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INTERACCIONES ENTRE BACTERIAS PROBIÓTICAS Y EL SISTEMA INMUNITARIO DE PECES TELEÓSTEOS

INTERACTIONS BETWEEN PROBIOTIC BACTERIA AND THE IMMUNE SYSTEM OF TELEOST FISH

Irene Salinas Remiro

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A mis padres,

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I. ABREVIATIONS

LIST OF ABREVIATIONS

ATCC: American type culture collection

BCA: bicinchoninic acid

BHI: brain heart infusión

BSA: bovine serum albumin

CACO: Caucasian colon adenocarcinoma

CECT: Colección Española de Cultivos Tipo

CTAB: cetyltrimethylammonium bromide

DAPI: 4',6-diamidino-2-phenylindole

DC: dendritic cell

DiO: 3,3'dioctadecyloxacarbocyanine perchlorate

DMEM: Dulbecco´s modified Eagle´s medium

DMSO: dimethyl sulfoxide

DTT: dithiothreitol

ECACC: European collection of cell cultures

EDTA: ethylenediamintetraacetic acid

EGTA: ethylene glycol tetraacetic acid

EPC: epithelioma carpis

FBS: fetal bovine serum

FDA: fluorescein diacetate

FITC: fluorescein isothiocyanate

FL: fluorescence

FSC: forward scatter

GALT: gut-associated lymphoid tissue

GI: gastrointestinal tract

HBSS: Hanks balanced saline solution

HKL: head kidney leucocyte

IEL: intraepithelial lymphocyte

Ig: immunoglobulin

IL: interleukin

IMR: Institute of Marine Research

LAB: lactic acid bacteria

LM: light microscopy

LPL: lamina propria leucocyte

MALT: mucosa-associated lymphoid tissue

MLN: mesenteric lymph node

NBT: nitroblue tetrazolium

PAMP: pathogen-associated molecular pattern

PB: peripheral blood

PBS: phosphate buffer saline

PI: propidium iodide

PMA: phorbol myristate acetate

PRRS: porcine reproductive and respiratory syndrome

S: spleen

SAF: seabream fibroblast

SEM: scanning electron microscopy

sRPMI: saline Roswel Park Memorial Institute medium

SRBC: sheep red blood cells

SSC: side scatter

SE: standard error

TEM: transmission electron microscopy

TLR: Toll-like receptor

TMB: 3′,5,5′-tetramethylbenzidine hydrochloride

TNF: tumoral necrosis factor

TRITC: tetramethylrhodamine isothiocyanate

TSA: trypticase soya agar

TSB: trypticase soya broth

II. ABSTRACT

Farmed fish are more susceptible to disease due to intensive rearing; therefore prophylactic tools are necessary to decrease disease derived losses. The use of probiotics in aquaculture has recently appeared as a promising biological control strategy. However, the effects of probiotic bacteria on the fish immune system were largely unknown until the last few years. The main purpose of this thesis was to investigate the interaction between teleost cells and probiotic bacteria from an immunological viewpoint.

Oral delivery of four different bacterial strains to gilthead seabream (*Sparus aurata* L.) revealed modulation of the main systemic innate immune parameters of this species. Notably, phagocytosis and natural cytotoxic activity of head kidney leucocytes showed the highest enhancement following probiotic bacteria administration. The assayed probiotic bacteria were non-fish (*Lactobacillus delbrüeckii* subsp. *lactis* and *Bacillus subtilis*) and fish (51M6 and Pdp11) derived as well as live and inactivated forms. *In vitro*, cellular innate immune responses of seabream head kidney leucocytes were increased dose dependently by the heat inactivated probiotics previously assayed *in vivo*.

Additionally, the morphological changes that take place in the Atlantic salmon (*Salmo salar* L.) gut epithelial barrier following exposure to pathogenic (*Vibrio anguillarum* and *Aeromonas salmonicida* subsp. *salmonicida*) or probiotic bacteria (*Carnobacterium divergens* and *delbrüeckii* subsp. *lactis)* were described. Probiotic bacteria alleviated the signs of tissue damage caused by pathogens in the salmon gut when incubated with such probiotic prior to pathogen exposure.

The lymphoid cells that constitute the gut-associated lymphoid tissue were studied in the seabream. Different available isolation protocols were assessed in order to optimally isolate the seabream gut leucocytes, which were then functionally characterised *in vitro*. Low phagocytic responses were recorded in seabream gut leucocytes compared with head kidney leucocytes. On the contrary, gut leucocytes had high natural cytotoxic activity. Different target particles including probiotic bacteria were used to investigate the phagocytic responses of seabream gut leucocytes, which revealed clear differential behaviour compared to head kidney phagocytes. Moreover, the nature of the assayed target particle was a determinant factor in gut leucocyte phagocytic behaviour. These

results will throw light on the modulation of teleost fish gut mucosal immunity by probiotic bacteria or other stimuli.

Finally, a procedure for the quantification of apoptosis in fish leucocytes by double staining flow cytometry was set up. This technique in conjunction with proliferation assays was employed for the study of the effects of probiotic bacteria extracts on the growth of two fish cell lines, SAF-1 from seabream and EPC from carp. Cytoplasmic extracts of *Lactobacillus delbrüeckii* subsp *lactis* and 51M6 probiotic bacteria had antiproliferative effects on the two cells lines and induced apoptosis, being the effects of the non-fish derived strain greater than the fish derived one with a general higher susceptibility of SAF cells compared with EPC cells.

Overall, these results are extremely important to understand the biological role of probiotic bacteria both present in the fish natural environment and those artificially administered to farmed fish in the diet and the way they interact with the teleost immune system.

III. INTRODUCTION

1. Probiotics

1.1 Definition

Since the Old Testament it is known that Abraham´s longevitiy was extraordinary thanks to the consumption of sour milk.

In the Microbiology age, Metchnikoff and others associated certain changes in human health with variations in the balance of the intestinal microbiota. Thus, it was advised to eat yoghourt containing lactic acid bacteria (LAB) and bifidobacteria, which would reduce the numbers of toxin producing bacteria in the gut and therefore increase the host´s life span.

The term probiotic comes from the ancient Greek and means "pro life", in contrast with the term antibiotic "against life". An extensive report on the usage and evolution of the word "probiotic" can be found in Schrezenmeir and de Vrese (2001).

The Medical Dictionary defines it as "Live bacteria ingested to supplement normal gastrointestinal flora, especially after depletion of flora caused by infection or ingestion of antibiotics". Fuller defined it in 1985 in a very similar way, limiting the use of probiotic bacteria to food additives and constraining their field of action to the gastrointestinal tract. More recently, other administration routes and other mucosal surfaces such as the vagina, the mouth or the skin have been incorporated to the definition.

Both in human medicine and in terrestrial animal veterinary medicine, the administration of probiotic bacteria implies the delivery of viable life bacteria to the host.

1.2 Probotics and animal health

Since the beginning of the XXth century, the number of investigations looking at the beneficial effects of probiotic bacteria on animal health keeps increasing. As therapeutic agents, there is more or less irrefutable evidence for probiotics to fight acute diarrhoea in children (Billoo et al., 2006), bowel disease (Marteau, 2006), diverticulitis (White, 2006), cholera (Focareta et al., 2006), Chron´s disease and allergic diseases such as rhinitis or Japanese polinosis (Xiao et al., 2006). Benefits are sometimes systemic, being its consumption associated, for instance, to a better mood in previously depressed patients (Benton et al., 2007).

In veterinary medicine there have been attempts to use probiotics in the treatment of colitis in mice, metritis in cattle (Otero et al., 2006), porcine reproductive and respiratory syndrome (PRRS) in pigs (Kritas and Morrison, 2007) and enteric infections in poultry (Dalloul et al., 2005). Interestingly, probiotics have commercially important properties different from therapeutical ones since cattle growth and production are increased following probiotic administration (Guo et al., 2006). Animal growth improvement may be achieved through the modulation of nutrient digestibility, the production of volatile fatty acids and the tuning of intestinal microbiota (Li et al., 2006b). Moreover, gastrointestinal motility is changed by probiotics as demonstrated in rodent models (Massi et al., 2006).

1.3 Modes of action

Despite the fact that positive effects attributed to probiotics are almost uncountable, the processes that take place at the cellular and molecular levels, in other words, how probiotic bacteria exert their function, are poorly understood (Ouwehand et al., 2002). Different modes of action for probiotic bacteria have been proposed:

- Metabolic or nutritional mechanisms, including the bioproduction of fatty acids like linoleic acid in the gut (Ewaschuk et al., 2006).

- Inhibition of growth and adhesion capacity of pathogenic microorganisms (Ruas-Madiedo et al., 2006). This inhibition is thought to occur via production of exopolysaccharides or inhibitory enzymes/metabolites. As an example, *Escherichia coli* nitrate reductase activity is inhibited by *Lactobacillus delbrüeckii* subsp. *lactis* (Hugo et al., 2006).

- Interacting with the gastrointestinal (GI) epithelial barrier (Otte and Podolsky, 2004; Parassol et al., 2005; Qin et al., 2005; O´Hara et al., 2006; White et al., 2006). Probiotic bacteria are capable of interacting with the cytoskeleton of enterocytes that form the epithelial barrier (Ait-Belgnaoui et al., 2006), regulating transepithelial Ca^{2+} transport (Gilman and Cashman, 2006), modifying mucin production (Otte and Podolsky, 2004) and preventing bacterial translocation from the gut lumen to the connective tissue by protecting the tight junctions (Qin et al., 2005; Zareie et al., 2006).

The latter is directly linked to the fact that expression of proteins like occluding and ZO-1 is regulated by probiotics (Qin et al., 2005).

- Modulation of immune responses, both locally (Duncker et al., 2006; Galdeano and Perdigon, 2006; Vinderola et al., 2006) and systemically (Erickson and Hubbard, 2000; Isolauri et al., 2001). It seems clear that probiotic bacteria modulate innate and specific immunity through a variety of mechanisms. First, they regulate the expression of Toll-like receptors (TLRs) such as TLR2 and TLR4 (Grabig et al., 2006) and, as a consequence, the way gut cells respond to pathogen associated molecular patterns (PAMPs) encountered in the GI environment. Second, cytokine production, as demonstrated by Takeda et al. (2006) for IL-12, and signal transduction (Ruiz et al., 2005) are modulated by probiotics. Adhesion molecules on lymphocyte surface are also decreased in cases of mice colitis, decreasing consequently the T lymphocyte recruitment at the inflammation site. Third, specific immune responses, including the balance between the so called Th_1 and Th_2 responses, are controlled by probiotics (Takahashi et al., 2006a). In some instances, Th_2 responses are supressed (Takahashi et al., 2006b). Humoral specific factors such as mucosal IgA in humans (Rautava et al., 2006), natural antibodies in the chicken (Haghighi et al., 2006) or antibody titres following vaccination, are increased by probiotic bacteria, pointing them as adjuvants (Tanasienko et al., 2005; Goldman et al., 2006).

- Antiproliferative and antitumoral properties. Different probiotic strains, mainly LABs, have been shown to inhibit the proliferation of mononuclear cells (Pessi et al., 1999) and cell lines (Lee et al., 2004). Moreover, probiotics are considered as cancer prophylactics especially in colorectal tumors and skin cancer (Commane et al., 2005; Malkov et al., 2005). In animal models, they succeeded in prolonging survival in tumour-bearing animals (Lee et al., 2004). Antitumor vaccine efficacy is enhanced by probiotics too (Tanasienko et al., 2005).

2. Probiotics in aquaculture

It is widely demonstrated that farmed fish are more susceptible to disease agents than their wild counterparts due to the artificial conditions posed by intensive rearing. When infectious outbreaks appear they may be fought by means of chemotherapeutants, vaccines or immunostimulants. More recently, the administration of probiotics either to fish (Gatesoupe, 1999, Gómez-Gil et al., 2000; Balcázar et al., 2006a), crustaceans (Farfanzar, 2006) or molluscs (Douillet, 1994; Gatesoupe, 1999) has appeared as a very promising control measure for aquaculture practices.

2.1 Definition

Aquatic animals and their symbiotic microbial communities are highly dependent on the microbiological composition of the aquatic environment. Thus, fish intestinal microbiota is temporary and unstable compared to that of terrestrial animals. These key differences have led to the redefinition of the term probiotic in aquaculture (Naidu et al., 1999; Salminem et al., 1999):

"Probiotics are microbial preparations that improve the well being and the health status of the host".

It is obvious that this is a wider concept than the one used in higher vertebrates which embraces those administered bacteria that, despite of not forming a stable microbial community in the gut, they provide benefits to the host. Several authors have shown that numbers of probiotic bacteria present in the GI tract start to decrease when the probiotic is not longer given in the diet (Robertson et al., 2000; Irianto, 2002; Balcázar et al., 2007), strengthening the labile character of microbial communities in the fish intestine.

This means that even inactivated forms of probiotics can be considered as probiotic preparations. The use of probiotics as prophylactic tools for fish farming implies the release of the cells of live bacteria into fish pens, which poses a potential risk to wild aquatic organisms since the bacteria may escape into the environment at large. The use of inactivated bacteria clearly solves such safety-related issues since they can no longer interact with other aquatic organisms. Moreover, fish continuously sample through their mucosal surfaces the microbes present in the water. This important difference with

terrestrial animals enables fish farmers to deliver probiotics directly to the culture water and not necessarily as food additives (Verschuere et al., 2000).

The types of probiotic formulations assayed in aquatic hosts can be classified as follows:

i) According to the origin of the bacterial strain:

- Strain isolated from the aquatic environment or from an aquatic host.
- Strains known to have probiotic properties in humans or terrestrial animals. This group is mainly formed by LABs.

ii) According to the number of strains administered in a formulation:

- Monospecific: a single bacterial strain is given to the host.
- Multispecific: several bacterial strain from different groups are combined in a single probiotic formulation also called microbial cocktail

iii) According to the viability of the bacterial cells:

- Alive/Viable
- Killed/Inactivated: this can be done either by heating, by UV radiation or by using chemical agents like formol.

