake*.

2.3.5 **Histological analysis**

Gut samples for histological analysis were fixed in 4% buffered formalin solution and processing for paraffin using routine procedures. Sections of 3 micrometer were stained for histological purposes with haematoxylin and eosin (HE). Average *villi* length, density (number of *villi*/area), goblet cells (number of cells per *villus* or fold) and muscular layer thickness were determined with a light microscope (Olympus BX51, cell^B software) on proximal intestine sections. To determine the *villus* length, ten *villi* of a proximal intestinal section of each animal were measured from the *muscularis mucosa* to the basal lamina of the epithelial cell (Pirarat *et al.*, 2011). The *villi* were chosen based on their integrity and higher length. The *villi* density was determined as the ratio between the total number of *villi* presented in each section and the section area (mm^2). The fused *villi* were considered as one, so a higher degree of fusion translates into a lower *villi* count density. Goblet cells were counted on the measured *villi*, on

HE sections based on their morphology: cells with clear and rounded cytoplasm. The overall leucocyte mucosa infiltration and widening were scored using an arbitrary scale: 0 - none, 1 - mild, 2 - moderate, 3 - noticeable, 4 - marked widening and infiltration, adapted from Bakke-Mckellep *et al*. (2007). The thickness of the muscular layer of intestinal section was measured from serosa to submucosa, in at least 8 sites for each section; an average per section and then per animal, was calculated (Batista *et al.*, 2015).

2.3.6 **Statistical analysis**

Statistical analyses were carried out using STATISTICA software (StatSoft, Inc., 2008, version 8, Tulsa, USA). Data were submitted to Kolmogorov-Smirnov and Levene tests, to verify normal data distribution and homogeneity of variances, respectively. Data were analysed by one-way (carcass composition, immune and histology parameters) or two-way ANOVA (growth performance). ANCOVA was also run for body composition and *villi* length, considering final weight and gut area as covariates. A post-hoc Tukey's or Dunnett test were used when significant differences between means were detected. A non-parametric test (Kruskal-wallis test) was used for non-normally distributed data. Significance differences were considered when $P < 0.05$. Results are presented as mean ± standard deviation.

2.4 RESULTS AND DISCUSSION

2.4.1 **Growth performance and whole body composition**

Rainbow trout (15.6 ± 0.5 g) and brown trout (15.3 ± 0.7 g) had similar initial weights. Along the feeding trial, rainbow trout grew faster than brown trout; rainbow trout biomass attained almost 300% of the initial weight, whereas brown trout reached near 170%, with significant differences between species body weight from 7 weeks onwards (Table 2-2). Growth rates (SGR and DGI) and feed intake (VFI) followed the same tendency. No dietary influence was observed on growth performance in both trout species.

There are scattered data on the effects of *Bacillus* on fish growth performance. Rainbow trout fry fed for 2 months diets supplemented with various levels of a commercial *Bacillus* blend (BioPlus2B: *B. subtilis* + *B. licheniformis*) showed improved growth rate, FCR and PER (Bagheri *et al.*, 2008), except on the lowest probiotic dose tested (4.8×10^8 CFU.g^{-1}), which

Table 2-2 Growth performance and nutrient utilization of brown trout and rainbow trout after 7 weeks of feeding probiotic-supplemented diets.

	Brown Trout (*Salmo trutta*)				Rainbow Trout (*Oncorhynchus mykiss*)				*Anova p-value*		
	Control	P1	P2		Control	P1	P2		*Species*	*Diet*	*Species x Diet*
Weight gain (g)	10.61 ± 3.93	13.77 ± 1.94	13.17 ± 2.40		27.82 ± 2.93	30.27 ± 6.36	27.49 ± 5.42		*< 0.0001*	*0.264*	*0.677*
SGR	1.09 ± 0.33	1.34 ± 0.14	1.28 ± 0.16		2.08 ± 0.15	2.17 ± 0.27	2.08 ± 0.27		*< 0.0001*	*0.201*	*0.543*
DGI	0.98 ± 0.32	1.24 ± 0.14	1.18 ± 0.17		2.07 ± 0.17	2.18 ± 0.32	2.06 ± 0.30		*< 0.0001*	*0.215*	*0.600*
FCR	1.32 ± 0.19	1.19 ± 0.14	1.15 ± 0.09		1.18 ± 0.18	1.11 ± 0.21	1.16 ± 0.14		*0.225*	*0.294*	*0.517*
VFI (% BW)	1.45 ± 0.12	1.53 ± 0.21	1.43 ± 0.22		2.24 ± 0.23	2.17 ± 0.20	2.21 ± 0.14		*< 0.0001*	*0.901*	*0.561*
PER	1.63 ± 0.27	1.79 ± 0.21	1.83 ± 0.14		1.82 ± 0.27	1.94 ± 0.35	1.82 ± 0.22		*0.189*	*0.417*	*0.587*

Values are means ± standard deviation, n = 6. At each row, different superscripts indicate significance differences between groups.

was higher than the concentrations used in the current study. In juvenile rainbow trout, the dietary supplementation of a *Bacillus* blend (*B. subtilis, B. licheniformis* trutas *E. faecium*) did not improve growth performance (Merrifield *et al.*, 2010b), except after oxolinic acid (antibiotic) treatment (Merrifield *et al.*, 2010a). Growth performance of rainbow trout (Gisbert *et al.*, 2013), common Dentex (*Dentex dentex*) (Hidalgo *et al.*, 2006) or silver catfish (*Rhamdia quelen*) larvae (Souza *et al.*, 2012) did not differ after feeding diet supplemented with *B. cereus toyoi.*

The described studies seem to indicate that *B. subtilis* may have a positive effect on fish growth, but are more effective in early life stages or after antibiotic treatments, probably due to easier gastro-intestinal colonization or establishment (Austin, 2006; Gómez & Balcázar, 2008). The probiotic concentration is also relevant to obtaining positive results. Whole body composition of both species (data not shown) was not changed by probiotic supplementation, in agreement with previous works (Gisbert *et al.*, 2013; Merrifield *et al.*, 2010a).

2.4.2 Innate immune parameters

Immune stimulation is a desirable characteristic in a probiotic. Lysozyme is an important enzyme present in mucus, serum, kidney, spleen, gut and eggs of most fish

species, capable of lysing the cell wall of Gram+ bacteria (Yano, 1996). Lysozyme activity is highly variable between fish species; rainbow trout seems to present one of the highest levels (Grinde *et al.*, 1988). The ACH50 is a measure of the humoral nonspecific mechanism of response to invading pathogens like bacteria, viruses or parasites (Song *et al.*, 2000). Peroxidase activity is mainly a measure of neutrophil myeloperoxidase (MPO) activity. The MPO is an enzyme released during the respiratory burst, producing oxidizing agents, like hypochlorous acid (HClO) with cytotoxic activity (Babior, 2000). None of the described humoral innate parameters were affected by probiotic supplementation in both species at either 3 or 9 weeks of the trials (Tables 2-3, 2-4).

Previous studies of *Bacillus* spp. supplementation to rainbow trout resulted in the improvement of some of those parameters, but the effects were not consistent. Serum lysozyme was improved after feeding 14 days with *Bacillus subtilis* at 10^7 CFU.g^{-1} (Newaj-Fyzul *et al.*, 2007) or *Bacillu*s sp. at 2 x 10^8 CFU.g^{-1} (Brunt *et al.*, 2007), while ACH50 remained unaffected. The same pattern was observed with longer supplementation (10 weeks) of *B. subtilis* plus *B. licheniformis* (10^8 CFU.g^{-1}), while their association with another microorganism (*Enterococcus faecium*) did not exert the same stimulating effect on serum lysozyme (Merrifield *et al.*, 2010b), reinforcing the idea that interactive aspects must be taken into account. ACH50 was elevated after 45 days of feeding with *B. subtilis* at 10^9 CFU.g^{-1} (Panigrahi *et al.*, 2007). It is possible that the absence of effects may be due to the current probiotic dosage applied, since the described studies used higher probiotic concentrations. However, another trial with *B. subtilis* plus *B. licheniformis* (10^8 CFU.g^{-1} for 10 weeks) resulted in no significant change in serum lysozyme activity (Merrifield *et al.*, 2010a), in accordance with our results.

Table 2-3 Immune parameters of brown trout after 3 and 9 weeks of feeding on experimental diets.

Weeks		**Control**		**P$_1$**		**P$_2$**	
3	Lysozyme (U mL^{-1} plasma)	87.8	± 26.7	85.3	± 17.7	79.2	± 26.9
	Peroxidase (U mL^{-1} plasma)	4.9	± 0.9	4.2	± 2.6	5.9	± 1.0
	ACH 50 (U mL^{-1} plasma)	151.7	± 94.3	120.6	± 80.3	118.5	± 35.4
9	Lysozyme (U mL^{-1} plasma)	168.4	± 30.3	176.4	± 7.0	164.6	± 24.9
	Peroxidase (U mL^{-1} plasma)	7.4	± 4.4	4.9	± 4.3	3.7	± 2.8

Values are means ± standard deviation (n = 10 at 3 weeks, n = 6 at 9 weeks). Values within each row without superscripts indicate no significance differences between treatments ($p > 0.05$).

Table 2-4 Immune parameters of rainbow trout after 3, 9 and 20 weeks of feeding on experimental diets.

Weeks		**Control**			**P_1**			**P_2**		
3	Lysozyme (U mL^{-1} plasma)	824.7	±	501.8	749.2	±	477.3	592.3	±	362.2
	Peroxidase (U mL^{-1} plasma)	4.5	±	2.4	4.8	±	3.9	6.0	±	3.2
	ACH 50 (U mL^{-1} plasma)	132.2	±	45.9	158.3	±	57.7	149.6	±	41.5
9	Lysozyme (U mL^{-1} plasma)	795.2	±	317.3	881.4	±	181.5	895.7	±	245.4
	ACH 50 (U mL^{-1} plasma)	30.9	±	9.2	33.1	±	23.7	40.8	±	21.6
20	Lysozyme (U mL^{-1} plasma)	752.1	±	131.5	870.4	±	261.9	775.7	±	153.1
	Peroxidase (U mL^{-1} plasma)	3.0	±	1.3	3.3	±	1.3	3.2	±	3.0
	ACH 50 (U mL^{-1} plasma)	64.9	±	40.6	68.3	±	11.7	75.2	±	20.3
	H-K nmoles O_2^-	3.8	±	0.6	3.8	±	0.4	3.7	±	0.3
	H-K Nitrite (µM)	3.8	±	1.3	2.8	±	0.6	2.3	±	1.0

Values are means ± standard deviation (n = 12 at 3 weeks, n = 6 at 9 and 20 weeks). Values within each row without superscripts indicate no significance differences between treatments ($p > 0.05$).

To our knowledge this is the first report about the impact of *Bacillus* administration on brown trout. The studies on the use of probiotics in this species are very scarce and limited to lactic acid bacteria, which were able to increase ACH50 and lysozyme activities (Balcázar *et al.*, 2007a) while the current probiotic P_1 and P_2 diets were not.

There is a lack of information about long term probiotic administration in these animals. Some studies report a time-course decrease of the immune response during probiotic administration (Cerezuela *et al.*, 2012; Sharifuzzaman & Austin, 2009; Sun *et al.*, 2010) and long term exposure to immune-stimulants can lead to immunosuppression (Merrifield *et al.*, 2010c).

Aiming to study the impact of a longer probiotic administration on immune parameters, the probiotic supplementation was prolonged for 20 weeks in rainbow trout, evaluating also HK cellular responses through the measurement of superoxide anion (O_2^-) and NO production (table 2-4). No significant differences were observed between probiotic groups and control, meaning that the longer probiotic supplementation was not immunosuppressive in case of rainbow trout.

2.4.3 **Intestinal morphology**

Dietary probiotic supplementation did not significantly affect the mucosal *villi* length in either fish species (Figure 2-1A). The *villus* length is related to absorptive surface and probiotics were capable to increase this parameter in the proximal intestine of tilapia (Pirarat *et al.*, 2011) and rainbow trout (Gisbert *et al.*, 2013) but not in gilthead sea bream (Cerezuela *et al.*, 2013).

The average number of goblet cells per *villi* was counted in both species and was not significantly influenced by dietary probiotic supplementation (Figure 2-1C) as observed in rainbow trout (Harper *et al.*, 2011; Ramos *et al.*, 2013) or Senegalese sole (Batista *et al.*, 2015) supplemented with commercial probiotics. Gisbert *et al*., (2013) reported an goblet cell increase after feeding *B. cereus* to rainbow trout, while Cerezuela *et al*. (2013) observed the opposite in sea bream receiving *B. subtilis* or inulin or both. Goblet cells are specialized in mucous production, the first line of defense against invading pathogens preventing their attachment to epithelial surfaces (Muiswinkel & Wall, 2006) and responsible for lubrication of undigested material, assisting their progression along the gut. Their number can be modified by feed ingredients, as soybean meal (Van den Ingh *et al.*, 1991) and additives as prebiotic and probiotics (Cerezuela *et al.*, 2013) possibly by the microbiota modulation (Bakke-McKellep *et al.*, 2007), as demonstrated in gnotobiotic fish studies (Rawls *et al.*, 2004).

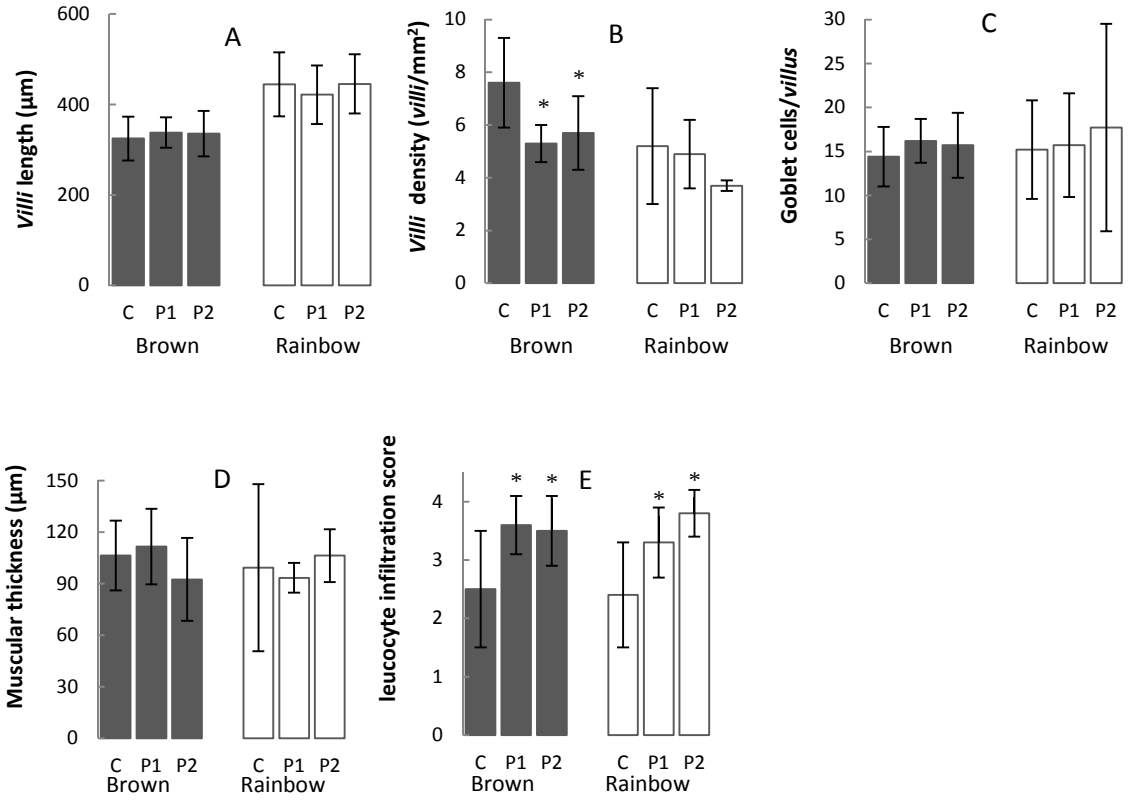

Figure 2-1 Intestinal morphology parameters of brown and rainbow trout after 9 weeks of feeding trial. Mucosal folds or *Villi* length (A), density (B), goblet cells per *villus* (C), muscular thickness (D) and leucocyte infiltration & widening scores (E) of brown (■) and rainbow trout (□) after 9 weeks of feeding C, P_1 and P_2 diets. Values are means ± SD (n = 6). * indicates difference towards the control (One way Anova and Dunnett test; $P < 0.05$).

Current morphometric measurements of *tunica muscularis* revealed no impact of probiotic in both species (Figure 2-1D). Gut muscular layer is involved in peristaltic movements, and is assumed to be modulated by feed components, as inulin, responsible for the hypertrophy of muscular layer in Atlantic salmon (Bakke-McKellep *et al.*, 2007) and rats (Greger, 1999) or probiotics (Batista *et al.*, 2015). However no effect was detected in the current case.

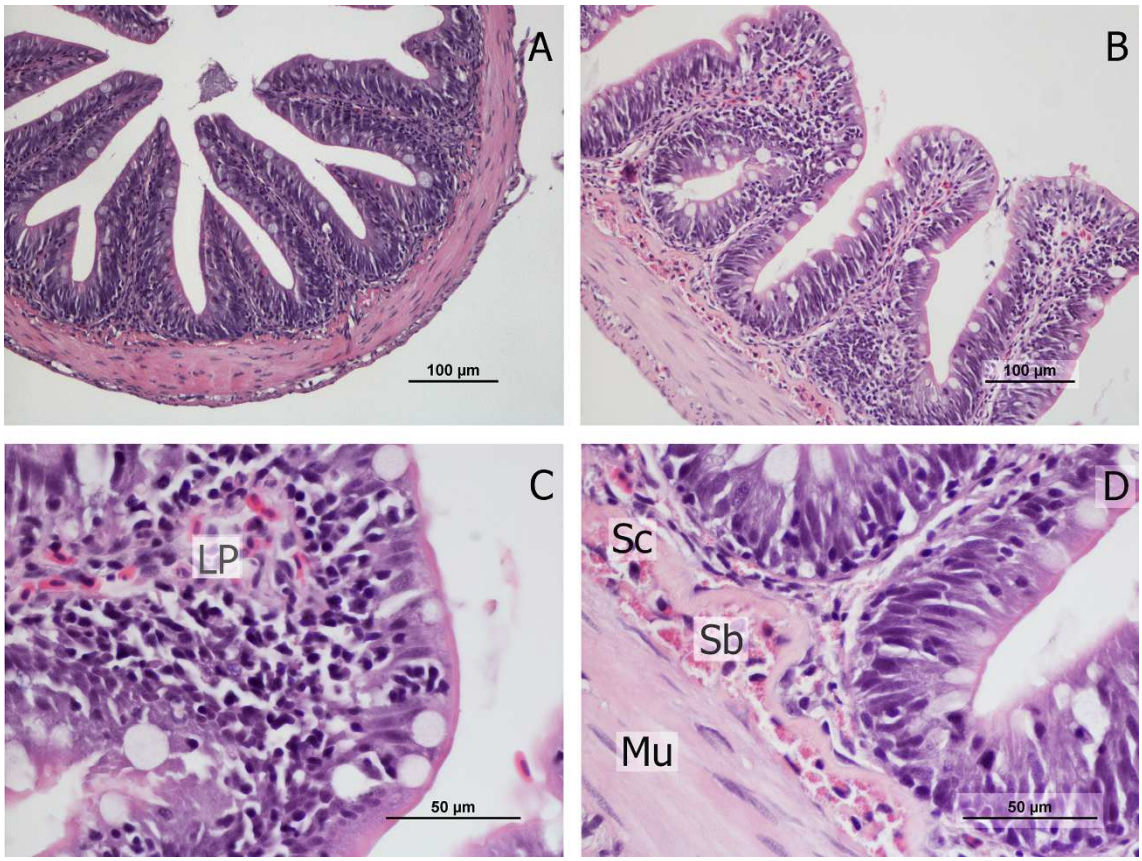

Figure 2-2 Representative images of proximal intestine section from brown trout fed control diet (A) and P1 diet (B, C & D) for 9 weeks. C and D are magnifications of image B.
Note the fusion of several villi. C) Mononuclear inflammatory cells are present in the lamina propria (LP) of the villi. D) Eosinophil's infiltrate in the stractum compactum (Sc) and submucosa (Sb) of proximal intestine of fish feed with P1 diet (HE-stained, Bar A and B =100 micrometer; C and D = 50 micrometer).

Brown trout fed probiotic diets, in particular, presented a higher degree of fusion between adjacent *villi*, with leucocyte infiltration of the lamina propria (Figure 2-2). To evaluate those fusions, the *villi* density was determined, counting fused *villi* as only one. After 3 weeks of feeding trial, *villi* density tended to decrease in brown trout fed dietary probiotics. After 9 weeks, however, *villi* density were significant lower in animals fed P_1 and P_2 diets, compared to control diet, as a result of a higher degree of fusion of adjacent *villi*. In rainbow trout, the

villi fusion was less pronounced, and as result no significant effect of dietary probiotics supplementation on *villi* density was observed at either 3 or 9 weeks of feeding (Figure 2-1B).

Several previous trials observed an increased intestinal epithelial leucocyte infiltration as a result of probiotic administration (Ferguson *et al.*, 2010; Gisbert *et al.*, 2013; Harper *et al.*, 2011; Pirarat *et al.*, 2011), reporting it as a signal of their immune stimulation properties. That response could explain the enhanced resistance to pathogen challenges observed after probiotic administration (Brunt *et al.*, 2007; Kim & Austin, 2006b; Vendrell *et al.*, 2008). In the same way, Balcázar *et al.* (2006b) observed an enhanced phagocytic ability of leucocytes isolated from the intestine of rainbow trout fed probiotics. Nevertheless, the increase of leucocyte infiltration when this is associated to *villi* fusion may express an increase in the mucosa inflammatory status and not an increased protection condition that could be afforded by rising immune system stimulus.