Table 1 shows all the probiotic strains that have been tested as candidate probiotic bacteria in finfish species. Other farmed aquatic animals like crustaceans or molluscs have not been included in this summary.

2.2 Effects of probiotics on aquatic animals

Research into the effects of probiotics on aquatic animals first began in the late 1980s, when most of the effort was put into the effects on larval culture and larval live-feed.

The list of beneficial effects prompted by probiotic bacteria on aquatic animals keeps getting longer every year. Several reviews are now available on the use of probiotics in aquaculture (Gatesoupe, 1999; Verschuere et al., 2000; Balcázar et al., 2006a); in marine larviculture (Vine et al., 2006) and in shrimp culture (Farzanfar, 2006).

Gram $^{+}$ Bacteria	**Fish Species**	**Reference**
Bacillus IP5832 spores	*Scophtalmus maximus*	Gatesoupe, 1991
Bacillus nº 48	*Centroponus undocirrelis*	Kennedy et al., 1998
Bacillus sp. (Biostart)	*Channel catfish*	Queiroz and Boyd, 1998
B. toyoi + *Enterococcus faecium*	*Anguilla anguilla*	Chang and Liu, 2002
B. subtilis + *B. licheniformis* (Bioplus 2B)	*Oncorhynchus mykiss*	Raida et al., 2003
B. circulans	*Labeo rohita*	Ramachandran et al., 2005
B. subtilis	*Labeo rohita*	Nayak et al., 2007
B. subtilis	*O. mykiss*	Panigrahi et al., 2007
Carnobacterium divergens	*Gadus morhua*	Gildberg and Mikkelsen, 1997
C. divergens B33	*O. mykiss*	Kim and Austin, 2006
Carnobacterium sp.	*Salmonids*	Jöborn et al., 1997
C. divergens	*S. maximus*	Ringo and Vadstein, 1998
Carnobacterium sp.	*Salmo salar*	Robertson et al., 2000
Carnobacterium BA211	*O. mykiss*	Irianto and Austin, 2002
C. maltaromaticum B26	*O. mykiss*	Kim and Austin, 2006
Clostridium butyricum	*Miichthys miiuy*	Song et al., 2006
Lactobacillus helveticus	*S. maximus*	Gatesoupe, 1991
L. plantarum	*S. maximus*	Gatesoupe, 1991
L. bulgaricus + *Sreptococcus lactis*	*S. maximus*	García de la Banda et al., 1992
L. plantarum	*G. morhua*	Strom and Ringo, 1993
L. plantarum	*S. maximus*	Gatesoupe, 1994
Lactobacillus sp.	*O. niloticus*	Suyanandana et al., 1998
L. rhamnosus	*O. mykiss*	Nikoskelainen et al., 2001
L. rhamnosus JCM1136	*O. mykiss*	Panigrahi et al., 2004
L. rhamnosus 53103	*O. mykiss*	Panigrahi et al., 2007
L. plantarum	*Sparus aurata*	Carnevali et al., 2004
L. fructivorans AS17B	*S. aurata*	Carnevali et al., 2004
L. sakei	*O. mykiss*	Balcázar et al., 2007
Lactococcus lactis subsp. lactis	*O. mykiss*	Balcázar et al., 2007
Micrococcus luteus A1-6	*O. mykiss*	Irianto and Austin, 2002
Paracoccus sp.	*S. aurata*	Makridis et al., 2005
Ruergeria sp.	*S. aurata*	Makridis et al., 2005
Shewanella sp.	*S. aurata*	Makridis et al., 2005
Streptococcus termophilus	*S. maximus*	Gatesoupe, 1991
S. faecium M74	*Cyprinus carpio*	Bogut et al., 1998
S. faecium SF68	*A. anguilla*	Chang and Liu, 2002
S. faecium	*O. mykiss*	Panigrahi et al., 2007
Gram $^{-}$ Bacteria		
Aeromonas hydrophila A3-51	*O. mykiss*	Irianto and Austin, 2002
A. sobria GC2	*O. mykiss*	Brunt and Austin, 2005
A. caviae	*S. maximus*	Ringo and Vadstein, 1998
Aeromonas sp.	*S. aurata*	Makridis et al., 2005
Pseudomonas fluorescens	*O. mykiss*	Gram et al. 1999
P. fluorescens AH2	*O. mykiss*	Gram et al., 2001
Pseudomonas sp.	*O. mykiss*	Spanggaard et al., 2001
P. fluorescens	*Salmo salar*	Smith and Davey, 1993
Roseobacter sp.	*S. maximus*	Hjelm et al., 2004
Roseobacter sp.	*S. aurata*	Makridis et al., 2004
Vibrio alginolyticus	*S. maximus*	Gatesoupe, 1991
V. pelagius	*S. maximus*	Ringo and Vadstein, 1998
V. mediterranei	*S. maximus*	Huys et al., 2001
Vibrio strain E	*S. maximus*	Gatesoupe, 1997
Vibrio PB1-11 y PB 6-1	*S. maximus*	Makridis et al., 2000
V. fluvialis	*O. mykiss*	Irianto and Austin, 2002

Table 1: Published *in vivo* studies conducted on probiotic bacteria and farmed finfish species.

These effects can be summarised into the following categories:

1. Water quality improvement. It has mainly been tested in crustacean culture systems by adding to the water Gram$^+$ bacteria belonging to the *Bacillus* genus (Vaseeharan et al., 2003).

2. Competition against pathogens for adhesion sites, energy or nutrient sources. The latter is a more theoretical than empirical statement since available *in vivo* evidence is not very solid. *In vitro*, some data was supplied by Vine et al. (2004).

3. Improvement of diet digestibility through the production of digestive enzymes. It has been demonstrated that juvenile rohu can digest leaf meal if the *Leucaena* leaves are treated with *Bacillus* ssp. (Bairagi et al., 2004).

4. *In vitro* inhibition of pathogen growth (Nikoskelainen et al., 2001; Sugita et al., 2002; Chabrillón et al., 2005). This is a commonly used feature when assessing the probiotic potential of bacterial isolates. However, *in vivo* demonstrations of this inhibition are lacking at present.

5. Enhancement of immune responses both at the local and systemic level including cellular and humoral parameters. This point has only been investigated in the past five years and in spite of the extensive list of bacterial strains considered as potential probiotic candidates for aquaculture and the wide range of aquatic animals used as host species, to our knowledge at the beginning of this thesis there had been no studies on any probiotic on the Mediterranean gilthead seabream (*Sparus aurata* L.). Due to the importance of this point for our interests, more information will be given in section 2.3.

6. Increased disease resistance. In the last years it has been reported that probiotic administration increases teleost fish resistance against red mouth disease (Raida et al, 2003), furunculosis (Irianto and Austin, 2002; 2003; Irianto et al. 2003), vibriosis (Austin y col., 1995; Spaangaard et al., 2001), saprolegniosis (Bly et al., 1997),

lactococcosis and streptococcocis (Brunt and Austin, 2005) and edwardselliosis (Chang and Liu, 2002; Pirarat et al., 2006).

2.3. Relationships between probiotic bacteria and the fish immune system

i. Systemic cellular responses: several investigations have recently shown that the activity of head kidney and blood leucocytes from different finfish species is modulated by probiotic administration. These activities include the respiratory burst, phagocytosis, peroxidase content, and natural cytotoxic activity (Nikoskelainen et al., 2003; Kim and Austin, 2006; Pirarat et al., 2006). Moreover, blood leucocyte counts are different in those individuals that receive probiotics compared with control fish (Irianto and Austin 2003; Irianto et al. 2003).
ii. Systemic humoral responses such as serum natural complement and lysozyme activities (Irianto and Austin, 2003; Panigrahi et al., 2004; Pirarat et al., 2006; Balcázar et al, 2007) are increased by probiotic supplementation in the diet.
iii. Cytokine expression: only very recently it has been demonstrated that rainbow trout fed different probiotic supplemented-diets (Kim and Austin, 2006; Panigrahi et al., 2007).
iv. Local cellular responses: less effort has been directed to this point due to methodological difficulties when studying gut leucocytes. However, a couple of publications reveal that, as in higher vertebrates, gut mucosal immunity is modulated by probiotic bacteria (Balcázar et al., 2006b; Picchieti et al., 2007).

Despite the broad body of papers that describe the effects of probiotic bacteria in farmed fish, mechanistic studies are scarce, even to a greater extent than in mammals.
Information is also biased in terms of the host species. When this thesis began, no studies had been conducted in adult seabream and therefore we aimed to address the potential use of probiotics in the context of Mediterranean aquaculture, where the seabream represents the main produced species and it has great economic importance in the Murcia region.

3. The gut epithelial barrier, bacterial translocation and mucosal immunity

3.1. Higher vertebrates

The GI tract is commonly regarded as an organ of digestion and nutrient absorption overlooking its extremely important function as a physical and immunological barrier. The intestinal mucosal surface is responsible for the containment of vast numbers of luminal antigens that reach the intestine. Commensal bacteria (up to 10^{12} organisms per gram of intestinal content) line the walls of the GI tract, where they encounter pathogenic bacteria which can potentially damage the structure of the "fence" formed by the intestinal epithelial cells or enterocytes. Under normal circumstances, the presence of this vast biotic mass is useful and benign in that it helps prevent colonization of the bowel by pathogens and does not in itself evoke inflammatory immune responses (the normal mucosal immune system is tolerant of its many antigens). Nevertheless, this flora can also cause disease, because there is now good evidence that excessive mucosal immune responses to components of the microflora, either due to abnormal or impaired effector or regulatory (suppressor) cell activity of the host, is the prime cause of inflammatory diseases (Strober, 2006).

Bacterial translocation is defined as the passage of viable indigenous bacteria from the GI tract to mesenteric lymph nodes (MLN) (reviewed by Berg, 1999).

Spontaneous translocation takes place in healthy individuals and consists of low numbers of indigenous bacteria that are then cleared by the host gut innate immune defences. There are two routes of bacterial translocation, the intracellular route (through the epithelial cells) and the paracellular route (between enterocytes).

Bacterial translocation in non-physiological scenarios represents the first stages of infection. In these cases, bacteria pass from the lumen to the underlying connective tissue in high numbers and the local immune defences are not able to control them *in situ*. The three primary mechanisms that lead to this pathological translocation are the intestinal bacterial overgrowth, the increased intestinal permeability and the host immunodepression. It is also possible to find scenarios in which more than one of these mechanisms is responsible for the excessive bacterial passage.

It is worth noting that commensal bacteria play a pivotal role in all of them, therefore they are capable of controlling intestinal pathogenesis. First, as it was mentioned before,

commensal bacteria can inhibit the growth of their pathogenic counterparts by producing a series of inhibitory substances and by competing for nutrient and energy sources. For this reason, oral antibiotic therapy results in an increased risk to bacterial translocation and infections originated from GI tract. Moreover, overgrowth is prevented by intestinal peristaltic waves since they discourage bacteria adherence (Rowlands et al., 1999) and commensal bacteria seem capable of modulating intestinal peristalsis (Massi, 2006).

Secondly, bacterial translocation via the paracellular route is not possible unless tight junctions between enterocytes allow such passage. Pathogenic bacteria and endotoxin can damage tight junctions increasing permeability between epithelial cells. They cause intestinal edema and separation between the lamina propria and the epithelial layer too. Enterocyte microvilli are also altered by pathogenic bacteria reducing its length and disrupting its continuity. The latter helps pathogens to translocate since commensals loose adhesion sites and no longer protect the mucosal surface. Altogether these morphological changes are known as the loss of epithelial integrity. Loss of epithelial integrity means, in other words, the loss of polarity of the epithelial cells which, under normal conditions, maintain differences between the apical (luminal) and basolateral (internal) domains. When polarity is broken the epithelial barrier is no longer a fence since the "outside world" stops being different from the host's realm. In addition, commensal bacteria are sensed as dangerous when intestinal epithelium is under stress and gut permeability is increased (Nazli et al., 2004).

The third mechanism for bacterial translocation is the host immunodeficiency. The relationships between commensal bacteria and host defences are intimate and complex. In the early stages of animal development, the appearance of intestinal mucosal immunity in rabbits depends on the gut microflora (Rhee et al., 2004). It also plays a role in preimmune antibody repertoire and B cell selection (Rhee et al., 2005). In humans, bacterial translocation is necessary for the gut-associated lymphoid tissue (GALT) to develop normally (Gebbers et al., 2004).

The most striking fact is that microbe-associated molecular patterns are obviously shared by commensal and pathogenic bacteria. However, the host innate immune system discerns between signals triggered by commensals and those coming from pathogens. This is thought to occur, for instance, during maturation of dendritic cells

(DCs) in the gut, which, depending on the type of bacteria would mature in a way or another (Niess and Reinecker, 2006). DCs are thus educated by being challenged to luminal antigens or commensal bacteria. For instance, gut DCs engulf commensal bacteria which persist inside them for days stimulating IgA production. These commensal loaded-DCs remain in the mucosal compartment thanks to MLNs and thus, no responses are elicited systemically (Macpherson and Uhr, 2004).

In conclusion, commensal bacteria orchestrate the epithelial barrier-bacteria-mucosal immunity triad and determine bacterial translocation in the gut. Obviously, this implies very complex ecological interrelationships which are still to be understood.

3.2. Fish

It is generally accepted that pathogenic microorganisms may enter the fish through the gills, the skin or the GI tract (Wang and Leung; 2000; Birkbeck and Ringø, 2005). Fish gut is, in fact, continuously bombarded with multiple antigens present in the aquatic environment and in the food. Due to the fact that it is also a digestive organ, the GI tract represents a relatively easy to access surface for many pathogens pathogens. Enteric pathologies either caused by parasites or by bacteria have been described in the literature (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Toranzo et al., 2005; Palenzuela; 2006).