In the current experiment, the lamina propria and submucosa were widened, with increased presence of inflammatory cells, suggesting an inflammatory process (Kortner *et al.*, 2012; Merrifield *et al.*, 2011b). Is well known that soybean meal can affect negatively the growth and the intestinal epithelium of salmonids, depending on fish species and strain (Venold *et al.*, 2012), the raw material processing method and the incorporation dosage (Kaushik *et al.*, 1995). The rainbow trout growth is not always depressed, even at high levels of soybean as 45% (Heikkinen *et al.*, 2006), but some impact on the intestinal mucosa seems to occur, namely the shortening of *villi*, disorganized vacuolization of enterocyte and leucocyte infiltration. Nevertheless the need to replace fish meal at an affordable cost, speciality before the reauthorization of the use of non-ruminant processed animal proteins in EU aqua feeds (European Commission, 2013), explains the commercial option to use it at somewhat high levels.

Probiotics can play a role in soybean tolerance since lactic acid fermentation was able to eliminate indigestible carbohydrates and antinutritional factors in soybean meal fed to Atlantic salmon (Refstie *et al.*, 2005) and a commercial multi species probiotic has improved the ability of rainbow trout to deal with high levels of soybean meal (Sealey *et al.*, 2009). Heat-killed *Lactobacillus plantarum* at 0.1% allowed to successfully replace fish meal by soybean meal at 30%, improving the feed ingestion and the growth rates of amberjack. Interestingly, in that study, at 15% substitution, the impact of the heat-killed probiotic on growth performances was not evidenced, pointing to an association effect of the diets ingredients (Dawood *et al.*, 2015). Also, probiotics enhanced the ability of Senegalese sole to deal with high levels of vegetable

oil in the diets (Rodrigáñez *et al.*, 2009). However, on the contrary, in the current trial, the gut mucosa barrier did not show morphological improvements and our observations indicates a deleterious effect in it.

In the same way, the dietary administration of *B. subtilis* or inulin or their association to gilthead sea bream resulted in gut mucosa damage: gut edema and inflammation (Cerezuela *et al.*, 2013); together with our data, the effect of diet supplementation with *Bacillus* spp. on fish intestinal morphology deserves more in-depth study.

Under the current rearing conditions, the dietary supplementation of *Bacillus subtilis* + *Bacillus cereus* var. *toyoi* did not improve growth performance or immune response in either species - to the contrary, probiotics had a deleterious effect, specially, on the gut of brown trout after 9 weeks of feeding. This study points to the necessity of evaluating gut integrity in future probiotic studies, and to consider evaluating autochthonous aquatic microorganisms, which may be better candidates for aquatic probiotics than terrestrial ones (Gatesoupe, 1999). The potential probiotic interaction with vegetable feed ingredients and the differential response of distinct strains of fish species to them, reinforce the necessity to evaluate case-to-case and not to generalize probiotic administration results.

3 *Growth, immune responses and intestinal morphology of rainbow trout (Oncorhynchus mykiss) supplemented with commercial probiotics*

Growth, immune responses and intestinal morphology of rainbow trout (*Oncorhynchus mykiss*) supplemented with commercial probiotics.

Key words: Probiotics, Rainbow trout, Growth, Innate immunity, Intestine histology

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3.1 ABSTRACT

The influence of two commercial probiotics on the growth, innate immune parameters and intestinal morphology of rainbow trout (O*ncorhynchus mykiss*) juveniles (initial weight: 16.4 ± 0.4 g) was evaluated. Two probiotic types: A, multi-species (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.) and B, mono-species (*Pediococcus acidilactici*) were tested at two levels each (A_1: 1.5 $g.kg^{-1}$, 8.6×10^5 $CFU.g^{-1}$; A_2: 3 $g.kg^{-1}$, 1.6×10^6 $CFU.g^{-1}$; B_1: 0.1 $g.kg^{-1}$, 2.6×10^4 $CFU.g^{-1}$; B_2: 0.2 $g.kg^{-1}$, 7.2×10^4 $CFU.g^{-1}$) versus an unsupplemented diet (C). Diets were distributed to sextuplicate tanks, three times a day to visual satiation for 8 weeks. Growth performance and immune responses (plasma lysozyme, ACH50, peroxidase and head kidney respiratory burst) were determined at 4 and 8 weeks of feeding. Body composition and intestine morphology were determined at the end of the feeding trial.

At 8 weeks, the lower dose of multi-species probiotic (A_1) improved growth rate, while both probiotic types improved feed conversion rate compared to the control animals, at the lower dose of multi-species (A_1) and at the higher dose of mono-species (B_2) probiotics. Body composition did not vary between treatments. At 4 weeks, ACH50 activity was significantly higher in fish fed higher dose of B probiotic (B_2, 123.7 ± 50.6 *vs* 44.1 ± 7.7 $U.ml^{-1}$in control). At 8 weeks, lysozyme activity was higher in fish fed A_1 (13.1 ± 5.2 $\mu g.ml^{-1}$) diet compared to fish fed control diet (7.8 ± 1 $\mu g.ml^{-1}$). Plasma peroxidase and head-kidney respiratory burst did not differ among the dietary treatments. *Villi* length and integrity and goblet cell counting of a cross section of the anterior intestine were not significantly different between groups.

Results suggest benefits in zootechnical performance and immune humoral responses using both probiotic types, in a dose dependent manner, without apparent alterations in intestinal morphology.

3.2 INTRODUCTION

Fish health status is greatly conditioned by surrounding environment owing to intimate contact with nearby microorganisms, capable of external and gastrointestinal (GI) tract colonization (Denev *et al.*, 2009; Ellis, 2001). A balance microbiota can contribute to resistance to pathogenic or opportunistic microbial colonization (Castex *et al.*, 2009). Mechanism of action include competitive exclusion of pathogen binding (Chabrillón *et al.*, 2005a; Vine *et al.*, 2004), competition for nutrients (Balcázar *et al.*, 2006a; Verschuere *et al.*, 2000) and support

to immune system development and maturation, specially at early stage of microflora establishment (Balcázar *et al.*, 2006a; Rawls *et al.*, 2004). Probiotics can contribute to the balancing of gut microbiota (Balcázar *et al.*, 2006a; Denev *et al.*, 2009; Merrifield *et al.*, 2010c). Therefore, it is no surprising the increasing number of studies testing probiotic supplementation in fish diets in recent years. The most extensively cited definition of probiotics is "living organisms, which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002), but the concept could be enhanced to "any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved, in part at least, by improving the microbial balance of the fish" (Merrifield *et al.*, 2010c). *Via* gut microbiota modulation and/or innate immune stimulation by bacteria or bacterial products (Irianto & Austin, 2002, 2003; Kim & Austin, 2006c), probiotics can contribute to enhance animal immune status. Several works report an increase of the activity of circulating lysozyme (Brunt *et al.*, 2007; Merrifield *et al.*, 2010b; Newaj-Fyzul *et al.*, 2007; Panigrahi *et al.*, 2004; Sun *et al.*, 2010), an important mechanism of defence against Gram-positive bacteria (Yano, 1996), peroxidase (Newaj-Fyzul *et al.*, 2007; Salinas *et al.*, 2008), a potent antimicrobial agent released during respiratory burst (Babior, 2000) or complement (Balcázar *et al.*, 2007a; Balcázar *et al.*, 2007b; Nikoskelainen *et al.*, 2003), an innate humoral mechanism of response to invading pathogens like bacteria, virus or parasite (Song *et al.*, 2000). Additionally probiotics can enhance intestine *villi* height (Pirarat *et al.*, 2011) increasing surface absorptive area and/or produce metabolic substrates like vitamins, organic acids, short chain fatty acids and digestive enzymes (Bairagi *et al.*, 2002; John *et al.*, 2006; Laiño *et al.*, 2013; LeBlanc *et al.*, 2011; Murillo & Villamil, 2011), helping to explain the enhancement in zootechnical performances, as better growth rates or feed conversion ratios, sometimes observed after probiotic supplementation (Díaz-Rosales *et al.*, 2009; Lara-Flores *et al.*, 2003; Merrifield *et al.*, 2010c; Zokaeifar *et al.*, 2012).

Extensive and recent reviews evidence the beneficial effects of probiotic supplementation in aquaculture (Balcázar *et al.*, 2006a; Kiron, 2012; Merrifield *et al.*, 2010c; Verschuere *et al.*, 2000). Overall results point to the necessity to adequate probiotics to each particular situation since several factors can affect their efficacy: dose (Alizadeh *et al.*, 2011; Brunt *et al.*, 2007), administration period (Shelby *et al.*, 2006) or timing (Merrifield *et al.*, 2010a), animals species and age (Gaggìa *et al.*, 2010; Nayak, 2010), association effects within microorganisms or feed components (Merrifield *et al.*, 2010b; Shelby *et al.*, 2007).

The present study intends to assess the impact of two commercial probiotics at two concentrations in the diet, on growth performance, innate immune parameters and gut morphology of juvenile rainbow trout (*Oncorhynchus mykiss*), an important salmonid species.

3.3 MATERIAL AND METHODS

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

3.3.1 Experimental diets preparation

A commercial diet (Alpis, from A. Coelho & Castro Lda., Póvoa de Varzim, Portugal) was milled to < 800 μm and the defined quantity of lyophilized probiotic in powder form was mixed to approximately one kg of the basal diet (table 3-1) and then mixed with the remaining diet amount by using an horizontal helix ribbon mixer (model Mano, 100 L capacity, CPM, San Francisco, USA). Diets preparation was sequential, starting with the control diets, and the probiotic diets, in an ascending order of probiotic inclusion.

Table 3-1 Probiotics composition, inclusion level and proximate composition of the experimental diets

	Experimental diets [a]				
Probiotic	Control	A_1	A_2	B_1	B_2
Inclusion ($g.kg^{-1}$)	-	1.5	3.0	0.1	0.2
$CFU.g^{-1}$ of diet	-	8.6×10^5	1.6×10^6	2.6×10^4	7.2×10^4
Microbial composition	-	*Bacillus sp., Pediococcus sp., Enterococcus sp., Lactobacillus sp*		*Pediococcus acidilactici*	
Proximate composition					
Dry matter (%)	94.4	94.5	94.6	96.9	97.1
Crude protein (%)	44.0	43.8	43.9	43.8	44.3
Crude fat (%)	15.0	14.9	15.5	15.5	15.3
Ash (%)	14.6	14.5	14.5	15.0	14.9
Gross energy ($kJ\ g^{-1}$)	19.8	19.9	19.9	20.3	20.2

[a] Main feed ingredients: fish meal (47.5 %); soybean meal 44 (28.5 %), wheat (12 %), fish oil (8 %), algae meal (3 %) and a vitamin and mineral premix (1 %).

All ingredients were cold pelleted using a laboratory pellet press with a 2.4-mm diameter (CPM, C-300 model, San Francisco, USA). After pelleting diets were dried in a ventilation oven (48h at 40ºC), and stored at 4ºC, in vacuum bags, until use.

3.3.2 **Animals and experimental conditions**

Juvenile rainbow trout (n=600) were obtained from Posto Aquícola do Torno (Marão, Portugal), and transported to the Aquatic Engineering Laboratory (ICBAS-UP, Porto, Portugal). Fish were kept under quarantine conditions for 3 weeks, during which their health conditions were monitored. The skin and gills colours were within the normal conditions for trout. There were no visible signs of external injuries or infections. The animals were distributed into 30 square fibre glass tanks (20 fish/tank), stocked at an initial density of 6.6 kg/m^3. Fish were reared in a controlled semi-closed recirculating freshwater system at 17 °C. After the acclimation period one of five experimental diets was randomly attributed to each tank, tested in sextuplicate. Diets were fed by hand 3 times a day (9:00, 12:30, 16:00 h), 6 days/week, until apparent visual satiety, for 8 weeks. Every 2 or 3 days the bottom of the tanks was cleaned, with approximately 30% of the recirculating water renewed each time.

3.3.3 **Experimental proceeding and sampling**

The growth trial lasted for 8 weeks. At the middle (4 weeks) and at the end of the feeding trial, animals were bulk weighed (after a 24h fasting), to determine growth and feed conversion performance. After the weighting, six animals per treatment (one fish per tank) were collected to evaluate cellular and humoral immune parameters. In the last sampling, gut samples were also taken to study intestinal morphology and additional twelve animals per treatment were sacrificed to evaluate carcass composition, as further described, and skin mucus was collected from those animals.

Animals were sacrificed by water immersion with ethylenoglycol monophenyl ether (1mL.L^{-1}). Blood was collected from the caudal vein, using heparinised syringes. Plasma was obtained after centrifugation (3000 × *g* for 10 min at 4ºC) and maintained at -80ºC for further analysis. Head-kidney was aseptically collected and immediately processed as described below. Gut samples were fixed in 4% formalin buffered solution and processed as described later for

histological assessment. The dorsal skin mucus of twelve animals per treatment (polled in three samples) was gently scraped with a scalpel and stored at -80ºC.

3.3.4 **Probiotic detection in diets**

The number of colony-forming units (CFU) per gram of diet was carried out for each of the probiotic bacteria at the beginning of the experiment. The determination of CFU was carried out using reference methods for isolation and enumeration of microorganisms in animal feeding stuffs: 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.).

3.3.5 **Diets and carcass chemical analysis**

At the beginning of the trial, 10 animals were sacrificed to determine initial body composition. After eight weeks of experimental feeding, 12 animals per treatment (2/tank) were sacrificed to evaluate proximate body composition. To diminish the number of samples, whole fish from two tanks from the same treatment were ground pooled, and moisture content was determined (drying at 105ºC for 24ºC). Samples were then freeze-dried. Then, proximate composition analysis of diets and whole fish was made according the following reference procedures (AOAC, 2006) in duplicate or triplicate: dry matter by drying at 105ºC for 24ºC (Binder ED 115, Tuttlingen, Germany), total ash by combustion at 550ºC for 6h in a muffle furnace (Selecta 2000366, Barcelona, Spain), crude protein, by multiplying nitrogen content by 6,25 (LECO nitrogen analyser, FP-528 model, St. Joseph, Michigan), crude lipid by petroleum ether extraction (Soxtec 2055, Foss, Hilleroed, Denmark) and gross energy by complete combustion in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany).

3.3.6 **Calculations**

Daily growth index (DGI) was calculated as $100 \times [(\text{Final body weight})^{1/3} - (\text{Initial body weight})^{1/3}] / \text{days}$. Feed conversion ratio (FCR) was determined as *dry feed intake / weight gain*. Voluntary feed intake (VFI) was calculated as (*dry feed intake / average body weight / days)*$\times$ *100*, where average body weight (ABW) is *[final body weight (*FBW*) + initial body weight (*IBW*)]/2*. Protein efficiency ratio (PER) was determined as *weight gain/protein intake.*

At the end of the feeding trial additional parameters were evaluated: Daily nutrient gain (*g/kg or kJ/kg ABW/day*) calculated as (*final carcass nutrient content - initial carcass nutrient content*)/*ABW*) / *days* and Nutrient retention (% intake) as [(*FBW* × *final carcass nutrient content - IBW* × *initial carcass nutrient content)/nutrient intake]* × *100*. Condition factor or K = *[weight (g)* × *100]/lenght*^{3} *(cm)*, Hepatosomatic index (HSI, %) = *100* × *(liver weight / body weight)*, Viscerosomatic index (VSI, %) = *100* × *(viscera weight / body weight)*.

3.3.7 **Innate immune parameters**

3.3.8 **Plasma alternative haemolytic complement pathway (ACH50), lysozyme and peroxidase activities**

ACH50 was determined following the method described by Sunyer and Tort (1995), using rabbit red blood cells (RaRBC) as target cells to haemolysis. The serially diluted plasma was incubated (100 min) with RaRBC (2.8×10^8 cell.ml^{-1}), centrifuged (122 × g, 2.5 min) and the optical density (OD) of the supernatant was measured at 414 nm. The reciprocal of the serum dilution causing 50% lysis of RaRBC was designated as ACH50 (Tort *et al.*, 1996). Results are presented as ACH50 units mL^{-1}.

Lysozyme activity was determined in plasma and skin mucus, measuring (450 nm) the decrease of the turbidity of a *Micrococcus lysodeikticus* suspension, as described by Ellis (1990) and Hutchinson and Manning (1996). Lysozyme concentration (μg mL^{-1}) was estimated using lyophilized hen egg-white lysozyme (Sigma®) serially diluted in sodium phosphate buffer (0.05 M; pH 6.2) to establish a standard curve. Skin mucus was prepared according methodology adapted from Fagan *et al*. (2003) and Fast *et al*. (2002). After sequential centrifugation (1500 × *g*, 20 000 × *g*, for 10 min at 4 °C), the supernatants were diluted with 3 parts of sodium phosphate buffer (0.05 M; pH 6.2) and analysed as previous described.

Total peroxidase activity in plasma was measured as described by Quade and Roth (1997). The peroxidase activity (U mL^{-1} plasma) was determined assuming that one unit of peroxidase produces an absorbance change of 1 OD. All analyses were conducted in triplicates.

3.3.9 Respiratory burst activity and nitric oxide production of head-kidney (HK) leucocytes

3.3.10 Isolation and culture of HK leucocytes

Rainbow trout HK leucocytes were isolated and maintained according to Secombes (1990). Briefly, the HK was collected aseptically, gently macerated trough a sterile nylon mesh and the cellular suspension separated through a Percoll (Sigma®) gradient (34%:51%) followed by a centrifugation (400 × *g*, 40 minutes at 4ºC), were the interface cellular suspension was collected. Viable cells were plated (2×10^6 cells per well), overnight incubated (18ºC), washed off and maintained with L-15, containing 5% FBS to conduct respiratory burst and NO assays, after 24h and 72h of incubation at 18ºC, respectively.

3.3.11 Respiratory burst activity and nitric oxide production

Respiratory burst activity of HK leucocytes was based on the reduction of ferricytochrome C method for the detection of extracelular O_2^- production as described by Secombes (1990). Optical densities (OD) were converted to nmol of O_2^- produced by multiplying by 15.87 as described by Pick (1986).

Nitric oxide (NO) production by HK leucocytes was assessed as described by Neumann *et al*. (1995) using the Griess reaction (Green *et al.*, 1982) to quantify nitrite content of the leucocytes supernatant, against a standard curve produced with known concentrations of sodium nitrite. To stimulate NO production, the adherent leucocytes monolayer was incubated for 72h, at 18ºC, with L15 medium containing 5% FBS and LPS (Lipopolysaccharide from Escherichia coli serotype 0111B4, Sigma; 1μM) in triplicate.

3.3.12 Histological analysis

After euthanasia, 6 fish per treatment were dissected and their entire digestive tracts exposed. Samples of anterior intestine were fixed in 4% buffered formalin solution for 24 hours.

After fixation, the samples were transversally sectioned, dehydrated and embedded in paraffin blocks according to standard histological procedures. Sections of 3 micra were stained for histological purposes with haematoxylin and eosin (H&E). In each animal, 3 transversal

sections of anterior intestine were examined with light microscope (Olympus BX51, cell^B software) and the follow parameters measured or counted in each section: area, the number of mucosal folds or *villi* present, *villi* length (measured since the *muscularis mucosa* to the basal lamina of the enterocytes and the total number of goblet cell. Afterwards, some elements were related with the section area, calculating *villi* density (number of *villi*/area), *villi* length per area and goblet cell density (number of cells per area and *villus*). The presence or absence of leucocyte infiltrations in the lamina propria and *muscularis mucosae* and *villi* fusion were examined.

3.3.13 **Statistical analysis**

Statistical analyses were carried out using STATISTICA software (StatSoft, Inc., 2008, version 8). Data were submitted to Kolmogorov-Smirnov and Levene tests, to verify normal data distribution and homogeneity of variances, respectively. Data were analysed by one-way ANOVA. Post-hoc Tuckey test was used when significant differences between means were detected. Non-parametric test (Kruskal-Wallis test) was used in non-normally distributed data. Significance differences were considered when $P < 0.05$. Results are presented as mean ± standard deviation (SD).

3.4 RESULTS

3.4.1 **Growth performance and whole body composition**

After 4 weeks of probiotic feeding, the growth and feed utilization of animals did not differ from the control group (table 3-2). As animals grew up, the density increased in the tanks with some expected competition and mortality. After 8 weeks of feeding no significant difference was observed in mortality, in average lower than 6%. At that time significant improvements due to probiotics administration were observed: A_1 animals weighed significantly more and had an increased daily growth index (DGI) than control animals; feed conversion ratio (FCR) was significantly lower than control in A_1 and B_2 groups while protein efficiency ratio (PER) was significantly higher in A_1, B_1 and B_2 diets. Voluntary feed intake (VFI) was significantly lower in animals fed A_2 and B_2 diets comparing to control fish.

Table 3-2 Growth performance of rainbow trout fed the experimental diets for 8 weeks

Diets	**Control**	**A_1**	**A_2**	**B_1**	**B_2**	***p-value***
IBW (g)	16.35 ± 0.30	16.55 ± 0.16	16.12 ± 0.85	16.46 ± 0.30	16.43 ± 0.33	*0.565*
4 weeks						
FBW (g)	39.36 ± 1.16 ^{ab}	41.95 ± 0.27 ^{a}	38.49 ± 1.88 ^{b}	40.93 ± 0.56 ^{ab}	38.77 ± 1.82 ^{b}	*0.010*
DGI	3.08 ± 0.13 ^{ab}	3.30 ± 0.25 ^{a}	3.04 ± 0.13 ^{ab}	3.22 ± 0.09 ^{ab}	3.01 ± 0.20 ^{b}	*0.024*
FCR	1.11 ± 0.05	1.07 ± 0.07	1.13 ± 0.06	1.10 ± 0.06	1.15 ± 0.08	*0.279*
PER	1.94 ± 0.09	2.03 ± 0.15	1.91 ± 0.10	2.01 ± 0.10	1.92 ± 0.13	*0.267*
VFI	3.27 ± 0.11	3.29 ± 0.08	3.30 ± 0.12	3.35 ± 0.13	3.30 ± 0.10	*0.720*
Mortality (%)	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	2.50 ± 2.70 ^{b}	*0.012*
8 weeks						
FBW (g)	82.28 ± 3.00 ^{a}	89.53 ± 3.65 ^{b}	84.39 ± 5.35 ^{ab}	86.97 ± 2.74 ^{ab}	86.28 ± 3.32 ^{ab}	*0.029*
DGI	3.23 ± 0.10 ^{a}	3.44 ± 0.11 ^{b}	3.32 ± 0.10 ^{ab}	3.37 ± 0.09 ^{ab}	3.35 ± 0.10 ^{ab}	*0.028*
FCR	1.02 ± 0.05 ^{a}	0.94 ± 0.05 ^{b}	0.95 ± 0.04 ^{ab}	0.97 ± 0.05 ^{ab}	0.92 ± 0.02 ^{b}	*0.006*
PER	2.11 ± 0.11 ^{a}	2.29 ± 0.11 ^{b}	2.27 ± 0.09 ^{ab}	2.29 ± 0.11 ^{b}	2.38 ± 0.04 ^{b}	*0.001*
VFI	2.43 ± 0.10 ^{a}	2.32 ± 0.08 ^{ab}	2.30 ± 0.07 ^{b}	2.36 ± 0.08 ^{ab}	2.23 ± 0.02 ^{b}	*0.002*
Mortality (%)	5.80 ± 8.00	5.80 ± 3.80	5.70 ± 5.00	4.20 ± 5.90	4.10 ± 3.80	*0.869*

Values are mean ± standard deviation (n=6). Different superscripts letters in the same row stand for statistical differences between diets (Tukey test)

IBW, Initial mean body weight, g. FBW, Final mean body weight, g. DGI, Daily growth index = $100 \times [(\text{Final body weight})^{1/3} - (\text{Initial body weight})^{1/3}] / \text{days}$. FCR, Feed conversion ratio = *dry feed intake / weight gain*. PER, Protein efficiency ratio = *weight gain/crude protein intake.* VFI, Voluntary feed intake = (*dry feed intake / ABW / days)*$\times 100$. ABW, Average body weight = *(IBW + FBW) / 2*. Mortality (%) = (*number of dead animal / number of total animals)*$\times 100$

Table 3-3 Body composition (%), hepatossomatic and viscerosomatic indexes (%) and condition factor (K) of rainbow trout fed the experimental diets for 8 weeks

Diets	**Control**	**A_1**	**A_2**	**B_1**	**B_2**	***p-value***
Body Composition (%)						
Moisture	73.76 ± 0.27	74.59 ± 0.6	74.15 ± 0.35	74.00 ± 0.26	73.65 ± 0.63	*0.172*
Crude protein	16.33 ± 0.41	15.82 ± 0.83	16.17 ± 0.28	16.02 ± 0.14	16.43 ± 0.25	*0.508*
Crude lipid	7.37 ± 0.22	7.00 ± 0.21	7.05 ± 0.23	7.35 ± 0.32	7.09 ± 0.41	*0.417*
Ash	2.21 ± 0.08	2.28 ± 0.21	2.31 ± 0.09	2.27 ± 0.04	2.26 ± 0.10	*0.895*
HSI (%)	1.20 ± 0.08	1.21 ± 0.25	1.14 ± 0.14	1.12 ± 0.13	1.10 ± 0.13	*0.241*
VSI (%)	10.83 ± 2.29	10.63 ± 3.49	10.50 ± 3.16	9.48 ± 2.23	8.56 ± 2.98	*0.250*
K	1.12 ± 0.25	1.16 ± 0.12	1.16 ± 0.13	1.13 ± 0.18	1.08 ± 0.10	*0.325*

Values are mean ± standard deviation (n=3 except for K: n= 27 and HSI and VSI: n= 15). Different superscripts letters in the same row stand for statistical differences between diets (Tukey test).

HSI, Hepatossomatic index, % = $100 \times (\text{liver weight / body weight})$. VSI, Viscerosomatic index, % = $100 \times (\text{viscera weight / body weight})$. Condition factor or K = $[\text{weight (g)} \times 100]/\text{lenght}^3\ \text{(cm)}$.

By the end of the feeding trial the whole carcass composition, liver and viscera somatic indexes and condition factor (K) were not affected by the dietary inclusion of probiotics (table 3-3).

Energy and nutrient intake and retention are displayed in table 3-4: lipid intake was lower in A_1 and B_2 diets, and protein intake was notably lower in all probiotic diets, regarding control groups. The energy intake of B_2 was lower than control. The retention of dry matter and protein was superior in animals receiving B_2 diet. Nutrient daily gain was not affected by probiotic inclusion (data not shown).

Table 3-4 Nutrient and energy intake and retention of rainbow trout fed the experimental diets for 8 weeks.

Diets	**Control**	**A_1**	**A_2**	**B_1**	**B_2**	***p-value***
Intake ($g.kg^{-1}$ or $kJ.kg^{-1}$ $ABW.d^{-1}$)						
Protein	11.33 ± 0.47 ^{a}	10.73 ± 0.36 ^{b}	10.68 ± 0.33 ^{b}	10.66 ± 0.37 ^{b}	10.18 ± 0.10 ^{b}	*0.0002*
Lipid	3.86 ± 0.16 ^{a}	3.65 ± 0.12 ^{bc}	3.77 ± 0.12 ^{ab}	3.78 ± 0.13 ^{ab}	3.51 ± 0.03 ^{c}	*0.0003*
Energy	510.33 ± 20.96 ^{a}	487.82 ± 16.39 ^{ab}	484.67 ± 14.91 ^{ab}	494.61 ± 17.36 ^{a}	465.06 ± 4.39 ^{b}	*0.001*
Retention (% intake)						
Dry matter	26.82 ± 1.41 ^{a}	27.81 ± 1.69 ^{ab}	28.22 ± 1.17 ^{ab}	27.82 ± 1.36 ^{ab}	29.78 ± 0.55 ^{b}	*0.010*
Protein	35.10 ± 2.31 ^{a}	36.64 ± 3.28 ^{ab}	37.30 ± 1.28 ^{ab}	37.11 ± 2.09 ^{ab}	39.91 ± 0.55 ^{b}	*0.016*
Lipid	50.54 ± 2.47	51.62 ± 2.96	49.83 ± 3.02	52.21 ± 2.44	53.82 ± 2.83	*0.144*

Values are mean ± standard deviation (n=6). Different superscripts letters in the same row stand for statistical differences between treatments (Tukey test)
Nutrient intake ($g.kg^{-1}.ABW.d^{-1}$ or $kJ.kg^{-1}.ABW.d^{-1}$) = [nutrient intake (g)/ABW (kg)]/days;
Nutrient retention (% intake) = nutrient recovered in carcass (g)/ nutrient intake (g) = [(FBW × final carcass nutrient content - IBW × initial carcass nutrient content)/nutrient intake] × 100.

3.4.2 **Innate immune parameters**

After 4 weeks of feeding, the plasma alternative complement pathway activity (expressed as ACH50 units) of animals fed the B_2 diet increased significantly compared to control group (table 3-5). Plasma lysozyme and peroxidase activities as well as the HK respiratory burst (evaluated through superoxide anion and nitric oxide production) did not change among dietary treatments. At the end of the feeding trial, plasma lysozyme activity of animals fed the A_1 diet were significantly higher than control ones. No other parameters differed among experimental groups.

Table 3-5 Innate immune parameters analysed over the experience.

	Control	**A_1**	**A_2**	**B_1**	**B_2**	***p-value***
4 weeks						
Plasma Lysozyme (μg ml^{-1})	10.77± 6.27	9.30 ± 4.15	8.57 ± 3.06	6.69 ± 1.91	11.02 ± 6.39	*0.580*
Plasma Peroxidase (U ml^{-1})	3.36 ± 1.45	3.25 ± 1.05	3.74 ± 1.69	3.22 ± 0.64	3.14 ± 1.52	*0.939*
Plasma ACH50 (U ml^{-1})	44.12± 7.65 ^{a}	70.11±23.89^{ab}	64.82±33.55^{ab}	86.97±54.16^{ab}	123.72±50.60^{b}	*0.034*
HK nmoles O_2^-	2.83 ± 0.57	3.19 ± 0.81	2.40 ± 0.67	3.28 ± 0.28	2.66 ± 0.63	*0.145*
HK Nitrite (μM)	4.01 ± 2.72	1.86 ± 0.76	2.76 ± 2.41	1.75 ± 0.70	2.71 ± 2.46	*0.480*
8 weeks						
Plasma Lysozyme (μg ml^{-1})	7.81 ± 0.95 ^{a}	13.14± 5.19 ^{b}	9.31 ± 2.67 ^{ab}	9.18 ± 1.21 ^{ab}	8.94 ± 0.90 ^{ab}	*0.044*
Plasma Peroxidase (U ml^{-1})	3.24 ± 1.27	2.91 ± 0.96	3.19 ± 2.24	2.75 ± 1.14	2.93 ± 0.28	*0.924*
Plasma ACH50 (U ml^{-1})	48.03±20.90	73.66±48.55	49.39±33.68	46.12±29.24	123.18±69.05	*0.166*
HK nmoles O_2^-	5.09 ± 0.61	5.08 ± 0.49	4.39 ± 0.38	4.41 ± 0.46	5.07 ± 0.70	*0.088*
HK Nitrite (μM)	5.80 ± 4.03	7.06 ± 2.78	5.22 ± 1.47	6.66 ± 3.22	4.84 ± 1.76	*0.697*
Skin Mucus Lysozyme (μg ml^{-1})	25.29± 4.65	29.50± 1.92	33.07± 3.94	35.50± 4.75	29.64 ± 2.32	*0.234*

Values are mean ± standard deviation (n=6; except skin mucus lysozyme, where n=3, each sample 4 animals pooled). Different superscripts letters in the same row stand for statistical differences between diets.

3.5 INTESTINAL MORPHOLOGY

Anterior intestinal morphology was evaluated at the end of the feeding trial. In order to standardize potential variation in intestinal section sizes and thickness muscular layer, measured parameters were then related to respective total area (Table 3-6). The animals fed the A_2 diet showed the larger section area of anterior intestine and higher number of goblet cells per *villi*. The *villi* length was improved in the A_1 group, that showed the least *villi* density and per section area. The dietary B_1 showed the least *villi* length, section area and goblet cells per *villi*, but the highest *villus* length per area and density. However, no significant differences between groups were detected in the parameters described. Attention was given to abnormal morphologies, such as fused *villi* and leucocyte infiltration in *lamina propria* and or *submucosa*, but no changes were detected in the intestinal sections evaluated.

Table 3-6 Intestinal histological parameters after 8 weeks of feeding the experimental diets

	Control	**A_1**	**A_2**	**B_1**	**B_2**
Section area (mm^2)	2.5± 0.1	2.9 ± 0.5	3.0 ± 0.9	2.2 ± 0.5	2.7 ± 1.2
Villi length (μm)	443.2± 25.1	510.5 ± 99.3	468.5 ± 110.4	402.2 ± 69.0	423.5 ± 98.7
Villi length/section area (μm/mm^2)	191.6± 25.8	183.5 ± 20.8	184.3 ± 41.1	209.5 ± 31.4	191.9 ± 72.3
Villi density (unit/mm^2)	8.7± 1.0	8.0 ± 2.2	9.3 ± 2.6	12.0 ± 2.6	10.7 ± 3.8
Goblet cells/*villus*	6.8± 1.6	5.5 ± 2.4	7.3 ± 3.7	4.6 ± 1.4	6.4 ± 2.4
Goblet cells/section area (unit/mm^2)	55.1± 17.0	44.1 ± 20.9	62.1 ± 22.1	44.6 ± 17.2	59.2 ± 16.1

Values are mean ± standard deviation (n=6). In each row absence of superscripts letters indicate no significant differences between diets ($p>0.05$).

3.6 DISCUSSION

In the first four weeks of the present study, the zootechnical performance was not noteworthy affected by probiotics. However, significant improvements of growth and feed conversion parameters were observed in the fish fed both probiotics types after 8 weeks of feeding. The multi-species probiotic blend improved DGI, FCR and PER only at the lowest dose tested (A_1). A higher CFU dosage (A_2) could imply a higher disturbance in GI microbiota or immune stimulation, with energy and nutrient wastage (Li *et al.*, 2012). *P. acidilactici* at the highest dose tested (B_2) improved FCR and PER. Proteolytic and lypolytic activities of *P. acidilactici* strains (Llorente-Bousquets *et al.*, 2008; Ramakrishnan *et al.*, 2013) could contribute to explain the higher feed efficiency observed in animals fed B_2 diet, given the significant lower nutrient intake with a similar or superior nutrient retention, comparing to control group. Previous studies, using superior bacterial counts of P. *acidilactici*, suggested no alterations in growth or feed conversion in rainbow trout (Aubin *et al.*, 2005; Merrifield *et al.*, 2011a).

In the current diets, the probiotic counts were not very high; however, animals were fed 3 times a day to satiation, meaning a more constant flow of those microorganisms and a possible better establishment in the gut. According to Merrifield *et al*., (2011a) a level higher than 10^6 $CFU.g^{-1}$, or a continuous supplementation of *P. acidilactici*, should be necessary to avoid the removal of resident cells due to mucus and brush border turnover.

At the end of the trial, no significant differences were observed in mortality or proximate whole body composition, equally to the observed previously (Aubin *et al.*, 2005; Merrifield *et al.*, 2010a; Merrifield *et al.*, 2011a; Merrifield *et al.*, 2010b). Similarly, the condition factor (k) remained unaffected by the present probiotic supplementation, while Merrifield *et al*. (2011a) observed a lower k of rainbow trout receiving *P. acidilactici* supplementation.

The stimulation of the host innate immune response is a key attribute of a probiotic. The respiratory burst activity of culture HK leucocytes was evaluated, measuring extracellular superoxide anion (O_2^-) and nitric oxide (NO) production, but no influence of mono or multi-species probiotic was observed. Similar results were observed in rainbow trout fed diets supplemented with Lactic Acid Bacteria like *Lactobacillus sakei* (Balcázar *et al.*, 2007b) or *Lactobacillus rhamnosus* (Panigrahi *et al.*, 2004; 2005; 2007) or in Nile tilapia supplemented with *P. acidilactici* (Ferguson *et al.*, 2010).

Peroxidase activity was measured in plasma, without significant alterations after 4 or 8 weeks of probiotic feeding. There are reports of the enhancement (Newaj-Fyzul *et al.*, 2007) of

this protection mechanism due to *Bacillus* administration, but also reports showing no influence in this parameter (Brunt *et al.*, 2007).

Plasma lysozyme activity was significantly elevated in A_1 animals after 8 weeks of feeding, indicating that multi-species blend probiotic (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.) can stimulate this innate immune response in a dose and time dependent manner. *Bacillus* can stimulate lysozyme activity in rainbow trout (Brunt *et al.*, 2007; Merrifield *et al.*, 2010b; Newaj-Fyzul *et al.*, 2007). However, association mechanisms seem to occur, since when *B. subtilis* + *B. licheniformis* were combined with *Enteroccoccus faecium* (Merrifield *et al.*, 2010b) or when were fed after an antibiotic treatment (Merrifield *et al.*, 2010a), lysozyme activity remained unaffected. Grouper receiving *B. pumilis* or *B. clausii* presented an increased lysozyme activity after 60 days of feeding but not after 30 days (Sun *et al.*, 2010), a pattern similar to our observations. *P. acidilactici* did not affect plasma lysozyme activity, according to the observed in previous experiments in rainbow trout (Merrifield *et al.*, 2011a) or channel catfish, alone or in combination with *Enterococcus faecium* (Shelby *et al.*, 2007).

Mucus covering the epidermal surface constitute the first line of defence against invading pathogens (Fast *et al.*, 2002), preventing the attachment of bacteria, fungi, parasites and viruses to epithelial surfaces (Muiswinkel & Wall, 2006). Additionally contains cytotoxic, antiviral, antifungal or antibacterial substances (Hellio *et al.*, 2002) like lysozyme. The information regarding probiotics influence in skin mucus lysozyme is yet scarce. By the end of the current experience, skin mucus lysozyme activity remained unaffected by probiotics.

In this study, alternative complement was increased by B_2 diet in a significant way for the first 4 weeks, remaining higher after 8 weeks of feeding. Is, to our knowledge, the first observation of *P. acidilactici* stimulation of this important innate defence mechanism in rainbow trout. Several other lactic acid bacteria (*Lactococcus lactis* ssp. *lactis*, *L. sakei*, *Leuconostoc mesenteroides and L. rhamnosus*; 10^6 CFU/g) fed for 2 weeks were able to elevate significantly ACH50 levels of rainbow (Balcázar *et al.*, 2007b; Nikoskelainen *et al.*, 2003) and brown trout (Balcázar *et al.*, 2007a). *Bacillus* and *Enterococcus faecium* increased ACH activity of rainbow trout at 10^9cfu/g (Panigrahi *et al.*, 2007) but not at 10^8cfu/g (Merrifield *et al.*, 2010b) or at 10^7cfu/g (Newaj-Fyzul *et al.*, 2007).

At the end of the experience, the anterior intestine morphology was analysed at light microscope, evaluating probiotics influence on gut integrity, *villi* length and density and goblet cell density. Those parameters were not affected significantly by mono or multi-species

probiotics, but a tendency to an increase in *villi* density by *P. acidilactici* was noticed, and could be related with an increase of nutrients absorption. There is scarce information in the literature about probiotics effects on intestinal morphology. Previous work of *P. acidilactici* fed to rainbow trout for two weeks, reported a trend to an elevation in goblet cell number, suggesting that it could stimulate the proliferation of these cells and the consequential mucus secretion and gut protection (Harper *et al.*, 2011). In tilapia, *Lactobacillus rhamnosus* improved *villi* length (Pirarat *et al.*, 2011) while no morphological changes were observed in anterior or posterior intestine after *P. acidilactici* feeding (Ferguson *et al.*, 2010).

Some authors have reported that multi-species probiotic supplementation could be more effective than mono-species or mono-strain ones (Nayak, 2010; Timmerman H.M. *et al.*, 2004), mainly due to: 1) a higher probability of a microorganism *consortium* to survive in a changing environment like the GI tract and to dominate the associate microbiota (Bezkorovainy, 2001; Verschuere *et al.*, 2000); 2) a potential stimulation of the immune system, given the diverse immune stimulation properties of strain specific properties (Nayak, 2010; Panigrahi *et al.*, 2007; Salinas *et al.*, 2005) and 3) a greater variety of antimicrobial properties associated with mixed formulations (*i.e.* production of organic acids, bacteriocins, hydrogen peroxide, biosurfactants, etc) preventing pathogen colonization and prosper in GI tract (Timmerman H.M. *et al.*, 2004). On the other hand, a single strain with unique properties can lead to patents whereas the clinical effectiveness of multi-strain probiotics is not easily patentable (Timmerman H.M. *et al.*, 2004). *P. acidilactici* (diet B) is to date, the only authorized dietary fish probiotic in the EU (Regulation (EC) 911/2009), improving skeletal conformation of rainbow trout (Aubin *et al.*, 2005) and sea bass (Lamari *et al.*, 2013).

The information concerning probiotic utilization in fish aquaculture is accumulating; nevertheless it is still difficult to define the best supplementation practices. This experiment confirms the potential interest of the administration for 8 weeks, of both commercial feed additives to improve immune status, FCR and PER, and of the multi-species probiotic in DGI. Also contributes to a dosage establishment, since the administration of two close counts of each product, translates into positive or nule results, with effectiveness observed at 1.5 $g.kg^{-1}$ (8.6×10^5 $CFU.g^{-1}$) of the multi-species and 0.2 $g.kg^{-1}$ (7.2×10^4 $CFU.g^{-1}$) of the mono-species additive. Furthermore our observations reinforce the necessity to test in the field several dosages of a determined commercial probiotic additive, within the range of the recomendeded levels by manufacturers, to achieve the funtional dosage to every particular situation, given the unique microbiota in the environment of each aquaculture farm.

4 *Dietary probiotic supplementation modulated gut microbiota and improved growth of juvenile rainbow trout (Oncorhynchus mykiss)*

Dietary probiotic supplementation modulated gut microbiota and improved growth of juvenile rainbow trout (*Oncorhynchus mykiss*)

Keywords: Probiotics, rainbow trout, growth, intestinal microbiota community

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4.1 ABSTRACT

In aquaculture, infectious diseases are the major cause of economic losses. Probiotic supplementation may change the microbiota of the digestive tract and modulate the immune defences and nutritional performance. This study was conducted to evaluate the dietary supplementation of multi-species (A: *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.) and single-species probiotics (B: *Pediococcus acidilactici*) on growth performance and gut microbiota of rainbow trout (*Oncorhynchus mykiss*). A basal diet was supplemented with probiotic A or B, at two concentrations each (A_1, A_2, B_1 and B_2) or not supplemented (control treatment). Diets were distributed to 30 groups of 20 fish, 3 times a day. The gut microbiota was analysed at the end of the feeding trial (96 days) with 16S rDNA denaturing gradient gel electrophoresis (16S-DGGE). Changes in gut microbial community were assessed by Shannon index (H´) and number of operational taxonomic units (OTUs). After 56 days of feeding, weight gain was significantly improved in fish fed diet A_1 when compared to the control group. Dietary probiotic supplementation changed the gut microbial composition. Number of OTUs (R) was higher in fish fed A_1 (multi-species at lower concentration) than in control group, while H´ was higher in fish fed A_1, B_1 and B_2.

4.2 INTRODUCTION

In intensive fish production, large quantities of disinfectants and drugs are released into the rearing water, which may affect the integrity of the gastrointestinal microbiota (Carnevali *et al.*, 2006), reducing growth and immune competence (Gomez-Gil *et al.*, 2000). To date, antibiotics have been the most widely applied agents in the treatment of fish diseases; however their use can result in the potential environmental and food contamination, as well as in the development and/or dissemination of pathogens antimicrobial resistance (Cabello, 2006; Sapkota *et al.*, 2008; Teuber, 2001; Witte, 2000). Vaccination can significantly reduce the incidence of many important diseases, although immunity is generally specific against a particular pathogen. In addition, several organisms may not respond to vaccination and new diseases or pathogen variants are constantly evolving (Olafsen, 2001).