Ever since Plehn (1911) suggested that the intestine was an infection route for *Aeromonas salmonicida*, mounting evidence points the GI tract as a entry site for some pathogens in fish (Birkbeck and Ringø, 2005). It is necessary for them to adhere to and penetrate through the epithelial cells to spread systemically. It seems that internalization and cytotoxicity are important virulence mechanisms in vibrio-fish cell interactions (Wang et al., 1998). Adherence and internalization are speculated to be interdependent events but regulated by different processes. Vibrios that have high adherence capability may not have a high invasion rate but poorly adherent strains had low invasion rates (Wang et al., 1998).

Neither the adherence mechanisms of fish pathogens to fish cells nor the morphological changes undergone by the epithelium following adherence of pathogenic bacteria have been extensively investigated. Only recently, it has been observed that fish pathogens like *Aeromonas salmonicida* ssp. *salmonicida* and *Vibrio anguillarum* act on the

epithelium causing extensive damage and disrupting the integrity of the intestinal barrier, which would facilitate bacterial translocation (Ringø et al., 2007).

Despite the fact that the epithelial barrier function appears enhanced by probiotic bacteria in murine and human models (Madsen et al., 2001), the latter has not been demonstrated in a fish model.

4. The gut-associated lymphoid tissue (GALT) of teleost fish

4.1. General characteristics

The vertebrate immune system includes lymphoid organs considered primary and secondary, according to their ontogenic and functional characteristics. Secondary lymphoid organs, whose lymphoid cell populations depend on the primary lymphoid organs, include the spleen, lymph nodes and mucosa-associated lymphoid tissue (MALT), which includes the gut-associated lymphoid tissue (GALT).

Teleost fish also possess primary and secondary lymphoid organs but there are major structural and morphological differences between fish and mammalian immune systems. Fish have a thymus but lack bone marrow, the kidney being the main lymphoid organ (Press and Evensen, 1999). As regards the secondary lymphoid organs the most obvious difference is the absence of lymph nodes in fish, although both, spleen and MALT are present. Fish MALT includes skin, gills and gut-associated lymphoid tissues.

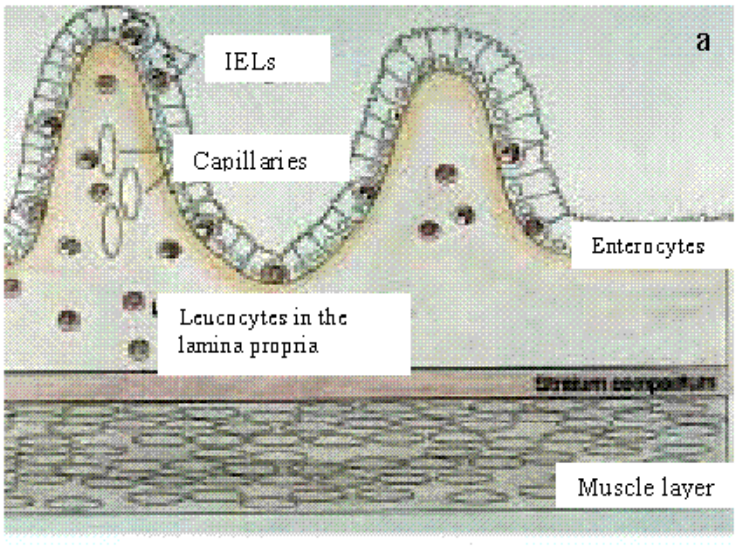

Figure 1a: Diagram showing the GALT in fish. Adapted from Press (1999)

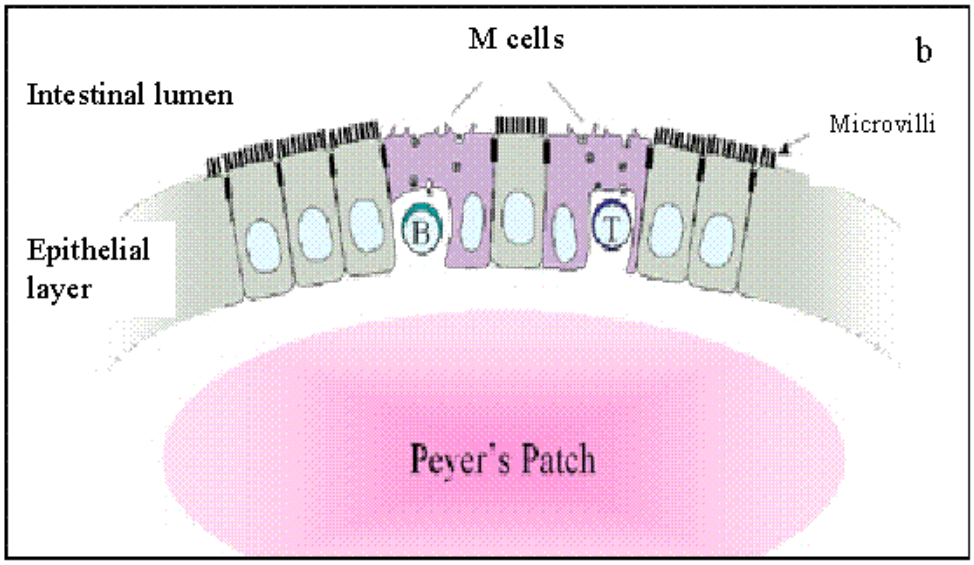

Figure 1b: Diagram showing the GALT in higher vertebrates. Obtained from www.rcai.riken.go.jp

The existence of mucosal immunity in fish was first realised in the early 80´s, when particulate antigens were given to fish and the uptake by intestinal cells was observed (Rombout et al., 1985; McLean et al., 1990). Additionally, orally immunised fish had detectable levels of Ig in the skin mucus, intestinal mucus and bile (Kawai et al., 1981). These findings had great importance because the possibility of orally vaccinating fish was for the first time regarded.

GALT has been described in both cartilaginous and bony fish species (Hart et al., 1988), where it does not appear to form structured aggregates such as the Peyer´s patches, which are characteristic of higher vertebrates (Fig. 1). Histologically, the gut tissue is composed by three layers: the serosa, muscularis and mucosa. The mucosal layer is made up by an epithelium with its corresponding basal membrane and underlying connective tissue named lamina propria. The lymphoid cells found in fish gut seem to be distributed in a diffuse manner along the lining of the alimentary canal both within the epithelium and in the lamina propria (Hart et al., 1988) although available data reveals great interspecies variability in this respect (Fournier-Betz et al., 2000).

In addition to this first difference, neither M cells, intestinal DC´s nor IgA nor an equivalent mucosal Ig isotype have been described in fish. In mammals, IgA is preferably secreted by B lymphocytes at mucosal sites and during local immune responses (Suzuki et al., 2006; McGhee et al., 2007). Isotype switching has not been described in lower vertebrates but it seems clear that the role of educated DCs and B lymphocyte selection are vital in mammalian models (Mora et al., 2006).

There are nevertheless two distinct populations of lymphoid cells in the fish gut in the same way they appear in higher vertebrates. They are known as intraepithelial lymphocytes (IELs) and leucocytes located in the lamina propia (LPLs). Both populations are found dispersed along the GI tract. Rainbow trout IELs mainly seem to be cytotoxic T lymphocytes (McMillan and Secombes, 1999). More recently, the TCR $\alpha\beta$ repertoire of rainbow trout IELs has been investigated showing greater variety than in higher vertebrates. This diversification was explained by the authors as a consequence of the absence of Peyer patches and MLNs in fish (Bernard et al., 2006). In other teleost species like the common carp, big macrophages are found intraepithelially in the gut. Concerning the number of antibody secreting cells, labelling of IELs with a monoclonal anti IgM antibody revealed that only 1% of total IELs are IgM^+ (Bernard et al., 2006) although other not yet characterised isotypes could be secreted by different B cell subsets in the same way s it happens in higher vertebrates. Future studies will throw light on this exciting field.

On the other hand, fish mucosal surfaces have a unique cell type known as rodlet cell. Rodlet cells are not consistently observed in all fish species or in all the individuals of a particular species. In the gut they are located within the epithelium from the basal to the apical side depending on their maturation stage. There is certain controversy concerning the biological significance of rodlet cells, firstly described as parasites and now more considered like fish leukocytes. Despite the greater consensus on latter hypothesis, more evidence is needed in order to characterise rodlet cells.

LPLs consist of lymphocytes, granulocytes, macrophages and plasma cells. The first studies conducted on carp and rainbow trout gut leucocytes already revealed low numbers of phagocytic cells in the gut. The phagocytic cells were able to ingest latex particles and to release free oxygen radicals (Davidson et al., 1991). Very little effort has been directed towards the functional characterisation and the role of fish GALT in

controlling bacterial translocation. The complex relationships between commensal bacteria and fish gut immunity are even less understood than in the case of mammals. For this reason, oral vaccines in aquaculture are still in its infancy, despite the fact that the first attempts for oral vaccination took place in the 80´s.

4.2. Methods for the isolation and purification of fish GALT

The diversity of fish ecology, biology, nutritional behaviour and physiology result in highly variable digestive system morphologies. Moreover, the alimentary canal exhibits adaptive flexibility in response to changes in dietary composition (Buddington and Hilton, 1987). Therefore, herbivorous, detritivorous, omnivorous and carnivorous fish species differ from each other in terms of the presence or absence of a stomach, the length of the intestine (from 1 to more than 20 times the body length), number and presence of pyloric caeca, intestinal loops and valves (Evans, 1998). In the particular case of the gilthead seabream, a carnivorous marine species, the digestive tract consists of an oesophagus, the stomach, few pyloric caeca and the intestine. The intestine includes a loop and it can be divided into foregut and hingut since it narrows from the stomach towards the anus. In other finfish species such as the carp, the intestine length is greater. It shows two loops and can be divided in up to four segments but no stomach or pyloric caeca are found (Fontaine, 1981). Salmonids, in turn, posses a short intestine that appears as a simple tube and the stomach is surrounded by hundreds of pyloric caeca. Thus, optimisation of the procedure is necessary in terms of time and cost due to the obvious anatomical and physiological differences in the gut between mammals and fish and between different fish species.

Fish gut immune cells merit investigation due to their direct implication in enteric diseases, antigen uptake, immunoglobulin production and, therefore, oral vaccination or therapy (Hébert et al., 2002). Different protocols have been reported to isolate and purify GALT cells, all of them based on one or more of the following treatments: mechanical, chemical or enzymatic (Lundqvist et al., 1992; Di Sabatino et al., 2007). Even more sophisticated alternatives are available involving the use of monoclonal antibodies and immunomagnetic techniques (Solano-Aguilar et al., 2000; Medina et al., 2004). Protocols developed to purify fish GALT cells (Rombout et al., 1993; Clerton et

al., 2001; Hébert et al., 2002; Bernard et al., 2006) have been adapted from those used routinely in mammalian species. No studies had yet applied them to the seabream.

It is normally assumed that lymphoid cells from gut tissue belong to two fractions: the DTT/EDTA-fraction, containing IELs, and the collagenase-fraction, containing the LPLs. IELs from rainbow trout were isolated without the use of DTT/EDTA by mechanically shaking gut fragments in culture media up to 5 or 6 times and collecting the cells from the supernatant after each agitation period.

Fish GALT has alternatively been isolated by some authors employing a purely mechanical procedure which implies the mechanical stripping of the gut epithelium from the underlying submucosa by means of a cell scraper (Rombout et al., 1993; 1998). This range of techniques yields cellular suspensions characterised by variable cell numbers, cell types and viability.

It is important to bear in mid that fish gut is a primary digestion organ and a secondary lymphoid with a heterogeneous nature. It produces variable amounts of mucus and it is subject to changes in diet, water microbiology and chemistry. Altogether, these factors make working with fish GALT a troublesome task and reduces the number of investigations dedicated to this subject.

5. Antiproliferative effects of commensal or probiotic bacteria

In higher vertebrates, suppression of intestinal inflammation (Riedel et al., 2006) and the generally accepted hyporesponsiveness of the intestine may be due to downregulatory effects by probiotic bacteria (Isolauri et al., 2002; Braat et al., 2004). In fact they have been shown to inhibit the proliferation of mononuclear cells (Pessi et al., 1999) and cell lines (Lee et al., 2004). Moreover, probiotics are considered as cancer prophylactics especially in colorectal affections (Rafter, 2002). In animal models they succeeded in prolonging survival in tumour-bearing animals (Lee et al., 2004). Antitumor vaccine efficacy is enhanced by probiotics too (Tanasienko et al., 2006).

Fish neoplasia has not been thoroughly investigated with the exception of those cases induced by chemical or toxicant exposure (Kinae et al., 1990). Indeed, fish models have been suggested as tools for screening and differentiating carcinogens since they could

develop liver tumours within only 2 months after treatment with diethylnitrosamine (Ding et al., 1989).

The antiproliferative effects of probiotic bacteria on fish cells (primary cultures and cell lines) have never been studied. Thus, it is unknown if probiotic bacteria are responsible, as it occurs in mammals, for controlling inflammatory responses in the host gut.

IV. OBJECTIVES

The general objective of this thesis is the study of the effects that probiotic bacteria produce in farmed teleost fish at the cellular level.
As individual or more specific objectives, we propose the following:

I. The systemic immune modulatory effects both *in vivo* and *in vitro* of probiotic bacteria on the seabream innate immune responses.

II. The role of probiotic bacteria in maintaining the morphology of the intestinal epithelial barrier in Atlantic salmon as well as the possible protective role against pathogen-caused tissue damage.

III. The isolation of the seabream gut leucocytes, the functional characterization of these cells and its modulation by probiotic bacteria.

IV. The evaluation of the antiproliferative potential of probiotic bacteria in fish cell lines and the mechanisms involved.

V. METHODOLOGY

OBJECTIVE I: IMMUNOMODULATORY EFFECTS OF PROBIOTIC BACTERIA ON SEABREAM INNATE IMMUNE SYSTEM

Bacteria

Two lyophilised bacteria strains were purchased from the *Colección Española de Cultivos Tipo* (CECT, Valencia, Spain). *Lactobacillus delbrüeckii* subsp. *lactis* (CECT 287) were grown in Man, Rogosa and Sharpe (MRS) Broth (*Laboratorios Conda*, Madrid, Spain) (pH 6.2; 37 °C) agar plates for 2–3 days. Colonies from cultured plates were then subcultured in liquid MRS Broth. *Bacillus subtilis* (CECT 35) colonies, which were grown in Nutrient Broth (Laboratorios Conda) (pH 6.8; 30 °C) plates, were subcultured in liquid Nutrient Broth with continuous stirring.