In the past decade, the reduction of chemical and drug use in aquaculture to obtain more ecological and acceptable practices to the consumer has been the target (Subasinghe, 1997). Nutritional and management approaches to disease prevention and health enhancement have

been under research and development (Oliva-Teles, 2012). Manipulations of the microbiota present in the fish gastrointestinal tract and live feed, is one way to reduce the incidence of opportunistic pathogens (Balcázar *et al.*, 2006a; Dimitroglou *et al.*, 2011). Many studies have pointed out that probiotics in fish diet and rearing water improved the resistance to colonization by pathogenic bacteria (Capkin & Altinok, 2009; Gaggìa *et al.*, 2010; Irianto & Austin, 2002), and improved growth performance and nutrient utilization (Lara-Flores *et al.*, 2003; Merrifield *et al.*, 2010a). Probiotics are live microorganisms that confer health benefits to the host, when administered in adequate amounts (FAO/WHO, 2002). The enhancement of animal health status can be a result from changes in the mucosal microbiota (Balcázar *et al.*, 2006a; Merrifield *et al.*, 2010c) through competitive exclusion (Chabrillón *et al.*, 2005a; Vine *et al.*, 2004), competition for nutrients (Verschuere *et al.*, 2000), production of antimicrobial substances (Cladera-Olivera *et al.*, 2004; Nes & Holo, 2000; Sugita *et al.*, 1998), and/or immunomodulatory effects (Balcázar *et al.*, 2006b; Kim & Austin, 2006a; Sharifuzzaman & Austin, 2009). Moreover, probiotic supplementation may provide vitamins, short chain fatty acids and/or digestive enzymes, and therefore may also contribute to host nutrition (Bairagi *et al.*, 2002; John *et al.*, 2006; Ringø *et al.*, 1995).

A large range of microorganisms are already commercially available worldwide as probiotics for use in aquaculture, including single species and multi species products (Cutting, 2011). So far only one probiotic was authorised for use in aquaculture in the European Union, namely *Pediococcus acidilactici* CNCM MA18/5M, a member of the lactic acid group bacteria (Regulation (EC) 911/2009). Probiotic efficacy is associated with the presence or multiplication of the probiotics in the environment and/or host (Lara-Flores, 2011). Potential colonization and replication within the host's gastrointestinal tract is considered an important probiotic property (Kesarcodi-Watson *et al.*, 2008; Merrifield *et al.*, 2010c) and studies of the composition of the dominant microbiota are an essential part in probiotic fish research (Spanggaard *et al.*, 2000). The application of culture-independent molecular techniques to evaluate the microbial community in fish gut as a result of probiotic supplementation is growing (Pond *et al.*, 2006). Many such methods rely on the 16S rRNA gene, using primers to determine dominant populations from amplified DNA product, followed by sequencing (Balcázar *et al.*, 2007c).

The present study was designed to evaluate the influence of dietary probiotic supplementation in gut microbiota profile and the subsequent affect on host growth performance of rainbow trout (*Oncorhynchus mykiss*), a salmonid species with economic

importance worldwide (FAO, 2012). Two commercial probiotics were tested in two doses, and fish intestine were analysed by denaturing gradient gel electrophoresis (DGGE), using the 16S rDNA sequences.

4.3 MATERIAL AND METHODS

4.3.1 Fish and husbandry conditions

Six hundred rainbow trout juveniles (initial body weight: 16.4 ± 0.5 g each) were evenly distributed into 30 square fibre glass tanks with 20 fish per tank. Fish were reared in a semi-closed recirculating freshwater system, stocked at an initial density of 6.6 kg/m^3 at a constant water temperature of 17 °C. All tanks were cleaned every two to three days with a 30% water renewal.

4.3.2 Diet preparation and feeding trial

A commercial diet (46% protein, 16% lipid, 21kJ g^{-1} gross energy; dry matter basis) was grounded and the defined quantities of the lyophilized probiotic commercial preparations A (1x10^9 colony forming unit (CFU) g^{-1} of dry powder) and B (1x10^{10} CFU g^{-1} of dry powder) were added to the basal diet (Table 4-1). After homogenization, the mixture was pelleted (2.4mm diameter) with 10% water incorporation, dried in a ventilation oven (48h, at 40ºC) and maintained at 4ºC in vacuum bags. During the trial, diets were hand-fed to apparent satiety for 96 days. The basal commercial diet was used as control, and passed through the same processing, excluding probiotic addition.

4.3.3 Experimental design, sampling and growth performance

After 21 days adaptation to experimental rearing conditions, each of the five experimental diets was randomly attributed to the six tanks. Thereafter, fish growth was monitored for 56 days. The fish were bulk weighed at the beginning of the experiment and after 28 and 56 days of probiotic feeding. Weight gain was calculated as the difference between final and initial biomass. Specific growth rate (SGR) was calculated as *[(ln Final weight) - (ln Initial weight)/days] ×100*. Diets with probiotic supplements continued to be fed in order to study gut

microbiota modulation of a longer period supplementation, namely a total of 96 days. At day 96, 10 fish per treatment were sacrificed with anaesthetics overdose (ethylene glycol monophenyl ether, 1mL L^{-1}) after a 12h fasting period. The whole gut was aseptically excised, immediately kept in liquid N and maintained at -80ºC until DNA extraction procedures.

4.3.4 **DNA extraction from trout intestines**

Total genomic DNA was extracted from 300 mg mucosa and digesta from the hind gut with a method adapted from Griffiths *et al*. (2000). In brief, mucosa and digesta were aseptically transferred into bead beating tubes containing a 1:1 mixture of glass beads and ceramic beads with a diameter of 0.5 mm and 1.4 mm, respectively (PEQlab). To isolate DNA, samples were incubated with 0.5 ml hexadecyltrimethylammonium bromide (CTAB) buffer and 0.1 ml lysozyme (125mg ml^{-1}) for 2 hours at 37°C. Then, 0.5 ml phenol-chloroform-isoamylalcohol (25:24:1; pH8.0; Sigma Aldrich) was added and the samples were lysed for 30s in a bead beater (Precellys – PEQlabs) with a speed setting of 5.5 ms^{-1}. The aqueous phase was separated by centrifugation (16,000xg) for 10 min at 4°C and treated with 40µl proteinaseK (25mg ml^{-1}) and 10µl RNaseA (10mg ml^{-1}) for 30 min at 37°C. Phenol remnants were removed by centrifugation with a corresponding volume of chloroform: isoamylalcohol (24:1). DNA was precipitated from the aqueous phase with 0.6 vol isopropanol and 0.1 vol 3M sodium acetate (pH5.2) for two hours at room temperature and subsequent centrifugation (14,000xg) for 30 min at 4°C. The DNA pellet was washed twice with 70% ethanol and dried prior to re-suspension in 60µl double distilled water. Quality and quantity of the extracted nucleic acids was determined by gel electrophoresis and concentration measurements with the Nanodrop photometer. To counteract individual variations, the DNA from two to four fish was pooled in each dietary group.

4.3.5 **Amplification of bacterial 16S rDNA V3 regions**

The variable region V3 of the 16S rDNA was amplified with the KAPA 2G Robust PCRkit from PEQlab (Germany), which includes 5X Buffer B, 5X Enhancer solution 1, 10 mM deoxynucleotide mix and KAPA 2G polymerase and universal primers 518r and 341f-GC (Muyzer *et al.*, 1993). The PCR reaction (50µl) contained 0,1 mM of each deoxynucleotide, 125 nM of universal primers 518r and 341f-GC (Muyzer *et al.*, 1993), 1x buffer B, 100 ng

genomic DNA and, 0,02 U KAPA 2G polymerase. Enhancer 1 was added to improve reaction efficiency and specificity according to the manufacturer's (PEQlab, Germany) instructions. The PCR program started with an initial denaturing step of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 56 °C for 20 s and 72 °C for 40 s and final elongation at 72 °C for 7 min.

4.3.6 **Denaturing gradient gel electrophoresis (DGGE) and cluster analysis**

DGGE was performed with the INGENYphorU system (Goes, The Netherlands). PCR products were separated for 16 hours at 60°C in a 30% - 60% gradient, 8% (w/v) polyacrylamide gel containing 32% formamide and 5.6M urea. The gel was stained with Sybr Green I Nucleic Acid Gel Stain (Sigma Aldrich) diluted 1:10,000 in 0.5x TAE buffer after electrophoresis. DGGE profiles (gel picture) were documented with Bio-Vision system and Vision-cap software (PeqLab) and assessed with the Gel compare II software version 6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium). The gels were normalized to a reference sample that consists of 16S rDNA V3 fragments amplified from genomic DNA of various bacteria with different G/C contents. The Pearson´s correlation describes the association between two samples and was thus used as a similarity matrix. The Unweight Pair Group Method with Arithmetic Mean (UPGMA), a hierarchical method, was applied to create a dendogram. Cophenetic correlation was included to measure the reliability of the dendogram. The number of OTUs (richness) and the intensity of each OTU were determined with the software Gel compare II version 6.0 (Applied Maths NV, Sint-Martens-Latem, Belgium).

4.3.7 **Statistical analysis**

Statistical analyses were carried out using STATISTICA program (StatSoft, Inc., 2008, version 8). Data was submitted to Kolmogorov-Smirnov and Levene tests, to verify normal data distribution and homogeneity of variances, respectively. Then data were submitted to a one-way ANOVA. Post-hoc Tukey test was used when ANOVA showed significance, to determine significant differences between means. Changes in diversity of the microbial community were assessed with Shannon index (H´) and richness (R). The Shannon index H´ was calculated with the formula $H' = -\sum_{i=1}^{S} Pi * \log(Pi)$, where S is the number of OTUs and Pi the proportion of a certain OTU in the sample. Pi was calculated by dividing the intensity of a peak with all

peaks in the sample ($Pi = \frac{ni}{N}$). The richness was calculated as the sum of all OTUs in a sample. Statistical analysis of H´ and R was performed in Origin 6.1 with a t-test. Significant differences were considered when $P < 0.05$. Results are presented as mean ± standard deviation.

4.4 RESULTS AND DISCUSSION

4.4.1 Growth performance

In this study, two commercial probiotics were separately incorporated into fish diet at two concentrations. The multi-species probiotic (A) consists of strains belonging to the genera: *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp. The single-species probiotic (B), consists of the lactic acid bacterium *P. acidilactici* (Table 1).

Table 4-1 Composition of the commercial probiotics, the dietary inclusion level in tested diets and CFU counts per gram of diet.

Probiotic	Dietary Supplementation				
	C	A_1	A_2	B_1	B_2
Bacillus sp. *Pediococcus* sp. *Enterococcus* sp. *Lactobacillus* sp.	-	1.5 g kg^{-1} (8.6×10^5 CFU)	3 g kg^{-1} (1.6×10^6 CFU)	-	-
Pediococcus acidilactici	-	-	-	0.1 g kg^{-1} (2.6×10^4 CFU)	0.2 g kg^{-1} (7.2×10^4 CFU)

Growth performance was monitored for 56 days after feeding diets with probiotics. After 28 days, no significant differences in weight gain and growth rate were observed, regardless of the diet. After 56 days of feeding the different diets, fish fed with diet A_1 gained significantly more weight compared to the control fish ($P < 0.05$). Weight gain of fish fed with diet A_2, B_1 or B_2 did not differ from the control group (Figure 4-1).

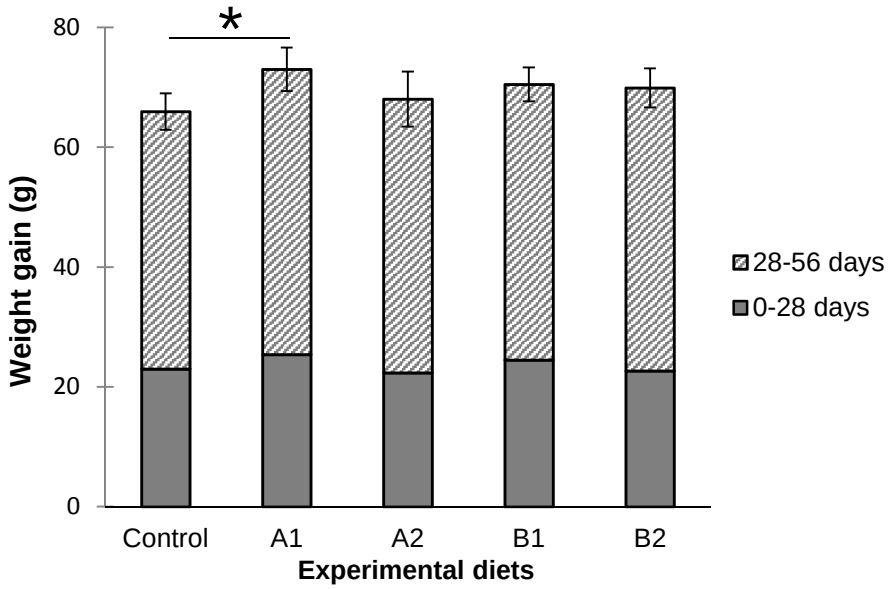

Figure 4-1 Weight gain of juvenile rainbow trout (IBW= 16.4 ± 0.5 g) fed for 56 days with diets containing probiotics.
(* indicates $p< 0.05$ relative to the control).Error bars represent standard deviation (of the weight gain verified in the whole period from 0 to 56 days).

Similar to weight gain, the specific growth rate of fish fed with diet A_1 was significantly better than fish fed with the control diet (Figure 4-2).

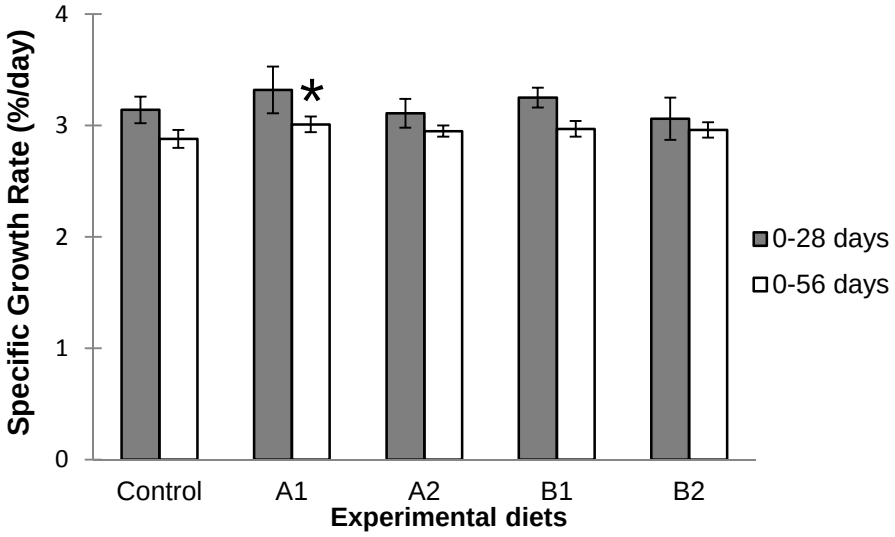

Figure 4-2 Specific growth rate (SGR) of juvenile rainbow trout fed for 56 days with diets containing probiotics. (* indicates $p< 0.05$ relative to the control). Error bars represent standard deviation.

These results indicate that diet A_1, containing the multi-species probiotic at 1.5 g kg^{-1} positively contributes to growth performance. The positive effect may dependent on the dose, since growth performance of fish fed with diet A_2 (multi-species probiotic at 3 g kg^{-1} diet) did not differ from fish fed with control diet.

The single-species probiotic, *P. acidilactici* (diet B_1 and B_2) did not affect weight gain or specific growth rate. In previous studies, *P. acidilactici* did not affect weight gain of juvenile rainbow trout fed for ten weeks (Merrifield *et al.*, 2011a) and for five months (Aubin *et al.*, 2005).

Other commercial probiotics that contain *P. acidilactici* or *E. faecium* did not improve growth performance of young channel catfish, *Ictalurus punctatus* (Shelby *et al.*, 2007). In fact, fish fed for 56 days with a combination of *P. acidilactici* and *E. faecium* at a 10^6 CFU/g diet, showed decreased weight gain compared to unsupplemented (control) group and to fish fed with either *P. acidilactici* or *E. faecium* (Shelby *et al.*, 2007).

Similarly, addition of *E. faecium* to the diet of Nile-tilapia larvae did not affect weight gain of the larvae, but addition of *P. acidilactici*, to the diet significantly decreased weight gain of the larvae (Shelby *et al.*, 2006).

In accordance with the studies above, diets with *Bacillus licheniformis* and *Bacillus subtilis* (BioPlus2B ®) had no significant positive impact on weight gain of juvenile rainbow trout (Merrifield *et al.*, 2010a; Merrifield *et al.*, 2010b), catfish (Shelby *et al.*, 2007) and tilapia larvae (Shelby *et al.*, 2006). The same was verified with *Lactobacillus* spp. supplemented to juvenile catfish (Shelby *et al.*, 2007) and *E. faecium* alone or associated to *B. licheniformis* + *B. subtilis* fed to rainbow trout (Merrifield *et al.*, 2010a; Merrifield *et al.*, 2010b).

However, in contrast with previous findings, and in agreement with our results, the addition of *B. subtilis* + *B. lichenifornis* to the diet improved weight gain and SGR in first-feeding trout larvae, in a dose dependent way (Alizadeh *et al.*, 2011; Bagheri *et al.*, 2008) and improved SGR in rainbow trout juveniles (Merrifield *et al.*, 2010a).

In the studies above, different doses of distinct probiotics were tested on rainbow trout in different developmental stages and under diverse conditions, which makes difficult to apply the underlying trends into the current study. Nevertheless, previous studies showed that the use of probiotics in fish larvae were more effective to improve growth than the same treatments during juvenile stages. Moreover, higher dosages not always translate into better results (Alizadeh *et al.*, 2011; Bagheri *et al.*, 2008). Additional factors that influence growth are rearing temperature and the daily feeding frequencies. Most of the previous studies were run at temperatures below

the temperature used in the current trial (17ºC) and the animals were fed twice a day, whereas in the present trial, animals were fed three times a day.

Usually, the main objective of probiotic administration is the improvement of fish resistance to opportunistic diseases, through immune stimulation and/or intestinal competitive exclusion within microbiota. The growth enhancement can be a result of the improvement of dietary nutrient assimilation, by for instance, an increase in the availability of metabolic substrates like vitamins (LeBlanc *et al.*, 2011) or digestive enzymes (Bairagi *et al.*, 2002) produced by microbiota. Given the obvious importance of growth in aquaculture activity (Bureau *et al.*, 2000), the potential as growth promoter is a significant attribute that can be consider to use probiotics as feed additives.

4.4.2 **DGGE analysis of intestinal microbiota**

The variable region V3 of the 16S rDNA was amplified from genomic DNA extracted from intestinal content and tissue of the hindgut and then PCR products were separated on a DGGE (Figure 4-3).

All samples contained three to four operational taxonomic units (OTU) with high intensity and several OTUs with low intensity in their DGGE profiles (Figure 4-3). Changes in the species composition of the intestinal microbiota were visualized with the dendogram (Figure 4-3). The dendogram consists of two clusters. Cluster one consists of samples from fish fed with control diet, diet B_1, or diet B_2, while cluster two contains all samples from fish fed with diet A_1 or A_2 (multi-species probiotics) as well as samples from fish fed with diet B_2, (single-species probiotic), and some control samples. Within cluster two, the high dose of the multi-species probiotic is well separated from the low dose of the multi-species probiotic. The first makes up sub cluster 2b, whereas the latter makes up sub cluster 2a. Composition and dosage of the probiotics influence the intestinal microbiota of rainbow trout. Separation of clusters is robust, as indicated by cophenetic correlation values at the nodes, which range from 84 through 100.

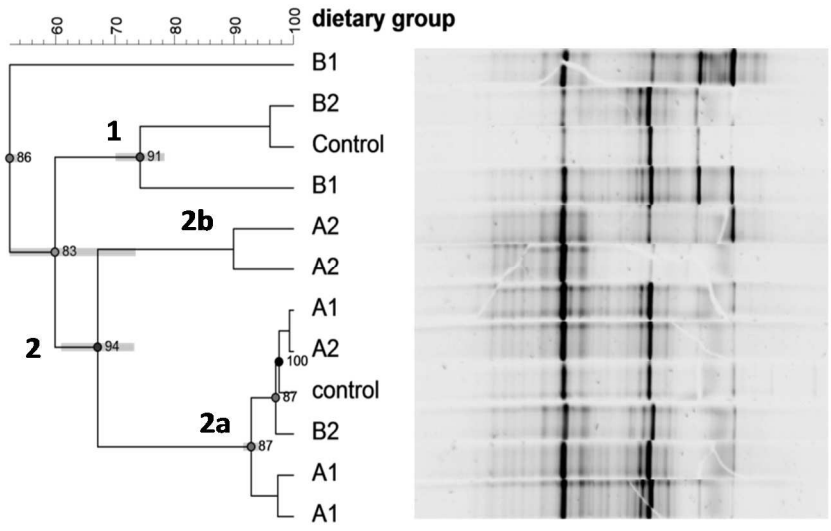

Figure 4-3 Cluster analysis and 16S rDNA PCR-DGGE profiles of hindgut in juvenile rainbow trout fed for 96 days with diets containing probiotics.
Samples from fish fed diet A1 (A1), samples from fish fed diet A2 (A2), samples from fish fed diet B1 (B1), samples from fish fed diet B2 (B2). Main clusters are represented by the bold numerals 1 and 2. The bold numbers 2a and 2b indicate the sub clusters of cluster 2.

To determine whether dietary probiotic supplementation may alter the number of OTUs in the gut, the richness (R) was calculated based on the numbers of OTUs in each DGGE profile. For each sample, the number of OTUs should reflect the number of detectable OTUs (Figure 4-4). In the current study, only samples from fish fed with diet A_1 had an increased number of OTUs compared to the other samples.

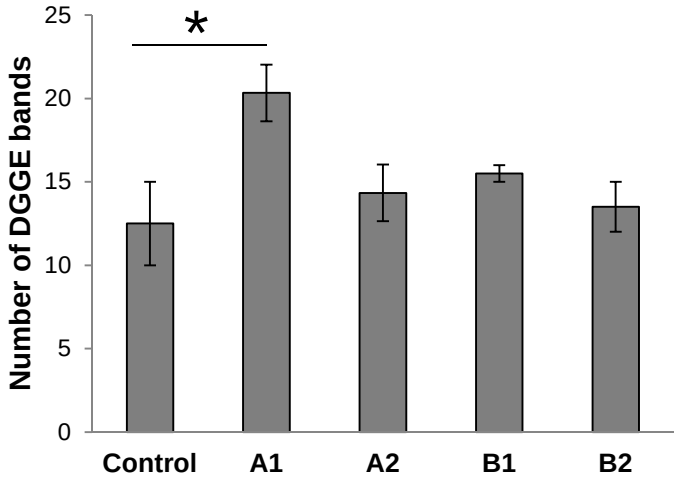

Figure 4-4 Number of OTUs (R) of the intestinal bacterial community in juvenile rainbow trout fed for 96 days with diets containing probiotics.
(* indicates $p < 0.05$ relative to the control). Error bars represent standard deviation.

Alterations of the diversity in the intestinal microbiota was calculated with the Shannon index H´, which is based on the presence of microorganisms and their relative abundance (Figure 4-5). The Shannon index was significantly increased for three groups of fish fed a diet supplemented with probiotics. However, samples from fish fed with diet A_1 showed a higher increase in diversity from 2.8 (control) to 3.7 (A_1) compared to the other dietary groups. Similar to richness (Figure 4-4), these data suggest that the multi-species probiotic at a dose of 1.5 g kg^{-1} significantly affected the microbiota in the hindgut.

The presence of a probiotic in the diet likely affects the gut microbiota of the fish and can help to improve growth performance of fish (Balcázar *et al.*, 2006a; Merrifield *et al.*, 2010c). Here, we evaluated the effects of a multi-species probiotic and a single-species probiotic on growth performance and intestinal microbiota, when these probiotics were added to the diet at two concentrations. The multi-species probiotic, which contains *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp., improved weight gain and specific growth rate in juvenile rainbow trout. Interestingly, not only the composition of the probiotic, but also the concentration of the probiotic was important.

When fish were fed 3 g kg^{-1} multi-species probiotic (diet A_2), changes were less apparent in specific growth rate and diverstiy of the microbiota, when compared to fish fed 1.5 g kg^{-1} multi-species probiotic (diet A_1). Higher dose of probiotic does not necessarily have a better effect than a probiotic administered at a lower dose. Likely, a high-dose of probiotics will be a greater disturbance for the microbiota and interfere with immune response (Li *et al.*, 2012), which leads to loss of energy that would normally be used for growth. However, too low probiotic doses could also be insufficient to promote performances of fish, and that could be the case of B diets, with lower bacterial count than A diets and below the manufactures recommendation.

Even though, A_2 and B_1 diets could increase diversity of the microbiota in the intestine compared to the control (Figure 4-5), these changes were not translated into increased weight gain or specific growth rate. Thus, changes in the diversity or richness of the gut microbiota may not per se reflect improvements in fish performance. It is important to analyse growth performance data and gut microbiota to determine the action of a given probiotic product. Gut microbiota modulation studies should include the determination of dominant species and how they were affected by probiotic administration.

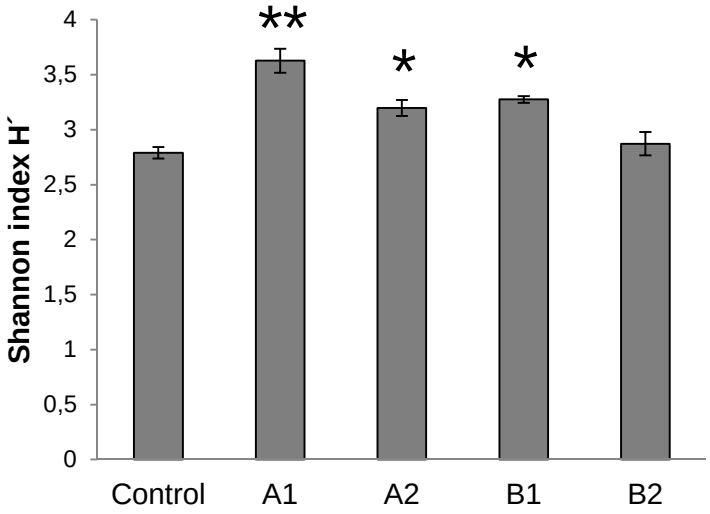

Figure 4-5 Shannon index H´ of the intestinal bacterial community in juvenile rainbow trout fed for 96 days with diets containing probiotics.
(** indicates $p<0.05$ relative to all treatments, including the control; * indicates $p< 0.05$ relative to the control). Error bars represent standard deviation.

The present study shows that probiotic A supplemented at 1.5 g kg^{-1} diet modulated gut microbiota and significantly improved growth performance of juvenile rainbow trout.

Multi-strain or multi-species probiotics might be more effective than single-strain probiotics, because different strains are more likely to survive and find their specific niche in the gut as suggested by Bezkorovainy (2001). The metabolic capacities of strains differ and some strains will enhance growth of other probiotic strains or even beneficial strains in the gut due to production of metabolites, acids. One function of the microbiota is to ferment non-digestible feed components. When probiotics aid the growth of the indigenous microbiota, digestibility of feed is likely increased (Timmerman H.M. *et al.*, 2004). In the event of an infection, the presence of several strains that have different antimicrobial spectra will ensure a better clearance of a pathogen (Lema *et al.*, 2001).

5 *Dietary probiotic supplementation improves growth and the intestinal morphology of Nile tilapia (Oreochromis niloticus)*

Dietary probiotic supplementation improves growth and the intestinal morphology of Nile tilapia (*Oreochromis niloticus*)

Keywords: Probiotics, Nile Tilapia, Growth rate, Innate immune responses, Intestine morphology, Oxidative stress

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5.1 ABSTRACT

Probiotic administration can be a nutritional strategy to improve the immune response and growth performance of fish. The current study aimed to evaluate the effects of a probiotic blend (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.) as a dietary supplement on growth performance, feed utilization, innate immune and oxidative stress responses and intestinal morphology in juvenile Nile tilapia (*Oreochromis niloticus*).

The probiotic was incorporated into a basal diet at three concentrations: 0 (A_0: control), 3 g/kg (A_1: 1.0×10^6 CFU/g) and 6 g/kg (A_2: 2.3×10^6 CFU/g diet). After 8 weeks of probiotic feeding, weight and growth rate (SGR) where significantly higher in fish fed A_1 diet than in fish fed A_0. Alternative complement in plasma was significantly enhanced in fish fed A_2 when compared to A_0. The hepatic antioxidant indicators were not affected by probiotic supplementation. Villi height and goblet cell counts increased significantly in the intestine of fish fed A_1 and A_2 diets compared to A_0.

The dietary supplementation of mixed species probiotic may constitute a valuable nutritional approach towards a sustainable tilapia aquaculture. The improvement of the immune responses and intestinal morphology play an important role in increasing growth performance, nutrient absorption and disease resistance in fish, important outcomes in such a competitive and developing aquaculture sector.

5.2 INTRODUCTION

Tilapias are among the most produced farmed fish in the world constituting the most widespread type of aquaculture in the world (FAO, 2014). Their success lies on their resistance towards diseases, the support of large aquatic conditions, adaptability to several production systems and omnivorous diet, feeding on a low trophic level (El-Sayed, 2006; Fitzsimmons *et al.*, 2011; Watanabe *et al.*, 2002). It is not surprising that the scientific investigation on tilapia species raised significantly in the last years and a good demonstration of their global importance are the international symposiums dedicated exclusively to tilapia aquaculture, currently in is 11^{th} edition.

Although tilapia presents the good supra cited attributes for aquaculture, the intensification of the production can be associated to poorest growth performances (Telli *et al.*, 2014) and to increased risk of diseases, requiring the application of more intense prophylactic methodologies (Watanabe *et al.*, 2002). Current strategies can include diet supplementation

with probiotics or "live organisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). Probiotics act in complex multifactorial ways, depending on species, strain and host (Lazado & Caipang, 2014), but mostly by gut microbiota modulation (Balcázar *et al.*, 2007a; Merrifield & Carnevali, 2014b) and their consequent influence on gut mucosal immunity.

Probiotics can alter the intestinal immunology and morphology towards a more tolerant and less inflammatory mucosa (Lazado & Caipang, 2014; Ringø *et al.*, 2007), ameliorating the epithelial barrier function (Madsen *et al.*, 2001). Probiotics can also provide nutrients (Iehata *et al.*, 2009; LeBlanc *et al.*, 2011), digestive enzymes (Bairagi *et al.*, 2002; Murillo & Villamil, 2011; Ray *et al.*, 2010) and enhance the absorptive surface area through longer *villi* (Gisbert *et al.*, 2013; Pirarat *et al.*, 2011) and longer (Merrifield *et al.*, 2010d) and denser microvilli (Standen *et al.*, 2015). Some experiments report the stimulation of systemic innate defenses as plasma lysozyme, complement or respiratory burst by probiotic microorganisms (Balcázar *et al.*, 2007a; Kim & Austin, 2006b) and their implication in the oxidative stress response (Castex *et al.*, 2009; Ozório *et al.*, 2016). As result, animals become more resistant to infectious diseases (Aly *et al.*, 2008a; Iwashita *et al.*, 2015; Selim & Reda, 2015) and/or present improved feed and growth performances (Pirarat *et al.*, 2011; Ramos *et al.*, 2015; Rodrigáñez *et al.*, 2009). The properties and effects described are not all displayed by a single microorganism, explaining why microbial consortia can be more effective (Verschuere *et al.*, 2000).

Probiotic are very promising in animal production but particularly in aquaculture, given the abundance of pathogen and other antigenic stimulus in aquatic environment (Foey & Picchietti, 2014) and their intimate contact with the animals. The proofs of their interest are accumulating, as reviewed in several specialized papers and books (Bidhan *et al.*, 2014; Dimitroglou *et al.*, 2011; Merrifield & Ringø, 2014; Newaj-Fyzul *et al.*, 2014).

Even so, those putative beneficial microorganisms must be tested at every situation. The probiotic efficacy is host, strain, dosage and timing dependent (Merrifield *et al.*, 2010c). In that way, the present work aimed to study two dosages of a commercial multi-strain probiotic formulation (AquaStar® Growout: *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.) on growth performance, immune and oxidative stress status and intestine histology of juvenile Nile tilapia (*Oreochromis niloticus*).

5.3 MATERIAL AND METHODS

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

5.3.1 Experimental diets

One commercial multi-species probiotic formulation (AquaStar® Growout: *Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp.*;* 1 x 10^{12} CFU kg^{-1}; CFU – colony forming unit) was incorporated at 3 $g.kg^{-1}$ (A_1, 1.0 × 10^6 colony forming unit, CFU, per g of diet) and 6 $g.kg^{-1}$ (A_2, 2.3 × 10^6 CFU per g of diet). A basal diet (composition presented on table 5-1) without probiotic was used as a control diet (A_0).

Table 5-1 Feed ingredients and proximate composition of the experimental diet.

Feed Ingredients (%)		***Proximate composition***	
Fishmeal 65	2.4	Dry matter (%)	93.4
Soybean meal 48[a]	15	Ash (% DM)	9.8
Corn gluten	3.8	Crude protein (% DM)	29.9
Rapeseed meal	19.9	Crude fat (% DM)	7.0
Sunflower meal	7	Crude fibre (% DM)	6.0
Corn meal	8.2	Neutral detergent fibre (% DM)	13.8
Wheat meal	27.2	Gross energy (kJ g^{-1} DM)	19.0
Rice bran	10.2		
Soybean oil	2.4		
Vitamin[b] & Mineral premix[c]	1		
Dicalcium phosphate	1.8		
Soy lecithin	0.5		
Guar gum	0.2		
L-lysine	0.25		
DL-methionine	0.15		

[a] Soybean meal 48: Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL SA

[b] Vitamins (in mg or IU kg^{-1} diet): Vitamin A (retinyl acetate), 20.000 IU; vitamin D3 (DL-cholecalciferol), 2000 IU; vitamin E (Lutavit E50), 100 mg; vitamin K3 (menadione sodium bisulfite), 25 mg; vitamin B1(thiamine hydrochloride), 30 mg; vitamin B2 (riboflavin), 30 mg; calcium pantothenate, 100 mg; nicotinic acid, 200 mg; vitamin B6 (pyridoxine hydrochloride), 20 mg; vitamin B9 (folic acid), 15 mg; vitamin B12 (cyanocobalamin), 100 mg; vitamin H (biotin), 3000 mg; vitamin C (Lutavit C35), 1000 mg; inositol, 500 mg; colin chloride, 1000 mg; betaine (Betafin S1), 500 mg;

[c] Minerals (mg or % kg^{-1} diet): Co (cobalt carbonate), 0.65 mg; Cu (cupric sulphate), 9 mg; Fe (iron sulphate), 6 mg; I (potassium iodide), 0.5 mg; Mn (manganese oxyde), 9.6 mg; Se (sodium selenite), 0.01 mg; Zn (zinc sulphate) 7.5 mg; Ca (calcium carbonate), 18.6%; KCl, 2.41%; NaCl, 4.0 %

The defined quantity of lyophilized probiotic preparation was dissolved in tap water (100ml kg^{-1} of diet) for one hour and coated the basal extruded diet, by slowly mixing in a kitchen mixer (Kenwood). The diets were prepared, one kg at the time, started with the control diet (A_0) without probiotic addition, then A_1 and A_2 at the end. After homogenization, diets were dried in a ventilation oven (48h at 35ºC) and stored at 4ºC until use. During growth trial, fresh diets were prepared every month to prevent the decrease of the probiotic viability.

The procedure of probiotic incorporation for the complementary digestibility trial was similar to the formerly described but, previous to probiotic incorporation, the basal diet was milled and 1% of chromic oxide (Cr_2O_3) was added. The homogenized mixture was pelleted (2.4 mm) using a laboratory pellet press (CPM, C-300 model, San Francisco, USA).

5.3.2 **Animals and experimental conditions**

Nine homogeneous groups of twenty male tilapia (initial body weight, IBW: 12.82 ± 0.02 g) were stocked at an initial density of 2.6 kg m^3 in square glass tanks (100 L), reared in a closed recirculating freshwater system at 24.5 ± 1.1°C and photoperiod 12:12 h (light: dark), at UTAD experimental research station. After the acclimation period of 15 days, each experimental diet was randomly attributed to triplicate tanks. Diets were fed for 8 weeks by hand three times a day (9:00, 12:30, and 18:00 h), six days/week, until apparent visual satiety.

The digestibility trial was performed after five months of feeding with the experimental diets (15 fish/treatment; 98.94 ± 25.81 g of BW) in duplicate cylindrical fiberglass tanks (75 L) adapted with faeces collection system (Choubert *et al.*, 1982). The water temperature and photoperiod were the same as the growth trial. Faeces were collected for seven days, after a two weeks period adaptation to diets with chromic oxide. At the end of the digestibility trial faeces were freeze-dried for chemical analyses.

5.3.3 **Sampling procedures**

The growth trial lasted for eight weeks during which fish weighting and tissue collection were done at two, four and eight weeks of feeding.

Prior to samplings, fish were fasted for 24 h and then sacrificed with an overdose of ethylene glycol monophenyl ether (MS 222; 250 mg L^{-1}). At each sampling time, two fish per tank were sacrificed and blood collected from the caudal vein, using heparinised syringes.

Plasma was obtained after centrifugation (3000 × *g* for 10 min at 4ºC) and maintained at -80ºC for further analysis. Liver was collected and stored at -80ºC for the analyses of the oxidative stress indicators. Head-kidney was aseptically collected and immediately processed as will be described hereinafter. Additional two fish per tank were sacrificed to collect blood for haematocrit and cell counting, and plasma. In addition, intestine were sampled and fixed in 4% formalin buffered solution for histological assessment.

Fish were individually weighed at the end of the trial for the calculation of growth performance parameters. Ten fish from the initial stock and three fish from each tank at the end of trial (nine fish per treatment) were sampled and stored at -20 ºC for body composition analyses.

At the end of the digestibility trial, eight animals per treatment were sacrificed for plasma analyses and intestine for digestive enzymes determination.

5.3.4 **Probiotic detection in diets**

A probiotic count of the diets was made at the beginning of the growth trial. Diets were homogenized with phosphate buffered saline (PBS), then serial dilutions were spread in MRS (de Man, Rogosa, Sharpe), TSA (Tryptone Soy Agar) and BEAA-TSA (Bile Aesculin Azide Agar - TSA) medium plates, respectively for *Pediococcus* and *Lactobacillus*, *Bacillus* and *Enterococcus* and incubated at 37ºC for 24 to 48h. Samples of the first diets were maintained at 4ºC, and new counts were made after two weeks of storage and after a year of storage (table 5-2).

Table 5-2 Probiotic counting of experimental diets ($CFU.g^{-1}$)

Probiotic of diets	**CFU.g^{-1}**	**Storage time (4ºC)**	
	Initial	**2 weeks**	**1 year**
A0	50×10	4×10	-
A1	1.0×10^6	8.8×10^5	2.0×10^5
A2	2.3×10^6	1.8×10^6	4.5×10^5

5.3.5 **Chemical analysis of diets, faeces and body composition**

Fish from the same tank were pooled and homogenized. Dry matter content was determined in fresh samples of diets, faeces and carcass, whereas the proximate composition

analyses was carried out in freeze-dried material. The proximate composition analysis of diets, faeces and carcass was made according the reference procedures (AOAC, 2006).

Dry matter by drying at 105ºC for 24ºC (Memmert UL40, Schwabach Germany); total ash by combustion at 550ºC for 6h in a muffle furnace (Thermolyne F6010, Dubuque, USA); crude protein, by multiplying nitrogen content by 6,25 (LECO nitrogen analyser, FP-528 model, St. Joseph, Michigan); crude lipid by petroleum ether extraction (Soxhlet behr extractor, R 106S, Düsseldorf, Germany); crude fibre after digestion with sulfuric acid and sodium hydroxide (Velp FIWE3); neutral detergent fibre using standard procedures (Van Soest *et al.*, 1991); gross energy by complete combustion in an adiabatic bomb calorimeter (Werke C2000, IKA, Staufen, Germany) and chromic oxide according to Bolin *et al*. (1952) by perchloric acid digestion.

5.3.6 **Growth performance and somatic indices**

Specific growth rate (SGR; % body weight gain.day^{-1}) was calculated as *[(ln Final body weight) - (ln Initial body weight)/days] ×100.* Feed conversion ratio (FCR) was determined as *dry feed intake / weight gain*. Voluntary feed intake (VFI) was calculated as (*dry feed intake / average body weight / days)× 100,* where average body weight (ABW) is *[final body weight (*FBW*) + initial body weight (*IBW*)]/2.* Protein efficiency ratio (PER) was determined as *weight gain/protein intake.*

Condition factor or K = *[weight (g) ×100]/lenght^3 (cm),* Hepatosomatic index (HSI, %) = *100 ×(liver weight (g) / body weight (g)),* Intestine somatic index (ISI, %) = *100 ×(intestine weight (g) / body weight (g)),* Spleen somatic index (SSI, %) = *100 ×(spleen weight (g) / body weight (g)).*

Apparent digestibility coefficients (ADC) were calculated with the follow formulae:

$$\mathrm{ADC}_{nutrient\ or\ energy}\ (\%) = 100\ \times \left(1 - \frac{(\%)\mathrm{marker}_{\mathrm{diet}}}{(\%)\ \mathrm{marker}_{\mathrm{faeces}}} \times \frac{(\%)\ \mathrm{nutrient\ \ or\ energy}_{\ \mathrm{faeces}}}{(\%)\ \mathrm{nutrient\ or\ energy}_{\ \mathrm{diet}}}\right)$$

ADC of dry matter (DM) were calculated as: $\mathrm{ADC}_{DM}\ (\%) = 100\ \times \left(1 - \frac{(\%)\ \mathrm{marker}_{\mathrm{diet}}}{(\%)\ \mathrm{marker}_{\mathrm{faeces}}}\right)$.

5.3.7 **Health and innate immune parameters**

5.3.7.1 Haematological parameters

Immediately after blood samplings, aliquots of blood were introduced into capillary tubes, centrifuged (microhaematocrit centrifuge, 10 000 g, 5 min, room temperature) and the haematocrit values were measured and read as % packed cell volume (%PCV).

Another aliquot was diluted in Natt Herricks stain solution for total erythrocytes and leucocytes counts using a haemocytometer.

The proceedings were done in duplicate per fish.

5.3.7.2 Plasma alternative complement pathway (ACH50), lysozyme and peroxidase activities

Alternative haemolytic complement pathway activity (ACH50) was determined following the method described by Sunyer and Tort (1995), using rabbit red blood cells (RaRBC) as target cells to haemolysis. The reciprocal of the serum dilution causing 50% lysis of RaRBC was designated as ACH50 (Tort *et al.*, 1996). Results are presented as ACH50 units mL^{-1}.

Lysozyme activity was determined in plasma, measuring the decrease of the turbidity of a *Micrococcus lysodeikticus* suspension at 450 nm, as described by Ellis (1990) and Hutchinson and Manning (1996). Lysozyme concentration (μg mL^{-1}) was estimated using lyophilized hen egg-white lysozyme (Sigma®) serially diluted in sodium phosphate buffer (0.05 M; pH 6.2) to establish a standard curve measured after 0.5 and 5 min.

Total peroxidase activity in plasma was measured as described by Quade and Roth (1997) technique. The peroxidase activity (U mL^{-1} plasma) was determined assuming that one unit of peroxidase produces an absorbance change of 1 OD. All analyses were conducted in triplicates.