Another two bacteria, 51M6 and Pdp11, kindly provided by Dr. Moriñigo (University of Málaga Spain) were grown in tubes containing 5 ml of trypticase soya broth (Oxoid) supplemented with 1.5% NaCl (TSBs) at 22 °C, with continuous shaking for 18 h. A volume of the culture was spread onto plates of trypticase soya agar (Oxoid) supplemented with 1.5% NaCl (TSAs), using a cotton swab to achieve bacterial mass, and the plates were then incubated at 22 °C for 24 h. The bacterial suspensions were prepared by scraping the cells from the plates and washing them in sterile phosphate-buffered saline (PBS, pH 7.4).

The number of bacterial cells present per millilitre of culture medium of the four bacteria cultures was measured by using a Z2 Coulter Particle Counter (Beckman Coulter, Barcelona, Spain), and adjusted to the required concentrations. Bacterial cultures were heat-inactivated for 1 h at 60 °C in the experiments that state so.

In vivo studies

Fish

Two hundred specimens (65 g mean weight) of the hermaphroditic protandrous seawater teleost gilthead seabream (*Sparus aurata* L.) obtained from Culmarex S.A. (Murcia, Spain) were kept in eight 260 l running seawater tanks (25 fish per tank) (flow rate 1500 l/h) at 20 °C with a 12 h dark/12 h light photoperiod and fed with a commercial pellet diet (ProAqua, Palencia, Spain). Fish were allowed to acclimatise for 15 days before the start of feeding trials. They were starved for 24 h prior to sampling.

Specimens were sacrificed by an overdose of benzocaine (4% in acetone) (Sigma), weighed and measured. All experimental protocols were approved by the Bioethics Committee of the University of Murcia.

First trial

Experimental diets containing 0 (control), *L. delbrüeckii* ssp. *lactis*, *B. subtilis* or both bacteria were prepared in the laboratory from a commercial pellet diet (ProAqua, Palencia, Spain). Briefly, normal pellet diet was crushed, mixed with tap water (where bacterial suspensions were added at the desired volume) and made again into pellets. Re-made pellets were allowed to dry and stored at 4 °C until use.

In a first experiment, fish in each aquaria received one of the four different diets: fish in one aquarium were fed a diet consisting of a non-supplemented commercial pelleted diet (control group); the second group was fed a diet supplemented with 10^7 cfu/g *L. delbrüeckii* ssp. *lactis*; the third group was fed a diet supplemented with 10^7 cfu/g *B. subtilis* and the last group a diet supplemented with 0.5×10^7 cfu/g *L. delbrüeckii* ssp. *lactis* and 0.5×10^7 cfu/g *B. subtilis*. Fish were fed at a rate of 10 g dry diet/kg biomass (1%) per day for 1, 2 or 3 weeks. Afterwards, all fish were fed the control diet for another week before being sacrificed.

Second trial

Experimental diets containing 0 (control), heat-inactivated Pdp11, heat-inactivated 51M6 or both killed bacteria were prepared as explained above. The fish in each aquarium received one of the four different diets: a diet consisting of the non-supplemented commercial diet (control group); the same diet supplemented with 10^8 cfu/g heat-inactivated Pdp11; a diet supplemented with 10^8 cfu/g heat-inactivated 51M6 and, finally, the fourth group received a diet supplemented with 0.5×10^8 cfu/g Pdp11 and 0.5×10^8 cfu/g 51M6, both heat-inactivated. In this case, fish were fed at a rate of 10 g dry diet/kg biomass (1%) per day for 1, 2, 3 or 4 weeks.

The biomass of fish in each aquarium was measured before the experiments and daily ration was adjusted accordingly after each sampling. No mortality was observed during the experiments.

Serum collection and head kidney leucocyte isolation

Six fish from each aquarium were randomly sampled 1, 2, 3 and 4 weeks after the beginning of the feeding trial. Blood and head kidney (HK) samples were obtained from each specimen and several immunological parameters were determined, as described below.

Blood samples were collected from the caudal vein with a 27-gauge needle, 1-ml syringe and allowed to clot at 4 °C for 4 h. After centrifugation, the serum was removed and frozen at −80 °C until used to assess natural haemolytic complement activity and the peroxidase level.

HKLs were isolated under sterile conditions according to Esteban et al. (1998). Briefly, the head kidney was cut into small fragments and transferred to 8 ml of sRPMI medium:RPMI-1640 culture medium (Gibco) supplemented with 0.35% sodium chloride (to adjust the medium's osmolarity to gilthead seabream plasma osmolarity, 353.33 mOs), 100 I.U./ml penicillin (Flow), 100 µg /ml streptomycin (Flow), 10 I.U./ml heparin (Sigma) and 5% fetal bovine serum (FBS, Gibco). Cell suspensions were obtained by forcing fragments of the organ through a 100-µm nylon mesh. After two washes (400 g, 10 min, 4 °C), HK leucocytes were counted in a Neubauer chamber and adjusted to 10^7 cells/ml of sRPMI.

Natural haemolytic complement activity

The natural haemolytic complement activity of the alternative pathway was assayed using sheep red blood cells (SRBC, Biomedics) as targets (Ortuño et al., 1998). SRBC were washed in phenol red-free Hank's buffer (HBSS) containing Mg^{2+} and EGTA and resuspended at 3% (v/v) in HBSS. Aliquots of 100 µl test serum as complement source, serially diluted in HBSS containing Mg^{2+} and EGTA to give final serum concentrations ranging from 10% to 0.078% were mixed with an equal volume (100 µl) of SRBC in a round-bottomed 96-well plate. After incubation for 90 min at 22 °C, the samples were centrifuged at 400 g for 5 min at 4 °C to remove unlysed erythrocytes. The relative haemoglobin content of the supernatants was assessed by measuring their optical density at 550 nm in a spectrophotometer (BMG, Fluoro Star Galaxy). The values of maximum (100%) haemolysis were obtained by adding 100 µl of distilled water to 100 µl samples of SRBC and minimum (spontaneous) haemolysis was obtained from SRBC without serum.

The degree of haemolysis (Y) (percentage of haemolytic activity with respect to the maximum) was estimated and the lysis curve for each specimen was obtained by plotting $Y/(1-Y)$ against the volume of serum added (ml) on a $\log_{10}$–$\log_{10}$ scaled graph. The volume of serum producing 50% haemolysis (ACH_{50}) was determined and the number of ACH_{50} units/ml obtained for each experimental group.

Peroxidase content

The total peroxidase content present in serum or inside leucocytes was measured according to Quade and Roth (1997). Briefly, 15 µl of serum was diluted with 35 µl of Ca^{2+}- and Mg^{2+}-free HBSS in flat-bottomed 96-well plates. Then, 50 µl of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB, Sigma) and 5 mM H_2O_2 (Sigma) were added (both substrates of peroxidase). To estimate the leucocyte peroxidase content, 10^6 HK leucocytes in sRPMI per well were dispensed into round-bottomed 96-well plates. The plates were centrifuged (400 g, 10 min, 22 °C) and the supernatants were removed. Leucocytes were then lysed with 75 µl of 0.02% cetyltrimethylammonium bromide (CTAB, Sigma) on a shaker at 40 cycles per min. Afterwards, 50 µl of 10 mM TMB and 5 mM H_2O_2 were added. Then, 150 µl of serum was transferred from each well to new 96-well plates. In both cases, the colour-change reaction was stopped after 2 min by adding 50 µl of 2 M sulphuric acid and the optical density was read at 540 nm in a multiscan reader. Standard samples without serum or leucocytes, respectively, were also analysed.

Phagocytic activity

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C) by gilthead seabream HK leucocytes was studied by flow cytometry according to Rodríguez et al. (2003). Heat-killed and lyophilized yeast cells were labelled with fluorescein isothiocyanate (FITC, Sigma), washed and adjusted to 5×10^7 cells/ml of sRPMI. Phagocytosis samples consisted of 125 µl of labelled yeast cells and 100 µl of HK leucocytes in sRPMI (6.25 yeast cells:1 leucocyte). Samples were mixed, centrifuged (400 g, 5 min, 22 °C), resuspended in sRPMI and incubated at 22 °C for 30 min. At the end of the incubation time, the samples were placed on ice to stop phagocytosis and 400 µl ice-cold PBS was added to each sample. The fluorescence of the extracellular yeasts was quenched by adding 40 µl ice-cold trypan blue (0.4% in PBS). Standard samples of

FITC-labelled *S. cerevisiae* or HKLs were included in each phagocytosis assay. All samples were analysed in a flow cytometer FACScan (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 5000 cells, which were acquired at a rate of 300 cells/s. Data were collected in the form of forward scatter (FSC), side scatter (SSC) and green fluorescence (FL1). The cytometer was set to analyse the phagocyte population identified by their high FCS and SSC. Phagocytic ability was defined as the percentage of cells with one or more ingested yeast cells (green-FITC fluorescent cells) within the phagocyte cell population. The relative number of ingested yeasts per cell (phagocytic capacity) was assessed in arbitrary units from the mean fluorescence intensity of the phagocytic cells.

Respiratory burst activity

The respiratory burst activity of gilthead seabream HKLs was studied by a chemiluminescence method (Bayne and Levy, 1991). One hundred µl (10^7 leucocytes/ml) in sRPMI and 100 µl of a phorbol myristate acetate (PMA)/luminol solution (HBSS with Ca^{2+} and Mg^{2+} containing 1 µg/ml PMA, Sigma and 10^{-4} M luminol, Sigma) were placed in the wells of a flat-bottomed 96-well microtiter plate. The plate was shaken and immediately read in a chemiluminometer (BMG, Fluoro Star Galaxy). Chemiluminescence measurements were performed in 30 cycles of 2 min each. The kinetics of the reactions were analysed and the maximum slope of each curve was calculated. Backgrounds of luminescence were calculated using reactant solutions containing luminol but not PMA. Control samples contained only leucocytes.

Natural cytotoxic activity

The natural cytotoxic activity of gilthead seabream HKLs was evaluated using a flow cytometry technique based on a double-fluorescent labelling (Cuesta et al., 1999). Each cytotoxic assay was carried out in duplicate. L-1210 tumour cells (mouse lymphoma, *American Type Culture Collection*, ATCC CCL-219) were cultured in sRPMI-1640 culture medium. They were incubated at 37 °C, with 85% relative humidity and 5% CO_2 atmosphere and maintained in exponential growth. Tumour cells were labelled by incubating with 10 µg/ml of 3,3'dioctadecyloxacarbocyanine perchlorate (DiO, Sigma) for 90 min in darkness. After labelling, free DiO was removed by washing three times in PBS and cell staining uniformity was examined by flow cytometry. Leucocytes

(10^7 cells/ml) in sRPMI (effectors) were mixed with DiO-labelled L-1210 cells (10^6 cells/ml) (targets) to obtain a final effector:target ratio of 50:1. Samples were centrifuged (400 g, 1 min, 22 °C) and incubated at 22 °C for 3 h. Cytotoxic samples incubated for 0 h (control) were used to determine initial target viability. At the end of the incubation period, 30 µl of propidium iodide (400 µg/ml, PI, Sigma) was added to each sample and mixed gently before analysis in a FACScan flow cytometer adjusted to obtain optimal discrimination of the target cell population. Data were collected in the form of two-parameter FSC, SSC, FL1 (green fluorescence, DiO) and FL2 (red fluorescence, PI) dot plots and histograms. Each analysis was performed on 3000 cells, which were acquired at a rate of 300 cells/s. The FACS only accepted the positive FL1 region, which corresponded to DiO-labelled target cells. The percentage of dead or non-viable target cells showing green and red fluorescence was related to the cytotoxic activity of gilthead seabream leucocytes. Cytotoxic activity, a parameter describing the percentage of non-viable target cells, was calculated using the formula:

$$\text{Cytotoxic activity } (\%) = 100\left(\%_{\text{sample}} - \%_{\text{control}}\right)/\left(100 - \%_{\text{control}}\right).$$

In vitro studies

HKLs were isolated from each fish (n = 6) under sterile conditions as explained before. After two washes, HKLs were counted in a Neubauer chamber and adjusted to 10^7 cells/ml of sRPMI. Heat-inactivated bacteria were adjusted in PBS to 10^6, 10^7 and 10^8 cfu/ml and 50 µl of each suspension was added to samples of 50 µl of HKLs (final concentration 5×10^5, 5×10^6 and 5×10^7 cfu/ml). After 30 min of incubation, cellular innate immune parameters were measured. Controls consisted of leucocytes incubated with 50 µl of PBS without bacterial cells.

After incubation, different cellular innate immune parameters (leucocyte peroxidase content and phagocytic, respiratory burst and cytotoxic activities) were evaluated using the same methodology as in the *in vivo* studies.

Statistical analysis

The results are expressed as the stimulation index (mean + standard error, SE), which was obtained by dividing each sample value by the mean control value at the same sampling point for each measured parameter. Values higher than 1 reflect an increase and lower than 1 a decrease in each parameter. The data from the flow cytometric assays were analysed using the statistical option of the Lysis Software Package (Becton Dickinson). Data were statistically analysed by one-way analysis of variance (ANOVA) and Tukey's comparison of means when necessary. Differences were considered statistically significant when $P < 0.05$ at all times.

OBJECTIVE II: STUDIES ON THE INTEGRITY OF THE GUT EPITHELIAL BARRIER

This objective was achieved by conducting two experimental trials both in Atlantic salmon.

First experimental trial

Fish

Unvaccinated Atlantic salmon (*Salmo salar* L.) of the Norwegian Breeding Programme were maintained, from the time of hatching, in freshwater under continuous light Aquaculture Research Station, Matredal, Norway until an average weight of 73 g was achieved. They were then transferred to the disease-challenge laboratory at the *Institute of Marine Research* IMR in Bergen where they were maintained in 150-l tanks (100 fish per tank). The water temperature was 10 °C, and the water flow was 1000 l/h. Fish were left to adapt to the new conditions for 15 days before the initiation of experiments. From initial feeding to the end of the experiment, they were fed to excess by using 24 h disc feeders loaded with commercial diets suitable to their relative size (EWOS Innovation). The experimental protocols and procedures were approved by the Animal Use and Care Committee at IMR, Bergen, Norway.

Bacteria

Aeromonas salmonicida ssp. *salmonicida* strain VI-88/09/03175 (culture collection, Central Veterinary Laboratory, Oslo, Norway) was used as this strain is pathogenic to salmonids (Samuelsen et al., 1998). Bacteria were stored in cryotubes with glycerol at −80°C and inoculated into brainheart infusion medium (BHI; Merck, Germany). After 24 h of incubation, 1% of this culture was inoculated into a new culture and incubated for another 24 h. This suspension was adjusted to a transmission at 600 nm (T600) of 3.5 by adding BHI. The bacteria were washed four times in phosphate buffered saline, resuspended in 10 ml salmon Ringer solution (140 mM NaCl, 2.5 mM KCl, 1.5 mM $CaCl_2$, 0.8 mM $MgSO_4$, 10 mM glucose, HEPES 5 mM, pH adjusted to 7.8 with 1.5 mM TRIS base) and stored refrigerated until used. The viability of the bacteria was

tested by plating out bacterial suspensions on BHI-Agar plates with added 1.5% NaCl. More than 90% of the bacteria were viable.

Carnobacterium divergens 6251 originally isolated from the foregut of Arctic charr (*Salvelinus alpinus* L.) and stored in cryotubes with glycerol at −80°C were incubated in TSB with added glucose (5 g/l) and NaCl (15 g/l), (TSBgs medium). After a 24 h incubation, 1% of the culture was inoculated into new TSBgs medium and incubated for 24 h. This culture was adjusted to a transmission at 600 nm (T600) of 3.5 by adding TSBgs. The washing procedure was similar to that reported for *A. salmonicida*. The viability of the bacteria was tested by plating out bacterial suspensions on TSA plates (Oxoid) with added 5 g glucose and 15 g NaCl (TSAgs plates). Approximately 90% of the bacteria were viable.

Vibrio anguillarum LFI 317 serotype O1 originally isolated from wrasse (cleanerfish) stored in cryotubes with glycerol at −80°C were incubated in TSBgs medium. The bacteria were cultured for 24 h. The cells were harvested, washed, and resuspended as described for *C. divergens*. More than 90% of the bacteria were viable.

In vitro exposure to bacteria

Fish raised at IMR were killed by a blow to the head, the intestine (from just posterior to the attachment of the pyloric caeca to the anus) was carefully removed, and the intestinal contents were washed by rinsing with salmon Ringer solution. Intestines were tightly tied at one end and filled up with approximately 1 ml appropriate bacterial suspension (see Table 2). The other end was immediately tied and placed into glass tubes containing sterile saline solution. Samples were incubated at 10 °C for 1 h in a cooling bath, after which the intestine was cut free, and a 0.5 mm segment from the anterior part of the intestine was excised. The exact bacterial exposure of the foregut was measured by plate counts. Stock solution was diluted in sterile 0.9% saline, and 0.1 ml volumes of appropriate dilutions were spread on the surface of BHI-Agar (*A. salmonicida*) and TSAgs plates (*V. anguillarum* and *C. divergens*).

Microscopic study

Foregut segments sampled from three fish from each treatment group were exposed only to *A. salmonicida*, *V. anguillarum*, or *C. divergens*, or to a mixture of *A. salmonicida* and *C. divergens* or *V. anguillarum* and *C. divergens* or were not exposed

to bacteria. They were immediately fixed in McDowell's fixative (McDowell and Trump, 1976) and prepared for analyses by light microscopy (LM), transmission electron microscopy (TEM), and scanning electron microscopy (SEM). For LM, segments were washed twice in Sørensen's buffer and then postfixed in OsO_4 (1% in Sørensen's buffer, 2 h). After serial dehydration steps in alcohol, samples were placed in 1,2-propylenoxide prior to being embedded in Agar 100 Resin. Sections (1 μm thick) were stained with 2% toluidine blue and examined under a Leica DMLB light microscope. Images were acquired by means of a Leica DC 300 digital camera.

For the processing of salmon intestine segments for TEM, gut pieces were washed in phosphate buffer (300–320mOsm, pH 7.4), postfixed for 1.5 h in OsO_4, rewashed in phosphate buffer and stained en bloc in 2% aqueous uranyl acetate. After dehydration in a graded series of ethanol concentrations, the specimens were embedded in Epon/Araldite via propylene oxide. Ultrathin sections were contrasted with uranyl acetate and lead citrate. TEM samples were examined under a Jeol JEM-1230 transmission microscope.

SEM samples were washed twice in Sørensen's buffer and then postfixed in OsO_4 (1% in Sørensen's buffer, 2 h). After serial dehydration steps in alcohol from 30% to 100%, samples were critical-point dried (Polaron, CPD7501), mounted on aluminum stubs, sputter-coated with gold by using a high-resolution fine coater (JEOL JFC-2300HR), and examined under a Jeol JSM-7400F scanning microscope.

To determine morphological differences in the salmon foregut of the various treatments, ten randomly sampled TEM images from each fish in each treatment group were taken. The impacts of treatments were monitored in terms of the presence of cell debris in the lumen, disorganized microvilli, protruding epithelial cells, edema, disintegrated tight junctions, dark cellular bodies under lamina propria, loss of epithelial integrity, and phagolysosome-like vesicles with bacteria and bacteria-like particles close to tight junctions. Differences were ranked as follows: 0=not observed, 1=low (1–3 out of 10 images), 2=moderate (4–6 out of 10 images), and 3=high (7 or more out of 10 images) frequency of occurrence. To compare different treatments, we applied a non-parametric Page's L trends test (Miller, 1975) with k treatment groups and N morphological variables. Data were handled by using Unistat v5.5 statistical software (Unistat, London). We tested the null hypothesis of no difference between treatments.

Treatment number	Bacterial strain and dose (cfu/ml)
1	Salmon Ringer solution
2	*A. salmonicida* 6×10^6
3	*V. anguillarum* 6×10^4
4	*V. anguillarum* 6×10^6
5	*C. divergens* 6×10^4
6	*C. divergens* 6×10^6
7	*A. salmonicida* 3×10^6 and *C. divergens* 3×10^6
8	*V. anguillarum* 3×10^4 and *C. divergens* 3×10^4
9	*V. anguillarum* 3×10^4 and *C. divergens* 3×10^6
10	*V. anguillarum* 3×10^6 and *C. divergens* 3×10^4
11	*V. anguillarum* 3×10^6 and *C. divergens* 3×10^6

Table 2: Experimental treatments applied to Atlantic salmon intestine during *in vitro* exposure to various bacterial strains.

Second experimental trial

Fish

Sixteen unvaccinated Atlantic salmon (*Salmo salar* L.) (Aquagen) reared from hatching until an average weight of 140 g at the IMR, Matre Aquaculture Research Station, Matredal, Norway were transferred to the disease challenge laboratory in Bergen. Here they were maintained in one 400 l tank supplied with aerated seawater at flow of 600 l/h. The water temperature was 9°C. Fish were acclimatised for 15 days before experimental start.

The fish were fed to excess using 24 h disc feeders loaded with a commercial diet (3.0 mm Spirit 50, Skretting Ltd) suitable to their relative size. The experimental protocols and procedures were approved by the Animal Use and Care Committee at IMR, Bergen, Norway.

Bacteria

Lactobacillus delbrueckii ssp. *lactis* were grown as previously described. Numbers of bacteria were estimated by measuring the optical density at 550nm and recounted by plating serial dilutions of the bacterial suspensions onto MRS plates.

Aeromonas salmonicida ssp. *salmonicida* pathogenic to salmonids (Samuelsen, 1998) was inoculated onto TSAgs plates and incubated at 20 °C for 48 h. After incubation, 1% of the growth culture was inoculated in TSBgs and incubated for 24 h. Numbers of *A. salmonicida* was tested by plating serial dilutions out bacterial suspensions on TSAgs plates.

In vitro exposure to bacteria

In the *in vitro* experiments three fish were used per treatment. Intestines were incubated either with TSB and saline solution (1:1) (controls), with *Lactobacillus* only, with *Aeromonas* only, or with *Lactobacillus* first and *Aeromonas* afterwards. The fish, anaesthetized, were killed by a blow to the head, and the ventral belly surface of the fish was open to exposure the peritoneal cavity, and spleen, gallbladder and liver were removed. The intestines (from just posterior to the attachment of the pyloric caeca to the anus) were carefully removed, emptied and thoroughly rinsed three times with salmon Ringer solution. Intestines were tightly tied at the posterior end and filled with approximately 1ml of *L. delbrueckii* ssp. *lactis* ($1.6x10^5$ cfu/ml) or sterile saline: TSBgs (1:1) (control). Thereafter the distal intestine was immediately tied up and the gut was placed into glass tubes containing sterile 0.9 % saline solution. After incubation at 9 °C for 30 min in a cooling bath, the intestines were cut free at both ends, rinsed with 5 ml of sterile saline: TSB (1:1), tied up again at the posterior end, and filled with 1 ml of either *A. salmonicida* ($7x10^7$ cfu/ml) or sterile saline: TSB (1:1) (control), closed at the distal end and incubated for 30 min. *A. salmonicida* was not washed prior to use. After the second incubation the intestines were open at both ends and rinsed with 5 ml sterile saline and TSBgs (1:1) solution. A 0.5 cm segments from the anterior part of the intestine were excised.

The exact amount of bacteria to which the foregut was exposed to was measured by plate counts. The stock solution was dilute in sterile 0.9 % saline and 0.1 ml volumes of appropriate dilutions were spread on the surface of TSAgs plates.

TRITC –labelling of L. delbrueckii ssp. *lactis*

L. delbruekii ssp. *lactis* was cultured and counted as described above, and the optical density measured at 550nm in order to quantify bacterial concentration. The bacterial suspension was washed four times (2800 g, 15 min, 4 °C) in conjugation buffer (NaCl 0.15M/ Na_2CO_3 0.1M; pH 9). The final concentration of the suspension was adjusted to $2.7x10^8$ cfu /ml in conjugation buffer. 10 µl of a stock solution of tetramethylrhodamine isothiocyanate (TRITC, Sigma) were added to the bacterial suspension and shaken gently at room temperature and in the dark for 1 h. 100 µl aliquots were stored at -20°C until use. Labelling was confirmed by use of a FACScan set to accept the positive (FL2) region, which corresponds to TRITC labelled bacterial cells. 99.9% of the bacteria were FL2-positive. Additionally, TRITC labelled- *L. delbrueckii* ssp. *lactis* was reconfirmed under confocal microscope Leica TCS SP2 AOBS (Leica Microsystems, Germany).

Confocal microscopy study

After incubation of salmon intestine for 30 min with 1ml of 10^7 cfu/ml TRITC labelled *L. delbrueckii* ssp. *lactis*, intestines were cut free, rinsed with 5ml of sterile saline and the anterior part of the intestine, 0.5 cm, was dissected and dried in filter paper prior to freezing. The tissue segments were placed in pre-cooled cryotubes floating in liquid nitrogen, and thereafter stored at -80°C until use. 10 µm-thick cryosections were obtained from each sample, fixed with 4% paraformaldehyde (10 min, room temperature), air dried and mounted onto coverslips with 10 µl of Vectashield Mounting Medium® with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Ltd, UK). Sections were observed under a confocal microscope using an argon ion (488 nm) and a He/Ne (594 nm) laser. TRITC emission was detected at 596-700 nm and fluorescent images were obtained with immersion oil x63 objective by an inverted light microscope connected to the confocal microscope. Original Leica TCS SP2 AOBS software was used for image acquisition and processing.

Microscopical sampling

Intestinal segments not exposed to bacteria (control), or intestinal segments exposed to *A. salmonicida* only, *L. delbrueckii* ssp. *lactis* only or *L. delbrueckii* ssp. *lactis* and then *A. salmonicida* were immediately fixed in McDowell's fixative and prepared for

analyses by light microscopy (LM) and transmission electron microscopy (TEM) as described in pages 29-30.

OBJECTIVE III: GALT ISOLATION AND MORPHOFUCTIONAL CHARACTERIZATION IN THE SEABREAM.

Fish

Fifty specimens of seabream (*Sparus aurata* L.) (150 g mean weight) were obtained from Culmarex S.A. (Murcia, Spain). Fish were allowed to acclimatised, and fed as explained in objective I, page 22. They were starved for 48 h before being sacrificed by an overdose of benzocaine, bled and then ventrally dissected to obtain their gut.

Protocols for the isolation of gut leucocytes

The digestive tract of each specimen from the pyloric caeca to the anus was collected. Head-kidneys was obtained as a positive control for the different assays. After weighing, the dissected tissues were placed in a Petri dish and washed in ice cold PBS to remove the connective tissue and gut contents. The whole sample was dissected into approximately 1 cm long fragments, which were then longitudinally opened by means of a scalpel. The range of protocols tested was the result of reviewing the current literature (Davidson et al., 1991; Lundqvist et al., 1992; Rombout et al., 1993; Rodrigues et al., 1995; McMillan and Secombes, 1997; Clerton et al., 2001; Hébert et al., 2002; Schwarz et al., 2005), with incubation time and enzyme concentration as the two variable parameters.

Mechanical

The isolation of gut leucocytes without the use of chemical or enzymatic treatments involved the mechanical stripping of the gut epithelium from the underlying submucosa by means of a cell scraper. Briefly, gut segments of about 1 cm long were placed in a Petri dish with 10 ml of cold sRPMI containing 10 I.U./ml heparin. Fragments were dissected longitudinally and the epithelium was carefully separated from the underlying mucosa. Then, after passing through a sterile 100 µm mesh, they were washed twice in sRPMI as before.