5.3.7.3 Respiratory burst activity and nitric oxide (NO) production of head-kidney (HK) leucocytes

The isolation and culture of HK leucocytes were done aseptically according to Secombes (1990). Briefly, the HK was collected aseptically, macerated with Leibovitz L-15 medium (L-15; Gibco®) and cells isolated trough a Percoll (Sigma®) gradient and centrifugation.

Microplates received 100 µL of $2x10^7$ viable cells.mL^{-1}, incubate overnight at 25ºC and the non-adherent cells were washed off and maintained with L-15 , containing 5% FBS to conduct respiratory burst and NO assays, after 24h and 72h of incubation at 25ºC, respectively.

Respiratory burst activity of HK leucocytes was based on the reduction of ferricytochrome C method for the detection of extracelular O_2^- production as described by Secombes (1990). Optical densities (OD) read at 550 nm (BioTek, Winooski, USA) were converted to nmol of O_2^- produced by multiplying by 15.87 as described by Pick (1986). Nitric oxide (NO) production by HK leucocytes was assessed as described by Neumann *et al*. (1995) using the Griess reaction (Green *et al.*, 1982) to quantify nitrite content of the leucocytes supernatant.

5.3.8 **Liver oxidative stress parameters**

Liver was diluted and homogenized with ultra-pure water (1:10) and an ultrasonic processor (less than 20 seconds per sample) on ice. Part of the liver homogenate was used to evaluate lipid peroxidation (LPO) measuring the presence of thiobarbituric acid reactive substances (TBARS) as described by Gravato *et al*. (2006) according to (Ohkawa *et al.*, 1979) and (Bird & Draper, 1984). To prevent lipid damage that portion was stored with 2% butylated hydroxytoluene (BHT; 4% in methanol). The rate of LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per gram of fresh tissue, using a molar extinction coefficient of $1.55 \times 10^5\ M^{-1}\ cm^{-1}$.

The remaining liver homogenate was centrifuged (10 000g, 20 min, 4ºC) to isolate de post-mithocondrial supernatant where catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) enzymes and total glutathione content (TG, GSSG+GSH), in its reduced (GSH) or oxidized (GSSG; glutathione disulfide) forms were determined.

CAT activity was determined by measuring the H_2O_2 consumption at 240 nm as described by Clairborne (1985). GPx activity was quantified by measuring the decrease of NADPH at 340 nm using H_2O_2 as substrate (Flohé and Gunzler 1984). GR activity was assayed according to Cribb et al. (1989), adapted to microplate measuring the decrease of NADPH levels at 340 nm. All enzymatic activities were determined at 25ºC and expressed as nmol min^{-1} mg $protein^{-1}$, except catalase that was expressed as µmol min^{-1} mg $protein^{-1}$.

TG (GSSG+GSH) and GSSG were quantified at 412 nm, using the recycling reaction of reduced glutathione (GSH) with 5,5′-dithiobis-(2-nitrobenzoic acid), DTNB, in the presence of

GR excess (Baker *et al.*, 1990). GSSG was quantified, after conjugation of GSH with 2-Vinyl-pyridine (Griffith, 1980). The GSH level was the result of subtracting GSSG from the TG level; then the GSH/GSSH ratio was calculated.

Protein content of both liver fractions was determined by Bradford (1976) method (Bio-Rad protein assay) using bovine serum albumin (BSA) or γ-Globulin as standard.

5.3.9 **Intestine histological examination**

After two, four and eight weeks of experimental feeding, intestinal samples from six fish per diet were collected for histological examination by light microscopy. From each animal, three samples, with approximately 2 cm were taken from the proximal, medial and distal intestine and fixed in 4 % buffered formalin solution. Fixed tissues were processed according to standard histological techniques. Sections of 3 micrometre were cut and stained for histological purposes with haematoxylin and eosin (H&E) and periodic acid-Schiff reagent (PAS). Histological examinations were made using light microscopy (Olympus BX51, cell^B software).

From each intestine section, two segments were analysed, measuring the following parameters: perimeter and area; *tunica muscularis* thickness, mucosal fold (or "villi") height and goblet cell presence.

The *tunica muscularis* thickness was measured in eight points of each analysed transverse section and an average was computed. The mucosal folds length or height was measured at ten highest folds. The measures were done from the fold tip to the bottom, following the curves of the fold. Goblet cells stained with PAS were counted on the previous selected highest *villi*, then the average number of goblet cell per *villus* was determined.

5.3.10 **Digestive enzymes**

Intestine samples were diluted and homogenized (1:5 w/v; 50 mM Tris-HCl buffer containing 200 mM NaCl, pH 8), on ice using a micro-homogenizer. The homogenates were centrifuged (13500 g for 30 min at 4ºC) and the supernatant kept frozen at –80ºC until analysis (Rungruangsak-Torrissen & Fosseidengen, 2007).

Amylase activity was carried according to Areekijseree *et al*. (2004) based on Bernfeld (1951), measuring the increment of maltose, using 5 % soluble starch dissolved in phosphate buffer (pH 7.0) as substrate, and comparing to a maltose standard (0 to 50 µmol).

Lipase specific activity was determined according to Winkler and Stuckmann (1979) and one enzyme unit was defined as the amount of enzyme able to produce 1 µmol of p-nitrophenol per minute. Briefly, 10 µl of enzyme gut extract were added to 200 µl of 0.01M p-nitrophenyl palmitate in 800 µl sodium phosphate buffer pH and incubated for 15 min at 30ºC. Incubation was stopped with 250 µl of 1 M Na_2CO_3 followed by a centrifugation at 15000g for 20 min at 4ºC. The absorbance of the supernatant was measured at 410 nm.

Trypsin and chymotrypsin activities were assayed following Rungruangsak-Torrissen *et al*., (2006) by incubating 100 µl of crude enzyme extract with 700 µl of trypsin substrate (1.25 mM benzoyl- L -arginine-p-nitroanilide for trypsin and 0.1 mM succinyl Ala-Ala-Ala-Pro-Phe-p-nitroanilide for chymotrypsin) at 25 ºC for 10 min. The reaction was stopped by the addition of acetic acid (800 µl; 30%); the nitroaniline produced was measured at 410 nm and compared with a nitroaniline standard curve.

The specific activity of the enzymes, as the activity of an enzyme per milligram of total protein (expressed in µmol min^{-1} mg^{-1}) was calculated dividing the sample enzyme activity by their protein content; protein content was determined by Lowry *et al*. (1951) methodology.

5.3.11 **Statistical analysis**

Statistical analyses were carried out using STATISTICA software (StatSoft, Inc., 2008, version 8). Data were submitted to Kolmogorov-Smirnov and Levene tests, to verify normal data distribution and homogeneity of variances, respectively. Data were analysed by one-way or two-way ANOVA. Post-hoc Dunnett or Tukey test was used when significant differences between means were detected. Non-parametric test (Kruskal-Wallis test) was used in non-normally distributed data. Significance differences were considered when $P < 0.05$. Results are presented as mean ± standard deviation (SD).

5.4 RESULTS

5.4.1 Growth performance and carcass composition

The growth, feed intake and feed conversion performances after the eight weeks of trial are displayed in Table 5-3.

Table 5-3 Growth performance of Nile tilapia fed the experimental diets for 8 weeks

Diets	Control	Probiotics		*Anova p-value*
	A_0	A_1	A_2	
IBW (g)	12.83 ± 0.03	12.82 ± 0.01	12.83 ± 0.02	*0.708*
FBW (g)	33.01 ± 1.35	39.10 ± 4.20 *	32.99 ± 0.38	*0.041*
Weight gain (g)	20.18 ± 1.34	26.28 ± 4.19 *	20.16 ± 0.36	*0.040*
length (cm)	13.07 ± 0.92	13.41 ± 0.82	13.36 ± 0.80	*0.430*
k	1.76 ± 0.17	1.72 ± 0.13	1.69 ± 0.12	*0.373*
SGR	1.69 ± 0.07	1.98 ± 0.19 *	1.69 ± 0.02	*0.033*
FCR	1.55 ± 0.06	1.41 ± 0.13	1.60 ± 0.07	*0.096*
PER	2.17 ± 0.08	2.39 ± 0.22	2.10 ± 0.09	*0.119*
VFI	2.43 ± 0.08	2.52 ± 0.08	2.51 ± 0.13	*0.528*
Mortality (%)	8.33 ± 7.64	6.67 ± 2.89	3.33 ± 2.89	*0.501*

Means ± SD (n=3; except length and k, n=12). In each row * indicates significant differences regarding control diet (Anova and Dunnett test p-value <0.05).

After two weeks of feeding trial, the voluntary feed intake (VFI) of A_1 group was higher (A_0 2.64 ± 0.04; A_1 3.07± 0.14; A_2 2.86 ± 0.14; P=0.01) regarding the control group and no other parameter differed between treatments. After four weeks none of the followed parameters were significantly different. However, after eight weeks of probiotic supplementation, the animals receiving A_1 diet presented an increased ($P<0.05$) weight and specific growth rate (SGR). The length of A_1 animals was higher but not significantly ($P>0.05$) and, therefore, the condition factor was similar between the all the experimental groups. Although the FCR and PER of A_1 group were numerically improved, towards the control group values, the difference had no statistical significance ($P>0.05$).

The carcass proximate composition showed no significant differences ($P>0.05$), as liver, spleen or intestine somatic indices (Table 5-4).

Table 5-4 Organosomatic indices and carcass proximate composition after 8 weeks of probiotic administration

	Control	Probiotics	
Diets	A_0	A_1	A_2
HSI	1.77 ± 0.45	1.92 ± 0.58	1.99 ± 0.65
SSI	0.36 ± 0.20	0.32 ± 0.16	0.40 ± 0.22
ISI	4.46 ± 0.74	4.51 ± 0.98	4.16 ± 1.05
Composition (% DM)			
Dry Matter	27.2 ± 0.9	24.0 ± 2.2	26.1 ± 0.8
Ash	12.9 ± 0.6	13.8 ± 0.5	13.1 ± 1.9
Organic matter	87.1 ± 0.6	86.2 ± 0.5	86.9 ± 1.9
Crude Protein	58.4 ± 1.0	58.7 ± 1.3	58.5 ± 1.3
Crude Lipid	28.1 ± 1.6	26.7 ± 2.9	27.7 ± 3.2

Means ± SD (n=12 for organ indices and n=3 for carcass composition). In each row absence of distinct letters or symbols indicate no significant difference between means (Anova p-value > 0.05).

5.4.2 **Haematological and innate immune status**

Haematological results (table 5-5) were not affected by dietary treatment. No significant impact of probiotic feeding was detected in haematocrit, leucocytes or erythrocytes count over the feeding period (P>0.05).

Table 5-5 Haematological parameters of Nile tilapia after 8 weeks of probiotic administration

	Control	Probiotics		*Anova*
Diets	A_0	A_1	A_2	*p-value*
Haematocrit (%)	22.74 ± 2.90	23.74 ± 3.18	24.55 ± 0.76	*0.402*
Erythrocytes (x 10^9 mL^{-1})	1.05 ± 0.77	1.57 ± 0.60	1.22 ± 0.70	*0.358*
Leucocytes (x 10^7 mL^{-1})	2.98 ± 1.63	3.55 ± 1.22	1.87 ± 1.07	*0.084*

Means ± SD (n=6). In each row absence of different superscripts letters or symbols indicate no significant difference between means (Anova p-value > 0.05).

Data from the innate immune parameters are presented in Figure 5-1. Respiratory burst activity and nitric oxide production of HK leucocytes were not significantly (P>0.05) affected by probiotic treatment at any sampling moment. Plasma lysozyme and peroxidase presented the same trend. After eight weeks of trial, alternative complement activity was significantly higher (P< 0.05) in fish fed the A_2 diet (38.3 ± 15.3 U ml^{-1}) when compared to fish fed A_0 diet (24.7 ± 9.7 U ml^{-1}); the remaining values did not differ from control ones.

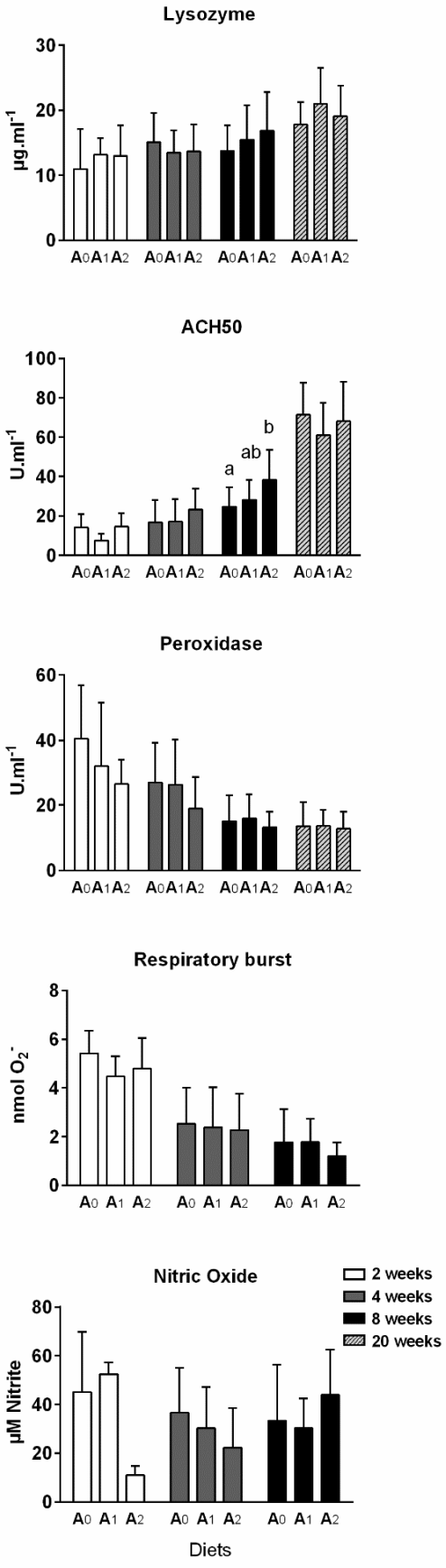

Figure 5-1 Lysozyme, alternative complement (ACH50) and peroxidase activities in plasma and respiratory burst and nitric oxide production by head-kidney (HK) leucocytes measured in Nile tilapia specimens receiving control (A0) or probiotic supplemented diets (A1 and A2) for 2, 4, 8 and 20 weeks. Results are expressed as means ± SD (n=12, except HK parameters where n=6, and 20 weeks where n= 16). At each sampling time, values with different superscripts letters indicate significant differences between treatments (one way ANOVA, Tukey test, $p < 0.05$).

5.4.3 **Oxidative stress indicators**

Along the growth trial, the lipid peroxidation level and some enzymatic and non-enzymatic antioxidant defences were followed at the liver (Figure 5-2). A large variation was observed among individuals fed the same diets, in the majority of the parameters analysed.

The lipid peroxidation increased significantly over time ($P<0.05$), since global values at eight weeks were higher than at four weeks. Although probiotic impact had no statistical significance, at the end of the trial, the peroxidation increased proportionally to the probiotic dosage.

The antioxidant enzymes studied were not significantly ($P>0.05$) affected by probiotic supplementation at any sampling period, but changed significantly over time: GPx decreased, while CAT increased and then decreased globally. GR presented no specific tendency over the trial and was very variable among specimens.

GSH levels after four weeks of administration were significantly increased and very variable in control group (A_0) comparatively to A_1 values. After two or eight weeks of supplementation, glutathione levels were not different between experimental treatments. GSH/GSSG ratio, although highly variable, did not differ between diets or with time (P>0.05).

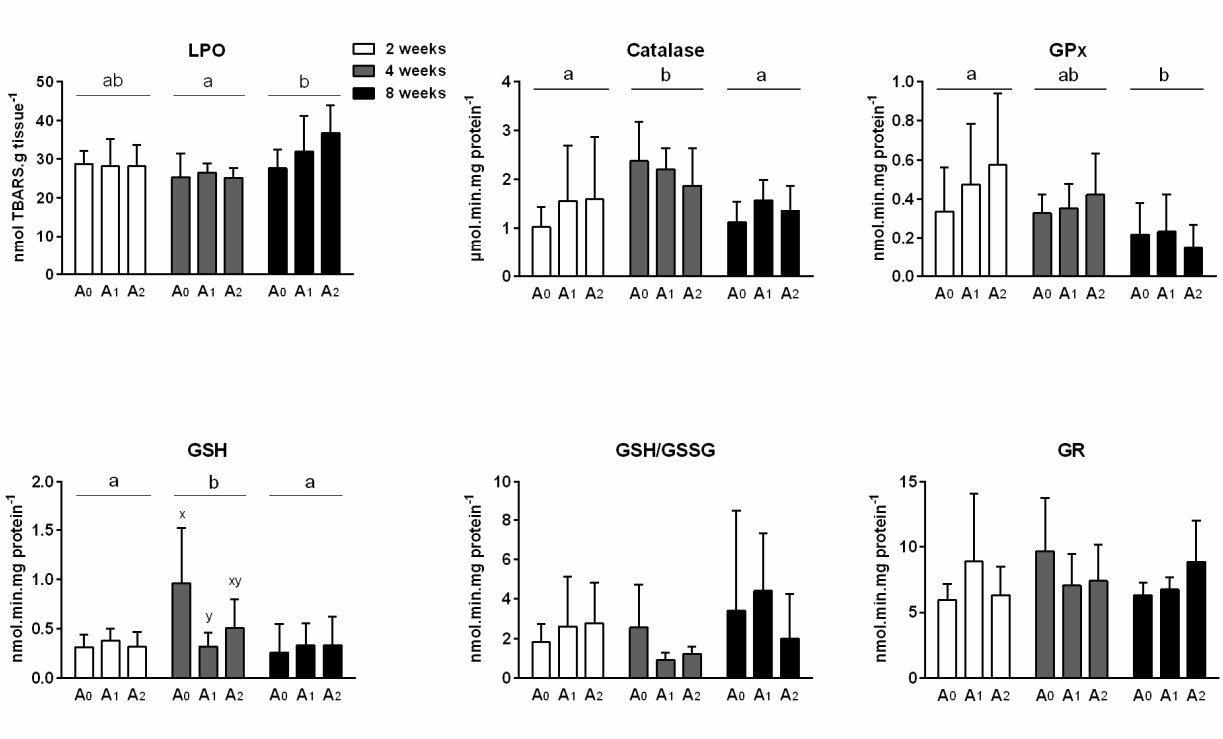

Figure 5-2 Liver oxidative stress indicators of Nile tilapia along the growth trial.
LPO lipid peroxidation, Catalase, GPx glutathione peroxidase and GR glutathione reductase activities; Glutathione (GSH) and the ration between reduced and oxidized glutathione levels (GSH/GSSG).
Results are expressed as means ± SD (n=6) measured in Nile tilapia specimens receiving control (A0) or probiotic supplemented diets (A1 and A2) for 2, 4 and 8 weeks. At each sampling time, values with different letters (x, y) indicate significant differences between treatments (one way ANOVA, Tukey test, $p < 0.05$). Distinct letters (a, b) between sampling times, indicate overall significant differences regardless of the diets.

5.4.4 **Intestinal histomorphology**

Table 5-6 displays the data regarding intestine morphometric evaluation at the end of the growth trial. The area of proximal (I), medial (II) or distal intestine was not affected by diet treatment at any sampling moment (P>0.05) and decreased significantly along the gut as expected in tilapia. The *tunica muscularis* did not differ significantly among experimental groups or intestine sections (P>0.05) at any sampling time.

The *villi* height was not affected by probiotic treatment after two or four weeks of supplementation. However after eight weeks of feeding, a significant difference (P<0.05) was

observed among control (A_0) or probiotic diets (A_1 and A_2), being the control globally shorter than probiotics, and markedly distinct at proximal intestine.

The presence of positive PAS goblet cells was similar among experimental groups after two and four weeks of trial. Yet, after eight weeks of probiotic feeding, the presence of goblet cells were significantly higher on fish supplemented with both probiotic diets independently of intestine section, comparatively to the control. Regarding the distribution along the gut, the goblet cells presence decreased in number.

Table 5-6 Intestinal histological parameters after 8 weeks of feeding the experimental diets, by gut part (I-proximal, I-medial, III-distal)

	Control	Probiotics		*Diet (D)*	*Gut section (G)*	*D×G*
	A_0	**A_1**	**A_2**			
area (mm^2)				*ns*	***	*ns*
I	3,1 ± 0,9	3,2 ± 1,1	4,1 ± 1,0		*a*	
II	1,6 ± 0,6	1,3 ± 0,5	1,4 ± 0,4		*b*	
III	1,3 ± 1,0	2,5 ± 1,9	1,0 ± 0,3		*b*	
tunica muscularis thickness (µm)				*ns*	*ns*	*ns*
I	57,1 ± 27,4	50,4 ± 15,5	42,5 ± 20,0			
II	55,1 ± 14,7	52,9 ± 6,8	49,6 ± 32,4			
III	46,3 ± 13,7	32,2 ± 4,3	49,5 ± 13,7			
Villi height (µm)	¥	§	§	**	***	*
I	389,2 ± 88,6 ^{yz}	644,2 ± 177,3 ^{x}	636,5 ± 215,9 ^{x}		*a*	
II	373,1 ± 74,2 ^{yz}	437,9 ± 67,1 ^{xz}	369,4 ± 43,9 ^{y}		*b*	
III	202,0 ± 85,0 ^{y}	220,9 ± 43,4 ^{y}	292,0 ± 31,7 ^{y}		*c*	
Goblet cell/*villus*	¥	§	§	**	**	*ns*
I	17.2 ± 8.5	41.6 ± 9.4	40.5 ± 25.5		*a*	
II	18.7 ± 16.2	39.0 ± 11.9	26.3 ± 13.1		*ab*	
III	10.0 ± 8.3	20.8 ± 12.1	19.5 ± 8.3		*b*	

Results are expressed as means ± SD (n=6). ns - no statistical significance (ANOVA p-value > 0.05); ANOVA p-value * < 0.05; ** <0.01; ***<0.001. Different symbols (¥, §) indicate significant differences between diets, independently of the gut section; Different letters (a, b, c) indicate significant differences between gut sections, independently of the diet; Different superscript letters (x, y, z) indicate significant differences between diets and gut section.