Chemical

Gut fragments were placed in 50 ml sterile test-tubes containing 15 ml of cold PBS with EDTA (0.37 mg/ml) and DTT (0.145 mg/ml, Sigma). DTT is a reducing agent frequently used to reduce the disulfide bonds of proteins and, more generally, to prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues of proteins. In the case of the gut tissue, it reduces the bonds present at the tight junctions present between the epithelial cells. The tubes were mechanically shaken (60 rpm, 10 min, 22 °C) so that cells located between epithelial cells were released from the tissue into the suspension. Supernatants were collected (S_1) and sieved through a 100 µm sterile mesh. Solid fragments were washed in cold HBSS supplemented with 5 % FBS, 100 I.U./ml penicillin (Flow), 100 µg/ml streptomycin (Flow), DNAse I (0.05 mg/ml; Sigma) to prevent possible inhibition of collagenase by DTT.

Enzymatic

Enzymatic treatment was carried out by stirring the sample (same conditions as before) in 15 ml of washing medium with 0; 0.15 or 0.37 mg/ml collagenase (Sigma) for 30, 60 or 120 min at room temperature. During the collagenase treatment, the connective tissue that houses LPLs is digested and cells are released into the media. Afterwards, the supernatants as well as the gut fragments were sieved through a 100 µm sterile mesh. Total cell suspensions (S_2) were washed twice in 10 ml sRPMI by centrifuging (400 g, 10 min, 4 °C). Tissue fragments from intestines were taken at different stages of the isolation protocols (chemical treatment consisting of 10 min in DTT-EDTA; enzymatic treatments after 30, 60 or 120 min incubation in washing media containing collagenase (0, 0.15 or 0.37 mg/ml) in order to visualise the different degrees of digestion undergone by the gut epithelium and lamina propria. Light microscopy samples consisted of fragments fixed immediately in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2, 3 h, 4 °C), washed in 0.1 M cacodylate buffer, then postfixed in OsO_4 and embedded in Epon. Thin sections were stained with toluidin blue and examined by a Leica (DMRB) light microscope.

Cell counts

At all times, it was necessary to thoroughly mix cell pellets with a Pasteur pipette prior to counting in order to avoid large cell clusters. Cell aggregation was minimised by the use of nylon wool columns. The purified seabream gut cells were counted in a Neubauer chamber and adjusted to 10^7 cells/ml.

Cell viability and flow cytometry study

Aliquots of 50 µl cell suspensions from the different isolation procedure assays were placed in 5 ml test-tubes and 30 µl of PI (400 µg/ml) were added to each sample. The tubes were gently mixed before analysis in a FACScan as previously described. Instrument setting were identical throughout the whole study. Dead cells were estimated as the percentage of cells with propidium iodide (red-PI fluorescent cells).

Nylon wool columns

Nylon wool columns were not used to separate a particular cell population of the total gut cell suspensions. They were made by tightly packing 0.5 g of nylon wool fibre (Kisker-Biotech) into a 5ml syringe. The column size was chosen according to the manufacturer's specifications. The columns were autoclaved and incubated for 1 hour at 24 °C with 5 ml sRPMI. Then 5 ml (10^7 cells/ml) of the gut cell suspension (S_2) were loaded into the column and incubated for 1 h at 24 °C after which, non-adherent cells were eluted by rinsing the column with sRPMI and washed twice as before. The resulting cell suspension was called NW. Viability, cell counts and flow cytometry analyses were conducted on this suspension. Additionally, nylon wool fibres from columns incubated with seabream gut cell suspensions from three specimens were fixed after being eluted. The latter aimed to further investigate which elements of the original suspension were being retained by the fibres. Briefly, samples were fixed in 3% glutaraldehyde and postfixed in 1% OsO_4, dehydrated in acetone, critical-point dried, sputter-coated with gold and studied by SEM.

Continuous Percoll gradients

A continuous Percoll gradient was established between 25% and 75% in a 50 ml plastic test-tube. Three millilitres of GALT leucocyte suspensions (S_2 or NW) were carefully layered over the Percoll gradients. Density beads (Sigma) were used to determine the

density of the bands found after centrifuging for 30 min at 400 g and 4 °C. After centrifugation, each band of cells present in the Percoll gradient was collected and washed twice in sRPMI prior to observation by light microscopy and flow cytometry. Cell viability was measured as before.

Microscopy

Light microscopy samples of the different cell suspensions obtained during the protocol were prepared by cytocentrifugation. Additionally, cytocentrifugation of aliquots of 5 x 10^5 cells (690 g, 10 min, 22 °C) of each band was also performed on glass slides. The slides were air dried, fixed for 3 min in pure methanol and stained with Giemsa (Merck; 1:10 in tap water) for 60 min. The slides were then washed to discard excess Giemsa, air-dried and mounted on DPX. Images were acquired with a Leica DC500 digital camera attached to a light microscope with x100 oil objective.

Respiratory burst (NBT) assay

Superoxide anion (O_2^-) production, by total cell suspensions obtained from seabream intestines by mechanical stripping, after 60 min in collagenase (0.15 mg/ml) or after 120 min in collagenase (0.37 mg/ml) was measured by the nitroblue tetrazolium (NBT, Sigma) method adapted from Muñoz et al. (2000). These three protocols were chosen because they theoretically represent different degradation levels of the gut lamina propria housing the phagocytic cells. Briefly, 100 µl of each cell suspension were plated in 96-well plates in triplicate. 100 µl of NBT (2 mg/ml in HBSS) with or without PMA (2 µg/ml) were added to each well. Positive controls consisted of suspensions of HKLs from the same fish. HK is known to contain high numbers of phagocytic cells in teleost fish that respond to PMA giving positive respiratory burst reactions. Plates were incubated for 60 min at 24 °C in a moisture chamber and then washed twice in PBS. PBS was carefully removed with a pipette and the plates were immediately examined under a Leica inverted light microscope coupled to a Leica DFC280 digital camera with an x40 objective. Cells containing formazan precipitates (NBT positive) were counted in each well.

Cell isolation

Gut leucocytes were obtained by chemical and enzymatical treatment. After the DTT/EDTA step, gut fragments were incubated in 0.15 mg/ml collagenase for 60 min at room temperature. The subsequent steps were conducted as explained in page 36. HKLs were isolated from each fish under sterile conditions as explained in page 24.

Phagocytosis

The phagocytosis of *Saccharomyces cerevisiae* (strain S288C), latex beads (2µm particle size, Sigma) or heat-inactivated *Lactobacillus delbrüecki* subsp. *lactis* by seabream GALT leucocytes was studied by TEM. Heat-killed and lyophilized yeast cells were adjusted to 5×10^7 cells/ml of sRPMI and added to GALT leucocytes in sRPMI (6.25 yeast cells: 1 leucocyte). Ten latex beads or *L. del. lactis* cells were added to each seabream gut leucocyte. *L. del. lactis* was grown and inactivated as explained before. Samples were mixed, centrifuged (400 g, 5 min, 22 °C), resuspended in sRPMI and incubated at 22 °C for 60 min except for the latex beads, which were incubated for 180 min. Pellets were obtained by centrifugation (400 g, 10 min. 22 °C) and processed as explained in page 30.

Respiratory burst

The respiratory burst activity of seabream GALT and HK leucocytes was studied by the Bayne and Levi chemiluminescence method explained in page 26. Additionally, GALT leucocytes were centrifuged (400 g, 10 min, 22 °C) and resuspended in sRPMI with 0.1 % FBS. Cells were allowed to adhere and then non adherent cells were collected and transferred to fresh wells. Adherent cells were washed twice in PBS. Both adherent and non-adherent samples were adjusted to a final volume of 100 µl sRPMI and respiratory burst was of whole HK or GALT leucocytes or adherent/non-adherent GALT isolated leucocytes was measured. The total area under the response curve was calculated. Backgrounds of luminescence were calculated using reactant solutions containing luminol but not PMA.

Peroxidase activity and natural cytotoxicity

Peroxidase activity present inside leucocytes and the natural cytotoxic activity of seabream GALT and HK leucocytes was measured as described before.

Protein A-FITC labelling

We used an experimental approach to determine the presence of Ig^+-cells by using fluorescein isothiocyanate (FITC)-labelled protein A based on the ability to bind immunoglobulins, including IgM. Prsence of IgM in fish gut has been analysed by other authors by means of monoclonal antibodies (Picchieti et al., 1997; Rombout et al., 1998; Bernard et al., 2006). Briefly, 10^5 leucocytes from GALT or HK were dispensed in triplicate in a 96 well plate. Cells were washed in PBS containing 2% bovine serum albumin (BSA) and 0.05 % sodium azide and adjusted to a final volume of 100 µl prior to the addition of 100 µl of FITC labelled- protein A (Sigma, 10 µg/ml) to each well. The plate was incubated at 4 °C for 30 min in the dark, after which cells were washed twice as before. Control samples consisted of unlabelled seabream cells. Samples were analysed in a flow cytometer.

Data analysis

Data are expressed as mean ± se.

OBJECTIVE IV: ANTIPROLIFERATIVE EFFECTS OF PROBIOTIC BACTERIA ON FISH CELL LINES

Bacteria and cytoplasmic extracts

Lactobacillus delbrüeckii ssp. *lactis* and 51M6 were grown and counted as explained before and then adjusted to 10^8 cfu/ml and kept at 4 °C.

The cytoplasmic extracts of the bacteria were obtained by disruption of cell walls in a French Press and ultracentrifugation at 70000 g for 30 min. Samples were then sterilized with a 0.2 µm filter, aliquoted and stored at -80 °C. One sample from each bacterium was used to quantify the amount of protein present in each cytoplasmic extract. The protein concentrations were estimated by the BCA protein assay reagent (Sigma) using bovine serum albumin (BSA) as a standard. Samples were adjusted to 100 µg/ml of protein.

Cell lines

SAF-1 cell line, a fibroblast-like culture derived from the gilthead seabream was purchased from the European Collection of cell lines ECACC (UK) and grown in sRPMI supplemented with 10% FBS at 23 °C , 5% CO_2 in 25 cm^2 culture flasks. Cells were trypsinised, washed twice in sRPMI (400 g, 10 min, 23 °C), counted in a Neubauer chamber and adjusted to 10^5 cells/ml. 96 flat bottomed culture plates were seeded with 100 µl of the cell suspension.

Epithelioma papulosum carpio (EPC) cells were maintained at 23 °C in DMEM 4.5 (Sigma) containing glutamine (2 mM; Gibco), piruvate (1 mM; Gibco), fungizone (2 µg/ml; Gibco) and gentamicine (50 µg/ml; Gibco) and supplemented with 10% FBS.

Cells were trypsinised, washed twice in DMEM (400 g, 10 min, 23 °C), counted and adjusted to $2x10^5$ cells/ml. 100 µl of the cell suspension were dispensed onto 96 flat-bottomed well plates.

One hundred µl of the bacterial cytoplasmic extracts adjusted to 25, 50 and 100 µg/ml in PBS were added in triplicates to the wells. The range of concentrations was chosen base don previous studies with other cells lines and bacterial strains. Controls consisted of wells with cells to which 100 µl of PBS were added. Plates were incubated for 4, 24, 48 or 72 h at 23 °C, 5% CO_2.

Proliferation Assay

The antiproliferative activity of probiotic bacteria cytoplasmic fractions was evaluated *in vitro* by means of the crystal violet assay as used in other studies (Pessi et al., 1999). Briefly, 100 µl of culture media were removed from each well and the same volume of Carnoy fixative was added. After 5 min, the entire volume was removed and 200 µl of Carnoy were added. Cells were fixed for 10 min and then the fixative was removed. Plates were air-dried and stained with 200 µl of 0.1% crystal violet solution (Panreac) for 30 min at room temperature. The plates were washed 3 times under tap water, carefully dried and the dye present inside cells solubilized with 100 µl of a 10% methanol and 5% acetic acid solution. After shaking vigorously for 3 min, absorbance at 570 nm was measured.

Setting up a double staining flow cytometry assay to measure apoptosis in seabream leucocytes

Cell isolation and cell culture

Six specimens were sampled for peripheral blood (PB), spleen (S) and HK leucocytes. The fish were anaesthetized with benzocaine (and bled from the caudal vein. 1ml of blood was place in 8 ml of sRPMI and carefully layered over a 51% Percoll density gradient. After centrifuging (400 g, 30 min, 4 °C), the leucocyte interface (PBL) was collected and washed twice in sRPMI (400 g, 10 min, 22 °C).

S and HK leucocytes were isolated from each fish under sterile conditions as explained in page 24. The leucocyte interfaces with S or HK leucocytes were collected and washed twice as before.

PB leucocytes, S leucocytes and HKLs were counted and adjusted to $2x10^6$ cells/ml of sRPMI.

Five hundred µl of each cell suspension previously obtained, were dispensed into flat bottomed 48-well plates and cultured for 0, 24 or 48 h (22 °C, 5% CO_2, 85% humidity). Additionally, 10 µl of resveratrol (22.8 µg/ml in absolute ethanol, Sigma), staurosporine (1 µg/ml in absolute ethanol, Sigma) were added in triplicate as positive controls for apoptosis. Additionally, controls consisting of absolute ethanol or dimethylsulfoxide (DMSO, Scharlau) only were tested. Positive controls for necrosis consisted of fixed cells. For that, aliquots of 500 µl of each cell suspension were centrifuged and the

supernatants discarded. The cell pellets were fixed by adding 2 ml of formaldehyde 10% in PBS (30 min, 4 °C). All assays were done in triplicate.

Apoptosis assay

A double staining flow cytometric assay combining fluorescein diacetate (FDA, Sigma) and PI was used by adapting the method used for the detection of apoptotic leucocytes in mammalian cells (Bartkowiak et al., 1999; Sandström et al., 2000) and fish cells (Saha et al., 2003) to the seabream leucocytes.

To optimise the FDA staining protocol for gilthead seabream leucocytes, 0.05 g FDA were resuspended in 4ml of DMSO. This solution was used to obtain different working solutions (12.5, 25, 50, 75 or 100 µg FDA/ml in sRPMI). 10 µl of each FDA concentration were added to each sample followed by 50 µl of PI (400 µg/ml, Sigma) 30 min prior to analysing the cells in the Coulter Epics XL flow cytometer (Beckman Coulter). Analyses were performed on 10000 cells, which were acquired at a rate of 300 cells/s. FCS, SSC, FL1 (green fluorescence) and FL3 (red fluorescence) for each cell population were represented in dot plots or histograms. To analyse the leucocyte cell subpopulations present in HKL cell suspensions, two regions were established according to the lymphocyte and phagocyte regions previously characterized (Esteban, 1998). This could only be undertaken at 0 and 24 h of culture because the defined subpopulations were no longer identifiable at 48 h and a significant new population consisting of debris from dead leucocytes appeared.

Microscopy study

Stained samples were directly observed from culture plates under a phase contrast microscope (Nikon). Photographs were taken by light and fluorescence microscopy using an alternating green (excitation 470 nm, 490 nm, barrier 520-560 nm) and red (excitation 510 nm, 560 nm, barrier 690 nm) filter combination. HKLs were also cultured in culture dishes (9 cm diameter) at the same cell density as above. Cells were incubated without (control) or with staurosporine (1 µg/ml) and samples were collected after 0, 24 and 48h of culture for examination by TEM. Cell suspensions were washed and the supernatants removed, while pellets were processed as explained in page 30. The sections were examined to identify normal, apoptotic or necrotic cells. Nuclear chromatin condensation was used as a typical criterion for apoptotic cells. Cells that

swelled and showed cytoplasmic vacuolisation as well nuclear destruction were characterised as necrotic.

Apoptosis induction by probiotic cytoplasmic extracts

Induction of apoptosis in fish cell lines prompted by the cytoplasmic fractions of *L. delbrüeckii* subsp. *lactis* or 51M6 was measured by a double staining flow cytometry assay using FDA and PI as explained above. Since the previous experiment revealed that 50 µg FDA/ml was the optimum one for labelling HKLs, this concentration was chosen for this study too. Given that cells were cultured in 96 instead of 48 well plates, FDA and PI volumes were adjusted accordingly. Staining was conducted for 30 min and then samples were analysed in the Coulter Epics XL flow cytometer

Statistical analysis

Data are expressed and analysed as it was done in the objective I (page 27).

VI. RESULTS

OBJECTIVE I: MODULATION OF THE SEABREAM SYSTEMIC INNATE IMMUNE RESPONSES BY PROBIOTIC BACTERIA

In vivo studies

Two *in vivo* experimental trials were conducted consisting of the oral delivery of probiotic bacteria to seabream. In a first trial, seabream were fed live *L. delbrüeckii* subsp. *lactis*, *B. subtilis* or both bacteria for 3 weeks and then switching to control diet for another week. The experimental diets resulted in significant changes in the cellular innate immune parameters of this farmed teleost species. The non-significant variations that were observed are not included in the results sections but they were thoroughly commented in published manuscripts.

The peroxidase content showed no significant differences during the first 2 weeks of feeding but a drop in this activity occurred at week 3 in all three experimental groups. By week 4, the group fed *L. delbrüeckii* ssp. *lactis* the previous 3 weeks showed a peroxidase content that was still significantly lower than that of the control group. However, the mean peroxidase content from the group fed *B. subtilis* or both bacteria for 3 weeks, partially recovered by week 4, when they were no longer different from that of controls (Fig. 2).

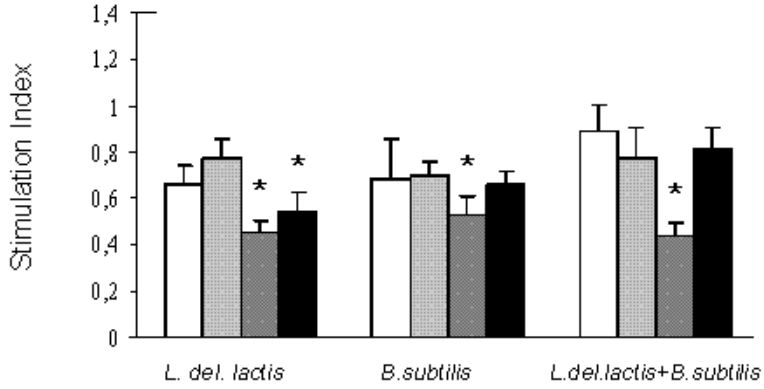

Figure 2: Leucocyte peroxidase content measured as optical density at 450 nm from gilthead seabream fed diets containing 10^7 cfu/g *L. delbrüeckii* ssp. *lactis*, 10^7 cfu/g *B.subtilis* or $0.5x10^7$ cfu/g *L. delbrüeckii* spp. *lactis* and $0.5x10^7$ cfu/g *B.subtilis* for 1 week (□), 2 weeks (▨), 3 weeks (▩) or 3 weeks of feeding the bacteria-supplemented diet and then the control diet for another week (■). Results are expressed as stimulation index (mean+SE; n=6) obtained by dividing each sample value by its mean control value. Asterisk denotes statistically significant differences ($p<0.05$) with respect to the control group.

Phagocytosis was the most affected parameter of all the cellular innate immune responses. Supplementation of seabream diet with *L. delbrüeckii* ssp. *lactis* produced a

significantly increased phagocytic ability of HKLs after 2 weeks of feeding, although the increment was no longer significant at weeks 3 or 4 (Fig. 3a). Phagocytic capacity of leucocytes from specimens fed *L. delbrüeckii* ssp. *lactis* supplemented diet remained unchanged after 1, 2 or 3 weeks of treatment. However, a statistically significant decreased phagocytic capacity was observed at week 4, that is, after administration of control diet for 1 week (Fig. 3b).

Fish fed the *B. subtilis* supplemented diet for a week showed a leucocyte phagocytic ability that was not different from that of control fish, but differences appeared in the second week of feeding, when HKLs from specimens fed supplemented diet showed a significantly greater phagocytic ability with respect to that of control specimens. Such stimulation was no longer significant by week 3 or 4 (Fig. 3a). HKLs from fish that received the *B. subtilis* supplemented diet for 1 week already showed significantly greater phagocytic capacity than the rest of the groups. After 2, 3 or 4 weeks of treatment, the phagocytic capacity of specimens from this group decreased, but such a decrease only became significant in week 4 (Fig. 3b).

Dietary administration for 2 or 3 weeks of both bacterial strains caused a significant stimulation of the phagocytic ability of HKLs compared to the values obtained from control fish (Fig. 3a). However, there were no differences among all three bacteria-supplemented groups. By week 3, the combination of *L. delbrüeckii* ssp. *lactis* and *B. subtilis* produced an increase in phagocytic ability of seabream HKLs when compared to control fish. Moreover, this increment observed in the phagocytic ability of seabream fed two bacteria-supplemented diets was also significantly different from the one observed in fish fed only *L. delbrüeckii* ssp. *lactis* diet (Fig. 3a).

No statistically significant differences were found in the respiratory burst activities of seabream HKLs from control and live bacteria-supplemented diets at any time of the experiment (not shown).

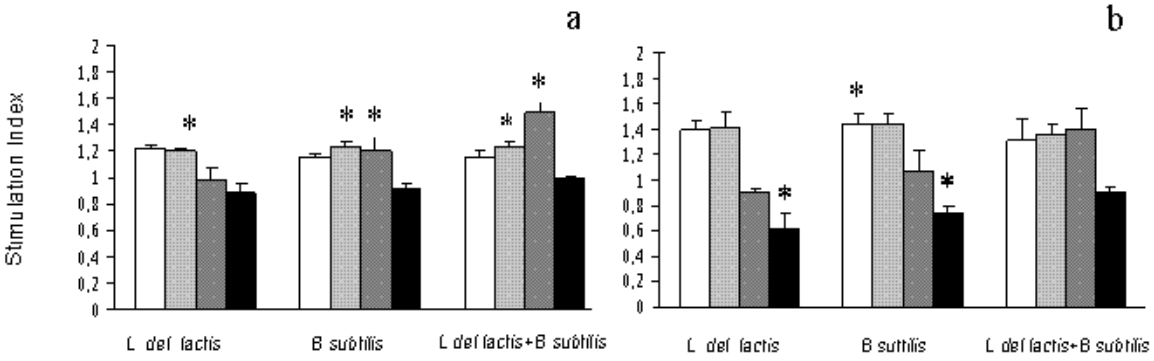

Figure 3: Phagocytic ability (a) and capacity (b) of HKLs from gilthead seabream specimens fed diets containing 10^7 cfu/g *L. delbrüeckii* ssp. *lactis*, 10^7 cfu/g *B.subtilis* or $0.5x10^7$ cfu/g *L. delbrüeckii* spp. *lactis* and $0.5x10^7$ cfu/g *B.subtilis* for 1 week (□), 2 weeks (▨), 3 weeks (▩) or 3 weeks of feeding the bacteria-supplemented diet and then the control diet for another week (■). Results are expressed as stimulation index (mean+SE; n=6) obtained by dividing each sample value by its mean control value. Asterisk denotes statistically significant differences ($p<0.05$) with respect to the control group.

The addition of both bacterial strains to the fish diet for 3 weeks resulted in a statistically significant increment of the natural cytotoxic activity of the specimens compared to the values obtained from fish in the control group. The observed stimulatory effect remitted when fish were swapped to the control diet for 1 week, at the final stage of the feeding trial (Fig. 4).

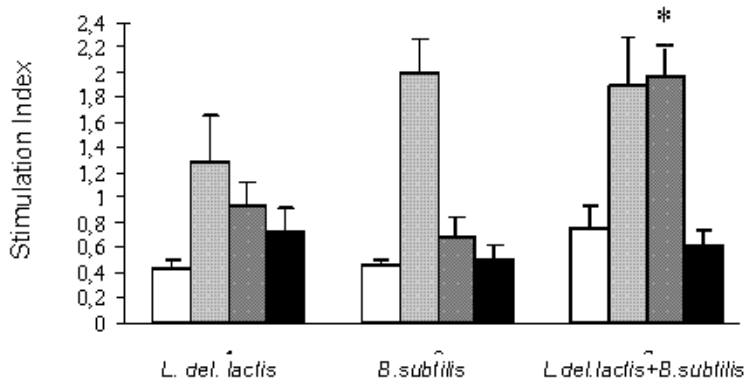

Figure 4: Cytotoxic activity of HKLs from gilthead seabream fed diets containing 10^7 cfu/g *L. delbrüeckii* ssp. *lactis*, 10^7 cfu/g *B.subtilis* or $0.5x10^7$ cfu/g *L. delbrüeckii* spp. *lactis* and $0.5x10^7$ cfu/g *B.subtilis* for 1 week (□), 2 weeks (▨), 3 weeks (▩) or 3 weeks of feeding the bacteria-supplemented diet and then the control diet for another week (■). Results are expressed as stimulation index (mean+SE; n=6) obtained by dividing each sample value by its mean control value. Asterisk denotes statistically significant differences ($p<0.05$) with respect to the control group.

In a second experimental trial we administered heat-killed 51M6, Pdp11 or a combination of both at equal amounts for 4 weeks to adult seabream. Modulation of both cellular and humoral innate immune responses occurred.

The supplementation of gilthead seabream diet with heat-inactivated Pdp11, 51M6 or both bacteria resulted in a similar pattern of effects upon natural haemolytic complement activity in serum. For Pdp11 or the multispecific formulations the highest activity was always recorded at week 4, although the observed differences were not statistically significant (Fig. 5).

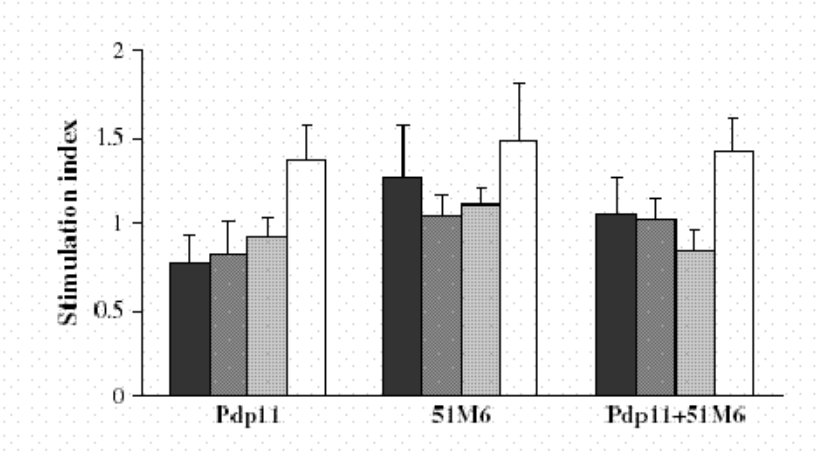

Figure 5: Natural haemolytic complement activity measured as optical density at 550 nm from gilthead seabream specimens fed diets containing 10^8 cfu/g heat-inactivated Pdp11, 10^8 cfu/g heat-inactivated 51M6 or $0.5x10^8$ cfu/g heat-inactivated Pdp11 and $0.5x10^8$ cfu/g heat-inactivated 51M6 for 1 week (■), 2 weeks (■), 3 weeks (■) or 4 weeks (□). Results are expressed as stimulation index (mean+SE; n=6) obtained by dividing each sample value by its mean control value.

Serum peroxidase content from fish fed the bacteria-supplemented diets was not statistically different from that of control fish at any given time of the trial as it occurred with the leucocyte peroxidase content.

Phagocytosis was the most affected activity of all the assayed immune parameters as occurred in the first trial. The phagocytic ability of HKLs was significantly increased after 2 weeks feeding with the Pdp11 supplemented diet, although this activity fell in a time-dependent manner after 3 or 4 weeks (Fig. 6). Similar results were obtained from the group fed the 51M6- supplemented diet, although the increases were not statistically significant until the third week of the treatment. However, when fish received the diet supplemented with both bacteria, increases were already statistically significant after 2 weeks of feeding and remained statistically higher than the control at week 3.