5.4.5 **Apparent digestibility coefficients (ADC) and digestive enzymes**

The ADC of nutrients and energy of experimental diets after twenty weeks of experimental feeding were not significantly different (Figure 5-3, bottom), even if the coefficients presented by A_1 diets were numerically higher towards the others. At that time, the specific activity of amylase, lipase, trypsin and chymotrypsin (Figure 5-3, top) were measured in the intestine, and no significant difference was observed between the specimens fed probiotic or control diets.

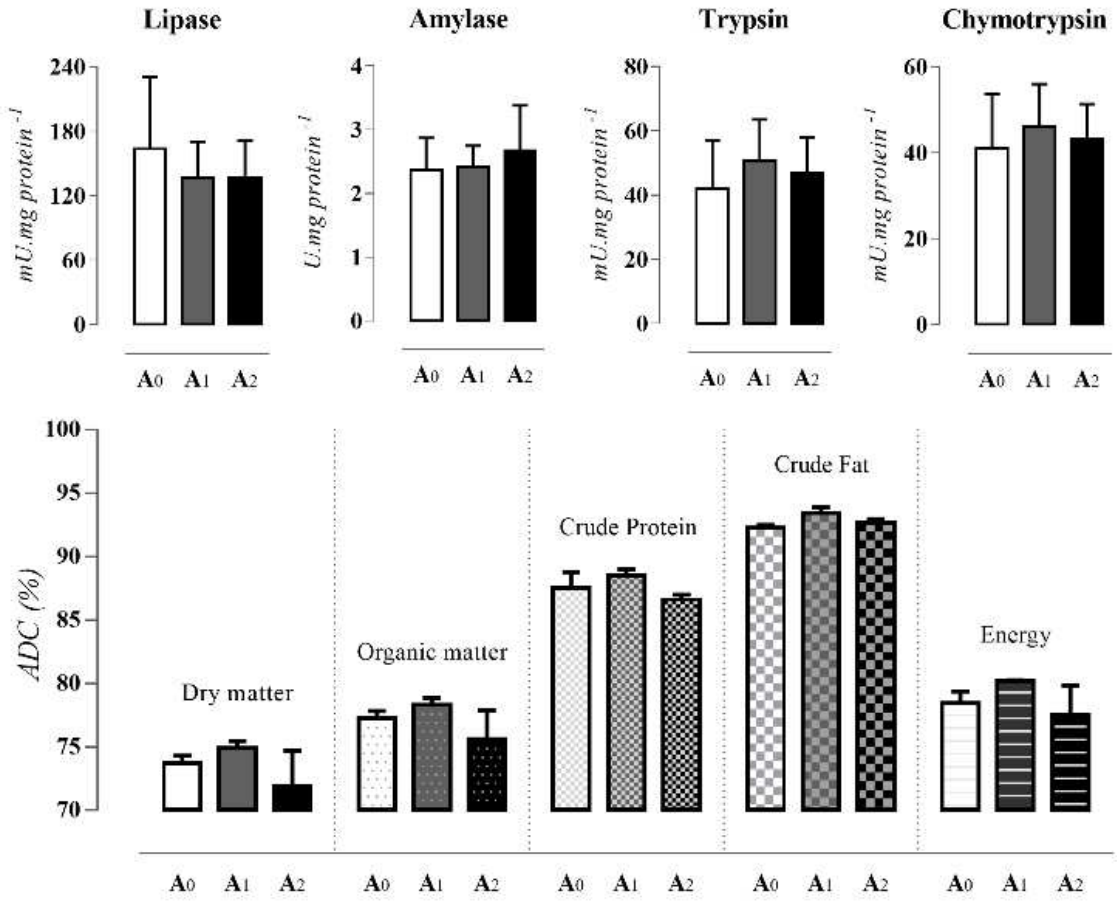

Figure 5-3 Top: Digestive enzymes specific activity after 5 months/20 weeks of probiotic supplementation. Values are means ± SD (n=8). 1 U = amount of enzyme that catalyses the formation of 1µmol of product per minute at specific conditions (by the respective order 35, 30, 25 and 25ºC and 7.4, 9, 7.5 and 7.5 pH). Bottom: ADC of nutrients and energy of experimental diets after 5 months of probiotic supplementation. Values are means ± SD (n=2). No significant differences were observed among the diet treatments in any of the parameters (Anova p-value > 0.05).

5.5 DISCUSSION

The improvement of growth performance after probiotic feeding has been observed previously in tilapias receiving several probiont species (Aly *et al.*, 2008a; Lara-Flores *et al.*, 2003; Pirarat *et al.*, 2011; Standen *et al.*, 2016). The current study is in agreement with Standen *et al*. (2016) supporting the use of the tested commercial product as growth promoter, when supplemented at $3g.kg^{-1}$ (A_1). Higher dosages may enhance changes in the microbiota and/or immune responses (Li *et al.*, 2012), consequently increasing the energy expenditure. Other recent studies on bacterial consortia in rainbow trout juveniles observed an improvement on

growth rate and FCR at 1.5 $g.kg^{-1}$ dietary probiotic supplementation, but not at 3 $g.kg^{-1}$ (Ramos *et al.*, 2015) and at 1$g.kg^{-1}$ but not at 5 $g.kg^{-1}$ (Giannenas *et al.*, 2015).

Similarly to previous findings (Standen *et al.*, 2016), we have not noticed influence on feed conversion ratio (FCR). Several management aspects may explain the lack of effects on FCR, such as lower water temperature and dietary protein level, the feeding frequency and the incorporation method of the probiotic. However, different hosts and rearing conditions, with their particular water and gut microbiota (Giatsis *et al.*, 2015; Nieto *et al.*, 1984), may be sufficient for some unpredictability of the results. In a previous study in rainbow trout, the authors observed a decrease in voluntary feed intake in fish fed probiotics when compared to the control group (Ramos *et al.*, 2015). Some reports indicated the possibility of the enhancement of appetite by probiotics (Robertson *et al.*, 2000), especially at the beginning of the feeding trial as reported by Irianto and Austin (2002) and in the current study. Those studies used coating as the incorporation method, which may explain the findings. Nevertheless, current trial applied water as suspension carrier, whereas Irianto and Austin (2002) used saline solution and Robertson *et al*. (2000) used fish oil. The possible influence of the incorporation method of probiotic on feed intake may deserve further attention.

Lara-Flores *et al*. (2003) observed an influence of probiotics on carcass composition in fingerlings. However, as the fish grow, the effect on body composition seems to attenuate (Ramos *et al.*, 2015; Standen *et al.*, 2016; Telli *et al.*, 2014) as in the current study.

Probiotics administered as dietary supplement, pass across the gut and are able to have an impact on the microbiota, even without colonization of the intestine (Merrifield & Carnevali, 2014a). The microbiota influences the mucosal and systemic immune system of fish (Lazado & Caipang, 2014). Recent study with the commercial probiotic used in the current study reported changes in intestine microbiota in tilapia, as well as up-regulation of intestine genes involved in immune responses (Standen *et al.*, 2016). In the current study, the alternative complement pathway was significantly higher in fish fed diets supplemented with 2.3×10^6 CFU/g diet (A_2). Alternative complement is a non-specific mechanism of protection against putative pathogens as bacteria, fungi, viruses or parasites (Sunyer & Tort, 1995). Previous studies on rainbow trout using the same probiotic blend reported an increase on the alternative (Ozório *et al.*, 2016) or total (Giannenas *et al.*, 2015) complement and no significant effect on lysozyme activity. Conversely Ramos *et al*. (2015) observed the significant enhancement of lysozyme while higher but not significantly alternative complement activity.

The immune system is closely linked to antioxidant system (Castex *et al.*, 2010). Recent findings of antioxidant activities of some lactic acid bacteria strains brings to attention the potential role of probiotic treatments on reactive oxygen species (ROS) clearance (Amaretti *et al.*, 2013; Lin & Yen, 1999). Oxidative stress results from an imbalance between oxidative processes and antioxidant mechanisms of defence, causing damage to cellular lipids, protein and DNA (Lushchak, 2011). Liver is highly adaptive to oxidative damage and main organ used for the studies on the effects of pollutants in fish (Ahmad *et al.*, 2004). Conversely to the findings of Ozório *et al*. (2016) in rainbow trout, the hepatic lipid peroxidation was not reduced in the present study. Similarly, no probiotic influence was observed on the antioxidant enzymes GR, CAT and GPx. After four weeks of feeding, fish fed control diet showed higher GSH levels when compared with fish fed probiotic diets, especially in fish fed A_1 diet. The reduction of GSH levels can hamper the ROS clearance, causing damage to cellular components (Lushchak, 2011). Several lactic acid bacteria were able to induce the biosynthesis of glutathione in rat ileal mucosa or pancreas (Lutgendorff *et al.*, 2009). Clear positive effects of probiotics have been often observed on immune or oxidative stress status when fish are subjected to stress situations. Telli *et al*. (2014) reported no differences regarding haematological parameters and plasma lysozyme levels between tilapia fed *B. subtilis* or the control diet, when stocked at lower densities. However, after stocking the animals at higher densities, the effect of probiotic administration was noticed, since probiotic treated fish showed increased lysozyme level, phagocytic activity and a decrease in the number of erythrocytes and in the haematocrit level, but increased levels of mean corpuscular haemoglobin. Inasmuch, Castex *et a*l. (2009) observed a lower oxidative stress level in the digestive gland of shrimps receiving *Pediococcus acidilactici*, after a *Vibrio nigripulchritudo* challenge. Previous to that infection, the positive impact of the probiotic was much less noted. Moreover, Reyes-Becerril *et al*. (2011) observed similar findings in leopard grouper fed yeast after *Aeromonas hydrophila* infection.

The evidences of probiotics implication in the maintenance of intestinal barrier homeostasis are accumulating in humans and animals (Madsen *et al.*, 2001). In fish, recent findings reported their role on intestinal morphology modulation, feature that may contribute to the improvement of zootechnical parameters.

In the current study, the *villi* length were increased in the anterior intestine of fish supplemented with probiotics for eight weeks. Longer *villi* enable an increase in absorption surface area, improving the dietary nutrient utilization, and ultimately improving the growth performance. A recent work in Nile tilapia observed no significantly effect on the mid-intestine

villi length using a distinct level ($5g.kg^{-1}$) of the same probiotic formula (Standen *et al.*, 2015), consistent with our findings at that gut section.

Pirarat *et al*. (2011) working with tilapia and Merrifield *et al*. (2010d) working with trout observed that intestinal morphology might be significantly affected by probiotic treatment, especially the proximal intestine. McGuckin *et al*. (2011) observed, in mammals, a less thicker mucus layer in small intestine relative to large intestine, correlating it with the facilitation of nutrient absorption. That feature, together with a lower microorganism content comparatively to more distal segments (Ringø *et al.*, 2003), might explain a higher impact of microbial feed additives in proximal intestine morphology, towards distal segments.

In the current study, goblet cells were more abundant in fish treated for eight weeks with both diets supplemented with probiotic. Goblet cells produce mucus that prevents dehydration, lubricates and protects the gastrointestinal tract against physical damage and injurious agents (Kim & Khan, 2013). The mucus, predominantly mucins (gel-forming glycoproteins), play an important role in innate immune defence mechanism (McGuckin *et al.*, 2011). In fish, the increase in goblet cell count as an effect of probiotics treatment has been previously observed (Pirarat *et al.*, 2011; Standen *et al.*, 2016).

Fish gastrointestinal microbiota produces several enzymes like amylase, cellulase, chitinase, lipase, phytase and/or proteases as extensively reviewed by Ray *et al*. (2012). That supplementary enzymes may help to digest substrates, especially those for which the host has no endogenous enzymatic equipment, as cellulose (Bairagi *et al.*, 2002). In that sense, gut microbiota can influence the digestibility of nutrients and/or provide additional nutrients to the host (Merrifield *et al.*, 2010c). In fish starting the exogenous feeding, lactic acid bacteria were able to stimulate an early maturation of digestive system, resulting in an increase of intestinal and pancreatic digestive enzyme activity (Askarian *et al.*, 2011; Suzer *et al.*, 2008). *Pediococcus acidilactici* stimulated trypsin and amylase activity in shrimps (Castex *et al.*, 2008). Wang and Xu (2006) observed an increase in amylase, lipase and protease specific activity after feeding photosynthetic bacteria, *Bacillus* sp. or their combination, to common carp juveniles (*Cyprinus carpio*), whereas Essa *et al.* (2010) observed similar outcome in tilapia juveniles supplemented with *B. subtillis* or *L. plantarum*. However, the findings of Wang and Xu (2006) and Essa *et al.* (2010) were not consistent with the current study and with Gisbert *et al*. (2013). The specific activities of amylase, lipase, trypsin and chymotrypsin, and diet digestibility were not significantly affected by long term probiotic supplementation. The diets used in the digestibility trial were prepared with external marker chromic oxide (Cr_2O_3). The

influence of Cr_2O_3 on intestinal microbiota, especially on midgut and hindgut sections, was previously observed (Ringø, 1993).

In summary, the dietary supplementation of multi-species probiotic at 3 $g.kg^{-1}$ (1.0×10^6 CFU) improved the growth performance, whereas 6 $g.kg^{-1}$ (2.3×10^6 CFU) stimulated the innate immune response in tilapia. Both dietary probiotic levels had a positive impact on intestinal morphology.

6 *General discussion, conclusions and future perspectives*

6.1 GENERAL DISCUSSION

The dietary administration of probiotics is considered as an "environmental friendly" nutritional strategy to enhance the aquaculture activity and efficiency (Dimitroglou *et al.*, 2011; Merrifield & Ringø, 2014; Zhou *et al.*, 2009).

The biological prophylactic and therapeutic tools were never so valuable and required for sustainable livestock and aquaculture activities as today. The growing of the world population demands additional fish and food production (FAO, 2014) and requires a better use of resources.

The antimicrobial resistance in microorganisms is developing and spreading, and is imperative the use of alternative ways to counteract the ubiquitous opportunistic pathogens (McGann *et al.*, 2016; WHO, 2014; Woolhouse *et al.*, 2015). Probiotic approach has the potential to improve both fish healthiness and zootechnical performance (Dimitroglou *et al.*, 2011; Kiron, 2012).

Probiotics act in several ways not completely clarified (Lazado & Caipang, 2014) but mostly strain-dependent. Still, the host immune system and the intestinal microbial community interact actively in the process (Montalban-Arques *et al.*, 2015). Diet composition and husbandry conditions are also active players in the outcome (Telli *et al.*, 2014).

The accumulation of studies with several fish models points to evidence the potential interest of several microorganisms. Even so, is not possible to extrapolate the outcomes with different microorganism strains, fish hosts or husbandry conditions (Balcázar *et al.*, 2007b; Merrifield *et al.*, 2010c). In this way, every microbial formulation has to be studied in defined conditions to assess its putative interest and define the functional dosages.

In this context, the general objective of this thesis consisted in studying the effects of commercial probiotics in selected aquaculture species, trying to optimize the diet supplementation towards a more sustainable and efficient aquaculture activity.

On our first trial (chapter 2) we administered a *Bacillus subtilis* & *Bacillus cereus* formulation through the diet. The interest of spore forming bacteria as *Bacillus* is justified by their long shell life and stability to high temperatures (Hong *et al.*, 2005). *Bacillus* may have immunomodulatory, nutritional and antioxidant properties (Cutting, 2011). Several *Bacillus* spp. formulations are authorized as terrestrial livestock feed additives by EFSA. In rainbow trout, other *Bacillus* formulations have demonstrated positive outcomes on growth (Bagheri *et al.*, 2008; Raida *et al.*, 2003), immune status, disease resistance (Brunt *et al.*, 2007; Raida *et al.*, 2003) and intestinal morphology (Gisbert *et al.*, 2013). Nevertheless, under the current trial

conditions, we have not found any beneficial effect in rainbow trout or brown trout (chapter 2). In fact, we observed a detrimental impact on intestinal morphology, possibly by interactive or associative effects with dietary vegetable protein ingredients. To our best knowledge, only one other study reported negative impact of probiotic on sea bream gut morphology and higher mortality after pathogen challenge (Cerezuela *et al.*, 2013). The authors observed a negative interaction with inulin. Vegetable ingredients can modulate the mucosal immunity and microbiota (Bakke-McKellep *et al.*, 2007; Dimitroglou *et al.*, 2009) and influence the probiotic outcomes. *Lactobacillus plantarum* (Dawood *et al.*, 2015) and a multispecies microbial blend (Sealey *et al.*, 2009) were able to improve the ability to cope with high levels of soybean meal in the diets of amberjack and rainbow trout, respectively.

Chapter 2 results reinforces the necessity to test carefully each microbial formulation, since the outcomes of microbial delivery can be null or negative and vary with host species and dietary factors/composition.

At the start of this thesis, several dietary probiotic incorporation methods were reported in the literature: spraying live cell suspension over the diet (Balcázar *et al.*, 2007a; Ferguson *et al.*, 2010) or mixing it with fish oil and then top-dress the diet (Harper *et al.*, 2011); suspension of lyophilized preparations in sterile water, phosphate buffered saline (PBS) solution (Merrifield *et al.*, 2011a) or fish oil (Aubin *et al.*, 2005; Castex *et al.*, 2009; Gisbert *et al.*, 2013) and then coat the diet with it. Vacuum coating with fish oil containing the lyophilised probiotics was also reported (Barroso *et al.*, 2014; Batista *et al.*, 2015). Panigrahi *et al*. (2005) compared viable *versus* lyophilized probiotic incorporation and, despite the higher response obtained with live cells, concluded that the freeze-dried incorporation could be an efficient mode of delivery. Aiming to test practical field options, in the first (chapter 2) and second trials (chapters 3 and 4) we included the lyophilized preparations directly into the ground basal diet, as other ingredient, prior to cold pelleting. The bacterial count (CFU/g) of the experimental diets of second trial (chapters 3 and 4) were somewhat inferior to the theoretically expected, in particular in B or mono-species probiotic (almost 10^2 difference), probably due to the probiotic incorporation methodology. Still, we detected effects of both probiotic administrations, validating it as a possible incorporation method. Recent studies adopted a similar methodology adding the lyophilized probiotic as a basal ingredient before cold press extrusion (Standen *et al.*, 2016; Standen *et al.*, 2015).

On tilapia trial (chapter 5) we used tap water to suspend the lyophilized probiotic and applied it over the basal extruded diet, posteriorly dried at 35ºC. That was a shortening of the

methodology recommended by the manufacturer, which also recommend finalizing the procedure by coating the feed with fish oil or molasses as top dressing. The simplified method was valid to deliver the probiotic and the CFU counts were in the range of the theoretically expected. Our results suggest that the tested probiotics can be included in the diets as another inert feed additive, *i.e* the incorporation of the lyophilized powder is possible. Also, a more reachable methodology, as tap water suspension and covering of the diet, is also possible with the multispecies product.

The gut microbiota influence on host development, metabolism, immune function, homeostasis, nutrition and growth is well recognized (Bauer *et al.*, 2006; Hord, 2008; Llewellyn *et al.*, 2014; Merrifield & Carnevali, 2014a; Rawls *et al.*, 2004; Ringø *et al.*, 2016; Walsh *et al.*, 2014). The microbiota modulation is considered to be a basilar aspect of probiotic action (Merrifield & Carnevali, 2014a; O'Toole & Cooney, 2008) and several studies document the intestine microbiota alteration in fish receiving probiotics (Balcázar *et al.*, 2007a; Dawood *et al.*, 2016; Ferguson *et al.*, 2010; Giannenas *et al.*, 2015; Gisbert *et al.*, 2013; Merrifield *et al.*, 2010b; Tapia-Paniagua *et al.*, 2010). The influence may be on the microorganisms load as well on composition, as comprehensively reviewed by Merrifield & Carnevali (2014a). Chapter 4 reports that the composition and dosage of the tested mono-species (B) and multi-species (A) probiotics influenced the microbiota of rainbow trout, increasing diversity. Rainbow trout receiving probiotic A (multi-species) at 1.5 $g.kg^{-1}$ presented higher number of DGGE bands, indicating a higher number of OTU in the intestine. The animals receiving probiotic A at 1.5 $g.kg^{-1}$ and 3 $g.kg^{-1}$ and probiotic B at 0.1 $g.kg^{-1}$ presented increased diversity of microbiota in the intestine compared to the control, most significantly at the lower dose of probiotic A (1.5 $g.kg^{-1}$).

The reports by other groups, examining the current commercial probiotics on gut microbiota by culture-independent methods, are distinct but not necessarily on disagreement with our findings. In tilapia, the supplementation with the same levels of the multispecies product as used by us in rainbow trout, resulted on absence of significant influence on richness and diversity of microbiota (Standen *et al*., 2016) while a higher dosage ($5g.kg^{-1}$) decreased it (Standen *et al.*, 2015). On rainbow trout, a middle dosage ($2g.kg^{-1}$) had limited impact on OTUs (Ozório *et al.*, 2016). The supplementation of the same strain of *Pediococcus acidilactici* (B probiotic) to tilapia, reduced significantly the gut microbiota species diversity and richness (Ferguson *et al.*, 2010), contrarily to our observations. That shorter trial (32 days) studied a higher dose (10^7) and, after reverting to a nonsupplemented diet, the diversity and richness were

increased. Those and ours findings highlight the host and dosage as major influencing factors and suggest that too high dosages may be too disruptive of gut microbiota. The diminishing of diversity suggests antagonism or competition with indigenous microorganisms. That aspect could only be of interest in the cases of reduction of putative harmful microorganisms. On the contrary, the enhancement of the gut microbiota diversity is generally recognized as a beneficial outcome, since is associated to a higher metabolic potential and a lower susceptibility of pathogens invasion and establishment (Blaut & Clavel, 2007; Lozupone *et al.*, 2012). Accordingly, disease conditions have been linked to a decreased microbial diversity in men (Lozupone *et al.*, 2012) and fish (Cerezuela *et al.*, 2013; Heikkinen *et al.*, 2006). Probiotics multi-species blends, like probiotic A, might present a higher range of actions and an increased ability to persist in the intestine, in relation to single strains (Nayak, 2010; Timmerman H.M. *et al.*, 2004). Nevertheless, we cannot conclude this on our trial since the $CFU.g^{-1}$ counts were lower in probiotic B diets rendering the true comparisons among the probiotic formulations impossible.

The microbiota and probiotics can influence positively the integrity, structure, morphology, motility and functionality of intestine (Awad *et al.*, 2008; Caipang & Lazado, 2015; Dimitroglou *et al.*, 2009; Matur & Eraslan, 2012; Sweetman *et al.*, 2010). On the second trial, at the tested conditions (chapter 3), the probiotics A and B did not induce any particular changes in rainbow trout proximal intestine histomorphology, under light microscopy examination. The intestine area, *villi* height, density or presence of goblet cells were not significantly affected by the tested probiotic dosages after eight weeks of feeding. Batista *et al*. (2015) observed similar results after testing the same probiotics on sole. *Pediococcus acidilactici* administration at higher dosages (10^6, 10^7) did not influence the absorptive area or goblet cell presence, either on rainbow trout (Harper *et al.*, 2011) or tilapia (Ferguson *et al.*, 2010; Standen *et al.*, 2013). Nevertheless, this bacterial strain is not devoided of ability to modulate the absorptive area, since Merrifield *el al*. (2010d), detected a significantly influence on microvilli length at the proximal (but not distal) rainbow trout intestine. We could not evaluate microvilli morphometry since is only possible with electron microscopy. In tilapia, the probiotic A was able to improve the intestine morphology after eight weeks of dietary supplementation (chapter 5). Two and four weeks were not enough to notice any significantly influence. Both probiotic dosages might increase the average goblet cell presence along the entire intestine sections and increase the *villi* height on proximal intestine section. Intestine

mucus is an important protective mechanism, crucial for the maintenance of epithelium integrity (Gomez *et al.*, 2013). Longer *villi* implies higher digestive and absorptive surface.

Probiotics, directly or indirectly (by microbiota influence) may act as immunomodulatory agents (Lazado & Caipang, 2014). Numerous trials demonstrate their potential to influence the systemic and local immunity of hosts as thoroughly reviewed by Nayak (2010) and Lazado & Caipang (2014).

Some *Bacillus* strains delivered orally were responsible for some immunomodulation on rainbow trout (Brunt *et al.*, 2007; Newaj-Fyzul *et al.*, 2007). The authors report the influence of the dosage on the outcomes. On our first trial (chapter 2), we could not find immunomodulatory activity of the tested *Bacillus subtilis* & *Bacillus cereus* blend, either on brown trout or rainbow trout, over a long period of delivery. Yet, our results corroborate the literature description on the different range of plasma lysozyme levels between the two salmonid species (Grinde *et al.*, 1988; Lie *et al.*, 1989).

On second trial (chapter 3), the studied probiotics revealed some immunomodulation ability in a dose and time-manner. Probiotic B or *Pediococcus acidilactici* at the highest dose administered (0.2 $g.kg^{-1}$), after four weeks of administration, was able to increase significantly the alternative complement pathway (measured as ACH50 units). The levels remained higher after eight weeks but without statistical significance. The importance of the dosage to achieve functionality was evidenced, since 0.1 $g.kg^{-1}$ of probiotic inclusion was not sufficient for detectable influence. To our knowledge, it is the first report of the potential for stimulating such important alert and defence mechanism (Boshra *et al.*, 2006) by this bacterial strain. Lysozyme was not affected by *Pediococcus acidilactici* as observed in other studies with this strain in rainbow trout (Merrifield *et al.*, 2011a), tilapia (Standen *et al.*, 2013), or Senegalese sole (Batista *et al.*, 2015). Conversely, the lower dose of multispecies probiotic (1.5 $g.kg^{-1}$) promoted an increase on lysozyme levels after eight weeks of supplementation, while the ACH50 levels remained unaltered. On the same fish model, the same bacterial blend did not influence lysozyme after eight (Giannenas *et al.*, 2015) or nine (Ozório *et al.*, 2016) weeks of delivery, but enhanced the complement response. Is necessary to highlight the higher probiotic CFU count of those diets.

On tilapia trial (chapter 5), the multispecies probiotic did not affect significantly lysozyme levels, but the higher dosage (6 $g.kg^{-1}$) enhanced the alternative complement after 8 weeks of supplementation. Just recently, the immune modulation of this commercial probiotic in tilapia has been reported concerning the intestine gene expression (Standen *et al.*, 2016).

Nonetheless, we are unware of reports studying the influence on the plasma immune parameters followed by us. As described above, in rainbow trout a similar trend was observed by others (Giannenas *et al.*, 2015; Ozório *et al.*, 2016). Taken that and our data into consideration, the results seen to suggest that higher multispecies probiotic dosages do not correlate with lysozyme enhancement, while ACH50 seems to require a superior CFU or dosage to be significantly elevated.

There are scarce information about the probiotics influence on skin mucus (Lazado & Caipang, 2014) but the dietary administration of *Bacillus amyloliquefaciens* was able to increase skin mucus antimicrobial proteins as lysozyme in *Catla catla* (Das *et al.*, 2013). Yet, under the conditions of our second trial (chapter 3), the tested probiotics failed to influence significantly the rainbow trout skin mucus lysozyme, although the numerically higher values presented by the fish fed probiotics.

At the three trials, we assessed the nitric oxide and superoxide anion production of head kidney leucocytes as indicators of leucocyte respiratory burst - a pathway of events that ends on pathogens clearance and on the generation of reactive oxygen and nitrogen species (Babior, 2000). We did not observe any influence on those parameters by any of the probiotics studied. Accordingly, Batista *et al*. (2015) reported no influence on those parameters after the administration of the same probiotics to sole and Ferguson *et al*. (2010) reported no alteration on blood respiratory burst after *Pediococcus acidilactici* administration to tilapia. In the literature it is possible to find distinct methods to evaluate the leucocyte respiratory burst (Newaj-Fyzul *et al.*, 2007; Nikoskelainen *et al.*, 2003; Secombes, 1990), so we cannot exclude the possibility of different outcomes with other methodologies.

On the last trial (chapter 5) we evaluated oxidative stress indicators on tilapia liver. The tested dosages of probiotic did not benefit the anti-oxidant system of Nile tilapia (chapter 5). Tilapia receiving probiotic A at 3 $g.kg^{-1}$ presented lower hepatic values of GSH than controls, after four weeks of administration. The other indicators evaluated were not significantly influenced by the administered dosages of the multispecies probiotic. Recent studies with rainbow trout observed some anti-oxidant influence after the same microbial consortia administration (Giannenas *et al.*, 2015; Ozório *et al.*, 2016) but current results suggest that, in tilapia under the tested conditions, the multispecies probiotic do not exhibit antioxidant properties. Nevertheless, given the amount of oxidative stress indicators and methods to evaluate them (Halliwell & Gutteridge, 2015), it is necessary to denote the limitation of our evaluation, implying cautious considerations.

The limited immune and antioxidant modulation observed on the current experiences could be due to an absence of a stressful situation, such as high density (Telli *et al.*, 2014), heat shock (Taoka *et al.*, 2006a) or pathogen challenging (Castex *et al.*, 2009) that may evidence the probiotic effects.

Probiotics A and B can modulate voluntary feed intake (VFI, chapters 3 and 5). In rainbow trout (chapter 3) the highest concentrations of both probiotics (A at 3 $g.kg^{-1}$ and B at 0.2 $g.kg^{-1}$) decreased VFI after eight weeks (not noticed at four weeks). Shelby *et al*. (2007) observed a negative influence on feed intake in one of the probiotic formulations under evaluation in catfish (*E. faecium* and *P. acidilactici*). In tilapia (chapter 5), an opposite tendency was observed in the beginning of the supplementation: after two weeks, the animals receiving 3 $g.kg^{-1}$ of probiotic presented higher VFI but the effect attenuated afterwards. Robertson *et al*. (2000) and Irianto and Austin (2002) observed the enhancement of appetite by probiotics. The intestinal microbiota modulation observed on rainbow trout (chapter 4) might result in augmented short-chain fatty acids (SCFA) levels such as acetate, butyrate or propionate. Acetate reduced appetite in mice (Frost *et al.*, 2014) and propionate might suppress the feeding behaviour in ruminants (Chambers *et al.*, 2015). Recent studies linked the appetite control with host gut microbiota (Alcock *et al.*, 2014; Norris *et al.*, 2013). It is not clear if probiotics are able to influence the diet palatability but the feed consumption, at some extent, can suffer apparently contradictory alterations (chapter 3 and 5). The methodology of introduction of the zootechnical additive to the diets was distinct in the two experiments but their influence on VFI deserves future attention.

The tested dosages of probiotics did not influence carcass chemical composition (chapters 2, 3 and 5). Previous studies on probiotic administration to fish larvae observed an influence in carcass composition (El-Haroun *et al.*, 2006; Sealey *et al.*, 2009). The changes on larvae body composition are possibly justified by the early maturation of the endogenous digestive enzymes by probiotics (Essa *et al.*, 2010; Suzer *et al.*, 2008) and/or to a greater modulation of gut microbiota, that is establishing at that phase (Makridis *et al.*, 2000; Verschuere *et al.*, 2000). In older fish, the impact loses significance in most cases (Gisbert *et al.*, 2013; Merrifield *et al.*, 2011a; Merrifield *et al.*, 2010b; Standen *et al.*, 2016; Standen *et al.*, 2013; Telli *et al.*, 2014). Our data corroborate these last observations.

The tested dosages of probiotics did not influence condition factor (K) of the animals and the evaluated organ somatic indexes (chapters 2, 3 and 5). The K condition factor relates with the animal body shape and nutritional status. Is a widely used indicator of fish “well being”

(Morgan & Iwama, 1997). The K condition factor varies with development and seasonal changes and may decline when energy reserves decrease or in stressfull situations, as high densities or other adverse situations (Goede & Barton, 1990; Morgan & Iwama, 1997). Yet, aquaculture fishes as salmonids, fed with high lipid diets, frequently accumulate abdominal fat and present higher K values. In this situation, the diminishing of K-factor could represent more attractive fish for the consumers, as stated by Merrifield *et al*. (2011a). We did not observed any influence of probiotic administration on any of the trials (Chapter 2, 3 and 5) similarly to Gisbert *et al*. (2013). Curiously, Merrifield *et al*. (2011a) administrating *P. acidilacti* to rainbow trout, incorporated on the diets at a vegetative or lyophilized form, observed the lowering of K-factor only in the animals that received the lyophilized probiotics.

Organo-somatic indices are metabolic indicators. When lower than normal, according to Morgan & Iwama (1997), may "indicate a diversion of energy away from organ growth in order to combat a stressor of some type". The hepatosomatic index (HSI) is positively related with the metabolism, nutritional state and growth rate (Schmitt & Dethloff, 2000), but can also increase after exposure to pollutants or toxins (Goede & Barton, 1990) Spleen is an hematopoietic organ; infections may increase their relative size while stress may decrease it (Schmitt & Dethloff, 2000). Feed restriction can reduce intestine mass and structure (Buddington *et al.*, 1997). Dietary factors are able to influence the intestine somatic index, as observed on Atlantic salmon distal intestine somatic index, decreased by soybean meal and increased by inulin (Bakke-McKellep *et al.*, 2007). On the current trials (chapters 2, 3 and 5) neither the condition factor nor the organo-somatic indexes were significantly affected by probiotic administration. Our findings are in accordance with Batista *et al*. (2016; 2015) that observed no influence on Senegalese sole HSI or visceral somatic index after administration of the commercial probiotics here designed as A and B (chapter 3 and 5).

Probiotics promotion of gut microbiota diversity (chapter 4), immune stimulation (chapter 3 and 5) and increase of the intestine absorptive surface (chapter 5) may result in improved growth and feed conversion ratio performances (chapter 3 and 5). On the second trial (chapter 3) the probiotics influenced the zootechnical performance, improved at the lowest dose of multi-species probiotic (1.5 $g.kg^{-1}$ *vs* 3 $g.kg^{-1}$) and at the highest dosage of mono-species probiotic (0.1 $g.kg^{-1}$ *vs* 0.2 $g.kg^{-1}$). The growth results mostly correlate with the immune and microbiota results. The growth and feed conversion promotion are major practical outcomes, validating the interest of the current A and B products. On tilapia trial (chapter 5), we tested higher dosages (3 $g.kg^{-1}$ and 6 $g.kg^{-1}$) of multispecies probiotic. Only the lowest dose enhanced

significantly the growth performance whereas the highest dose stimulated the alternative complement pathway. Both probiotic diets enhanced the intestinal absorptive surface and the mucous production.

Our findings suggest better results on growth performance and microbiota diversity using lower dosages, especially regarding the multispecies product (chapter 3 and 4) and tilapia (chapter 5). Giannenas *et al*. (2015), in accordance, observed better growth rates and FCR at the lower dosage tested in rainbow trout. Possibly higher dosages are more disturbing of gut microbiota and immune function, as suggested by others works were the microbiota diversity was reduced after high dosages. The direct antagonism towards the microbial community is plausible (O'Toole & Cooney, 2008). Higher probiotic dosages may also imply an extra supply of energy and nutrients for immune responses (Humphrey, 2010). The activation and responses of immune cells demands fuel substrates as glucose, glutamine and arginine (Humphrey, 2010; Li *et al.*, 2009). Mucus production requires substantial energy and amino acids (mucins are rich in threonine, McGuckin *et al.*, 2011). The shift of nutrients from growth to immune activation possibly contributes to justify why higher probiotic dosages frequently do not correspond to the best zootechnical performances, as observed in Chapters 3 and 5. Nevertheless, a minimal amount of probiotic is required for functionality, possibly explaining why B_1 diet presented no significantly influence on rainbow trout growth performance.

After the observation of better growth and feed conversion rates after probiotic administration (chapter 3 and 5) we hypothesized the possible probiotic influence on the apparent digestibility of diets and/or digestive enzymes activities, as observed previously in common carp (Wang & Xu, 2006) or tilapia (Essa *et al.*, 2010). It is well recognized that the microbiota provide the host with metabolites, nutrients or enzymes (Merrifield & Ringø, 2014). Nevertheless, tested dosages of probiotics failed to improve tilapia digestive enzymes specific activity or the apparent digestibility coefficients of the diets, after twenty weeks of supplementation (chapter 5). We could not exclude the possibility of the chromic oxide addition to the diets or the longer supplementation period being influences in the outcomes.

Current results, along with literature data, suggests that the functional dosage and administration time might be different for distinct goals. In poultry science, it is well establish that the immune system stimulation occurs at expense of growth (Humphrey, 2010). It is not unreasonable to expect the same in fish. The growth promoting effect seems to be achieved with relatively lower probiotic amount (multispecies consortia 9×10^5 to 1×10^6 CFU/g; *Pediococcus acidilactici* 7×10^4) for a minimal time (eight weeks, maybe less, but more than

four). We did not run pathogens challenge trials, but several studies from other groups (Brunt & Austin, 2005; Capkin & Altinok, 2009; Newaj-Fyzul *et al.*, 2007) report the observation of higher survival rates against pathogens exposure after administration of higher amounts of probiotics (10^7 or 10^8 are frequently the more functional dosages), for shorter supplementation periods (as two weeks). Possible higher probiotic dosages would impact more intensely the intestine microbiota and the host immune system, increasing the fish endurance towards pathogens outbreaks, but may not be ideal for growth promotion. This type of supplementation would possibly be most adequate before stressful moments as management operations or environmental conditions prone to pathogens surge. Future studies would allow further knowledge.

6.2 CONCLUSIONS

The results presented along the previous chapters allow us to drawn the following conclusions:

- Microbial commercial formulations should be carefully tested, since outcomes vary with host species and dietary factors, and can be negative (chapter 2).

- Studies evaluating probiotics *in vivo* should include a histomorphology observation of the intestine. The exclusive examination of zootechnical parameters, might not be sufficient to detect eventual negative physiologically impacts (chapter 2).

- The tested probiotics can be included in the diets as another inert feed additive (chapter 3). A simplified process, consisting in the covering of the diets with a water suspension of the lyophilized multispecies product, is also efficient with the multispecies product (chapter 5).

- Composition and dosage of the tested probiotics influenced the microbiota of rainbow trout, increasing the microbial diversity (chapter 4).

- The tested amounts of probiotics A (multi-species) and B (mono-species) did not induce any particular change in rainbow trout proximal intestine histomorphology, under light microscopy evaluation (chapter 3).

- Probiotic A improved Nile tilapia intestine histomorphology after eight weeks (chapter 5). Both probiotics dosages may increase the goblet cell presence along the entire intestine sections and increase the *villi* height on proximal intestine.

- The tested dosages of probiotics do not induce any particular stimulation (on extracellular $O_2^{\bullet -}$ or NO production) on head kidney leucocytes of trouts or tilapia (chapter 2, 3 and 5).

- Probiotics A and B can stimulate to some extent the plasma lysozyme and alternative complement pathway (ACH50) of rainbow trout, in a time and dose dependent manner (chapter 3): probiotic B at 0.2 $g.kg^{-1}$ increased ACH50 after four weeks, remaining high after eight weeks; probiotic A at 1.5 $g.kg^{-1}$ increased lysozyme activity after eight weeks.

- Probiotic A can stimulate to some extent the plasma ACH50 of Nile tilapia, in a time and dose dependent manner (chapter 5): probiotic A at 6 $g.kg^{-1}$ increased alternative complement activity (ACH50) after eight weeks.

- The tested dosages of probiotic A did not benefit the hepatic anti-oxidant indicators of Nile tilapia (chapter 5).

- Probiotics A and B can modulate voluntary feed intake but the mechanisms remain to be elucidated (chapters 3 and 5).

- The tested dosages of probiotics did not influence carcass chemical composition (chapters 2, 3 and 5).

- The tested dosages of probiotics did not influence condition factor (k) of the animals and the evaluated organ somatic indexes (chapters 2, 3 and 5).

- Probiotics A and B can improve zootechnical performance of rainbow trout after eight weeks of administration (and not significantly after four weeks, chapter 3): probiotic A at 1.5 $g.kg^{-1}$ can promote growth rates; probiotic A at 1.5 $g.kg^{-1}$ and probiotic B at 0.2 $g.kg^{-1}$ can promote the feed conversion ratio.

- Probiotic A can improve zootechnical performance of Nile tilapia after eight weeks of administration (and not significantly after two and four, chapter 5): probiotic A at 3 $g.kg^{-1}$ can promote growth rates. FCR was lower, but not to a significant level.

- The tested dosages of probiotics failed to improve digestive enzymes specific activity or apparent digestibility coefficients of the diets, after twenty weeks of supplementation to tilapia (chapter 5).

The present conclusions, drawn from our observations, must be cautious. The dietary probiotics administration results may change, even replicating the current trials conditions, since the background microbiota from water and host will be different. These factors, along with diet composition are powerful elements conditioning the outcomes (Ingerslev *et al.*, 2014a; Ingerslev *et al.*, 2014b).

6.3 FUTURE PERSPECTIVES

Probiotic administration through the water or incorporated into diets can constitute a strategy to increase aquaculture productivity. The growth promoting ability and the improvement of animal health and resistance to diseases are desired aspects of a probiotic. The growth promotion was confirmed in probiotics A and B but the protection against diseases was not assessed. We observed some systemic immunomodulation ability in both fish species and a superior number of goblet cells in tilapia, possibly indicating a superior mucus presence, a major defence mechanism. Nevertheless, challenges with biological agents (virus, bacteria or parasites) and stress factors (manipulations, inadequate densities or water parameters) would constitute the ultimately proof of improved resistance drove by probiotics. Challenge trials require specific installations to run the experiments. Also imply the sacrifice of a large number of animals, raising ethical concerns. Still, they constitute valuable studies, after trials like the current ones. *In vitro* studies - like the screening of inhibition or antagonism against pathogenic agents - and cell culture studies would also provide useful information. It is expected an increase in the availability of fish cellular lines in the following years.

The study of immune parameters locally, at the gut, skin and gills mucosa, would be very important to a deeper understanding of probiotic action, especially in the gut. The complexity

of the mucosal immune system modulation by commensal microorganism and probiotics is far from being total understood (Gomez *et al.*, 2013). In particular, the studies of key messengers as cytokines may give a better outlook of the immunomodulation ability resulting from probiotic administration (Lazado & Caipang, 2014).

Mucus is an important defence barrier and highly modulated by microbiota. The knowledge of mucus and mucins in fish is limited comparing to terrestrial vertebrates and their study can also enlighten the probiotics mode of action (Gomez *et al.*, 2013).

The antioxidant response mechanisms are crucial to preserve the integrity of cell components. The potential involvement of some probiotics strains promoting or preventing oxidative stress require in deep study.

The molecular technologies allow a greater knowledge on complex microbial ecosystems as the gastrointestinal microbiota. The evolution of techniques will allow a better insight of gut microbial diversity and function. It is expected an easier access to the next-generation sequencing techniques (Llewellyn *et al.*, 2014). Technologies like genomics, transcriptomics and proteomics are being applied to study microbiota communities and microbiota modulation (Zoetendal *et al.*, 2004). Metagenomic studies will give a superior knowledge of probiotic modes of action (Gueimonde & Collado, 2012) and metabolomic studies focusing on the host and microbial metabolome will expand our understanding on impact of stress, diets or probiotic and pathogenic microorganisms in fish (Llewellyn *et al.*, 2014). The microorganism interplay with each other and with host cells, and the signals involved in that communication (Zoetendal *et al.*, 2004) maintaining or disrupting homeostasis (Guinane & Cotter, 2013; McFall-Ngai *et al.*, 2013), will progressively be revealed. The integration and development of techniques will contribute to elucidate the microbiota roles on diverse physiological functions (McFall-Ngai *et al.*, 2013). Further knowledge will allow to use the more suitable probiotic strains for specific situations or goals.

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