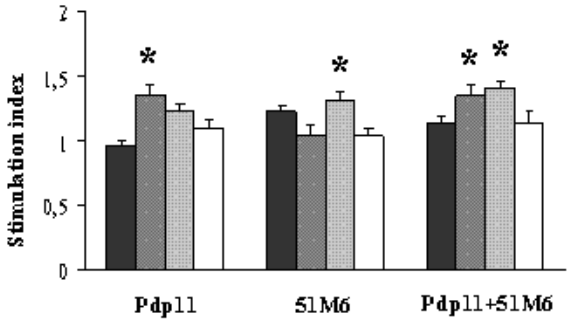

Figure 6: Phagocytic ability of HKLs from gilthead seabream specimens fed diets containing 10^8 cfu/g heat-inactivated Pdp11, 10^8 cfu/g heat-inactivated 51M6 or $0.5x10^8$ cfu/g heat-inactivated Pdp11 and $0.5x10^8$ cfu/g heat-inactivated 51M6 for 1 week (■), 2 weeks (▩), 3 weeks (▣) or 4 weeks (□). Results are expressed as stimulation index (mean+SE; n=6) obtained by dividing each sample value by its mean control value. Asterisk denotes statistically significant differences ($p<0.05$) with respect to the control group.

No statistically significant differences were found in the respiratory burst activities of seabream HKLs between the control and inactivated bacteria supplemented diets at any time of the experiment.

Fish fed either of the two diets containing heat-inactivated 51M6 showed no significant differences in the cytotoxic activity of HKLs during the first 2 weeks of treatment but administration of these two diets for 3 weeks significantly increased the cytotoxic activity of HKLs, although such enhancement was not observed after 4 weeks of treatment (Fig. 7).

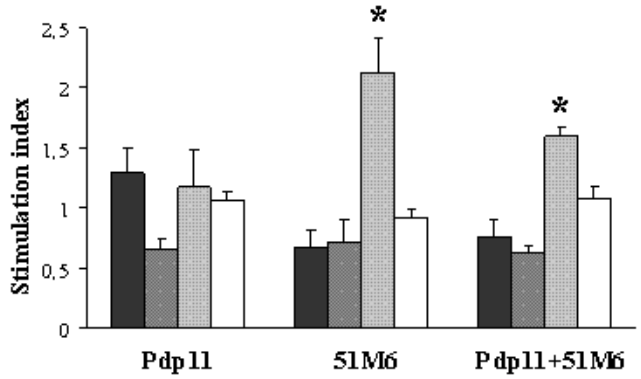

Figure 7: Cytotoxic activity of HKLs from gilthead seabream fed diets containing 10^8 cfu/g heat-inactivated Pdp11, 10^8 cfu/g heat-inactivated 51M6 or $0.5x10^8$ cfu/g heat-inactivated Pdp11 and $0.5x10^8$ cfu/g heat-inactivated 51M6 for 1 week (■), 2 weeks (▩), 3 weeks (▣) or 4 weeks (□). Results are expressed as stimulation index (mean+SE; n=6) obtained by dividing each sample value by its mean control value. Asterisk denotes statistically significant differences ($p<0.05$) with respect to the control group.

In vitro experiments

Cellular innate immune parameters of seabream HKLs were studied *in vitro* following short incubation with the heat-inactivated forms of the four bacterial strains studied *in vivo: Lactobacillus delbrüeckii* subsp. *lactis, Bacillus subtilis,* 51M6 and Pdp11. Three different doses were assayed and clear effects took place in a dose depending manner as shown by the following results.

The peroxidase content of seabream HKLs slightly decreased after incubation with any of the four assayed bacteria at $5x10^5$ cfu/ml. Similar results were obtained after incubation of HKLs with $5x10^6$ cfu/ml, except for *B. subtilis*, which slightly increased the peroxidase content of leucocytes. However, when $5x10^7$ cfu/ml was added, stimulation indexes higher than 1 were observed in all the cases. Only this bacterium produced dose-dependent increments in the leucocyte peroxidase content. Statistical analysis revealed that differences were not significant for any treatment (not shown).

Seabream HKLs consisted of monocyte-macrophages, granulocytes, thrombocytes and lymphocytes and only the monocyte-macrophages and acidophilic granulocytes showed phagocytic activity when studied by TEM (Fig. 8).

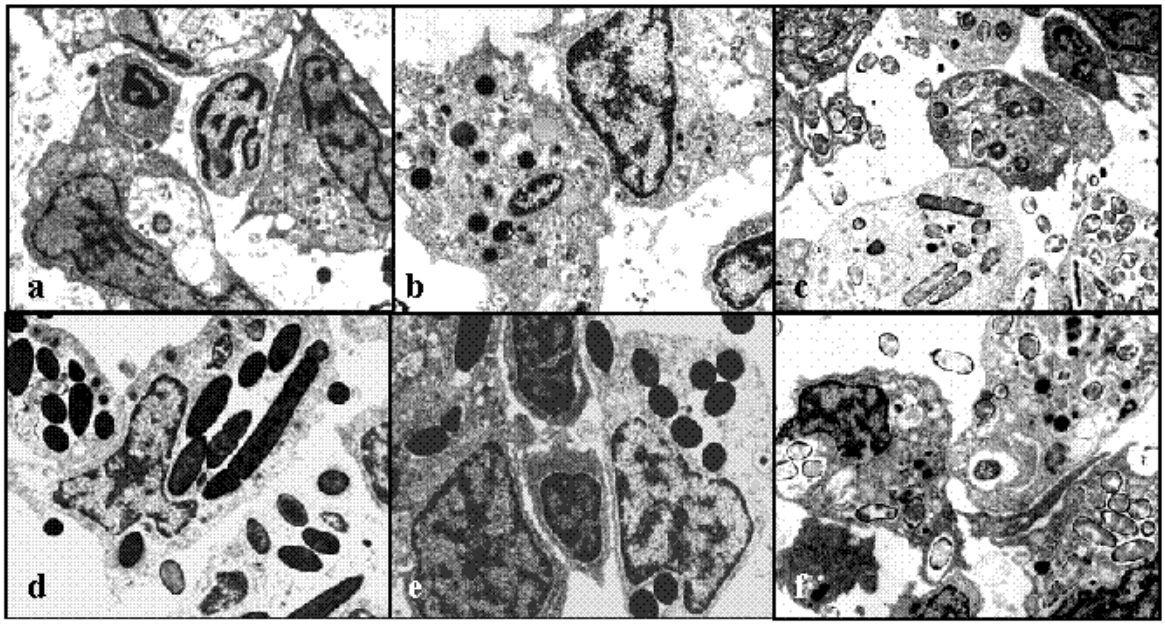

Figure 8: Gilthead seabream HKLs incubated for 30 min with probiotics (10 bacterial cells per leucocyte). a) 51M6 (x7500); b) Pdp11 (x7500); c, f) *Bacillus subtilis* (x5500) ; d-e) *Lactobacillus delbrüeckii* subsp. *lactis* (x7500).

Both types of phagocytic cells contained variable numbers of bacteria cells inside endocytic vesicles of different sizes and shapes. Of the four different bacteria species studied, *L. delbrüeckii* subsp. *lactis* and *B. subtilis* were more readily ingested by the seabream phagocytes than 51M6 and Pdp11 as evidenced by the greater number of ingested bacteria per leucocyte.

Maximum slope of respiratory burst was significantly higher after addition of $5x10^7$ cfu/ml heat inactivated *L. delbrüeckii* subsp. *lactis* or *B. subtilis*. The same concentration of the two fish-derived bacteria strain also resulted in an increase of this parameter, although the observed increment was not statistically significant due to great fish-to-fish variability. Lower doses of all bacteria slightly stimulated this activity but in a non-significant manner (Fig. 9).

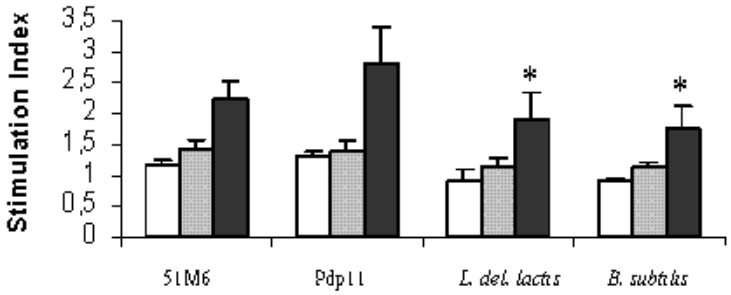

Figure 9: Respiratory burst activity of seabream head-kidney leucocytes after incubation with $5x\ 10^5$ cfu/ml (□), $5x\ 10^6$ cfu/ml (▨), $5x\ 10^7$ cfu/ml (■) heat-inactivated bacteria. Results are expressed as stimulation index (mean+SE; n=6) obtained by dividing each sample value by its mean control value. Asterisk denotes statistically significant differences ($p<0.05$) with respect to the control group.

Heat-inactivated bacteria stimulated *in vitro* in a dose-dependent manner the cytotoxic activity of HKLs (Fig. 10). In general, a dose dependent response was recorded for Pdp11, 51M6 and *B. subtilis*. Statistical analysis revealed that significant differences appeared after incubation with 51M6, *L. delbrüeckii* subsp. *lactis* or *B. subtilis* at the highest assayed concentration. The greatest stimulation occurred following treatment with 51M6 (stimulation index = 2.77 ±0.5).

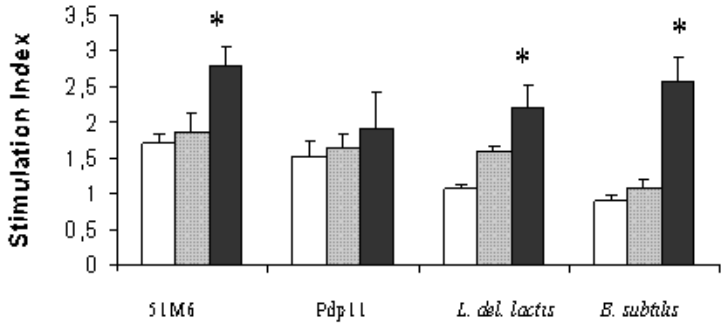

Figure 9: Cytotoxic activity of seabream head-kidney leucocytes after incubation with 5x 10^5 cfu/ml (□), 5x 10^6 cfu/ml (▨), 5x 10^7 cfu/ml (■) heat-inactivated bacteria. Results are expressed as stimulation index (mean+SE; n=6) obtained by dividing each sample value by its mean control value. Asterisk denotes statistically significant differences ($p<0.05$) with respect to the control group.

OBJECTIVE II: STUDIES ON THE INTEGRITY OF THE GUT EPITHELIAL BARRIER

The first experiment consisted of the exposure of Atlantic salmon foregut to pathogenic or probiotic bacteria as well as the combination of both in order to investigate how the morphology of the gut epithelial barrier changes in the presence of these different bacterial treatments *in vitro*. A summary of all the different morphological changes observed after each treatment is presented in Table 3.

Control samples, of Atlantic salmon foregut exposed to only Ringer solution (not bacteria) showed normal appearance of enterocytes lining the gut both under LM and TEM. The epithelial cells examined by LM had undamaged apical tight junctions as well as well organized and uniform microvilli (Fig. 11a). Similar findings were observed when the foregut was incubated only with *Carnobacterium divergens* at $6x10^4$ and $6x10^6$ bacteria (Fig. 11b). These results were confirmed by TEM (data not shown).

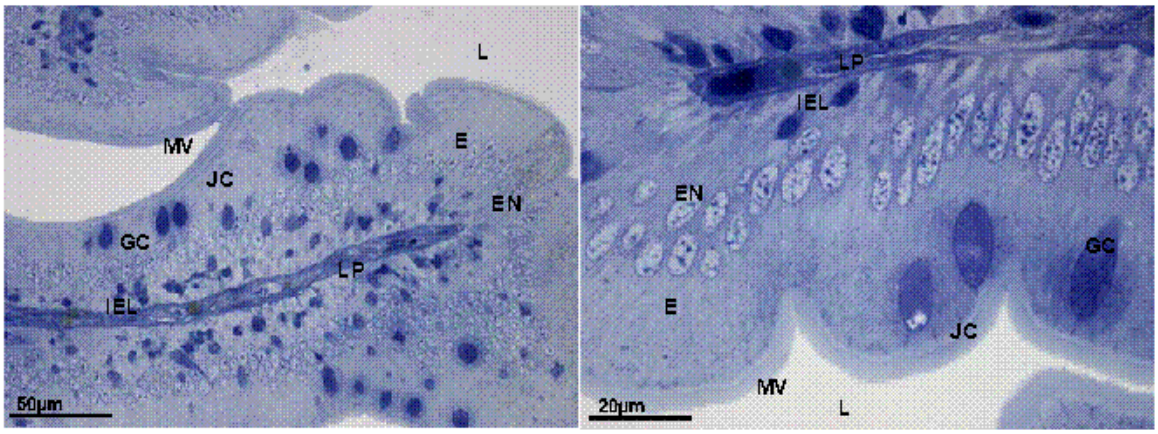

Figure 11: Light microscopy (LM) of the foregut (anterior part of the intestine) of Atlantic salmon (*Salmo salar* L.). a) incubated with Ringer solution (not exposed to bacteria) showing normal enterocytes. Bar: 50 µm. b) incubated with $6x\ 10^6$ cfu/ml *Carnobacterium divergens* showing normal enterocytes. L Lumen, LP lamina propia, MV microvilli, JC junctional complex, E enterocytes, EN enterocytes nucleus, GC goblet cells, IEL intraepithelial lymphocytes. Bar: 20 µm.

In contrast with these results, it was clearly demonstrated that when the foregut was exposed to *Aeromonas salmonicida,* damaging effects such as the presence of cell debris in the lumen were observed (Fig. 12a). Furthermore, microvilli, although not severely damaged, seemed to have lost physical contact with the underlying enterocytes.

A low electron density band parallel to the lumen (light band) was present at the apical part of the enterocytes right below the microvilli (Fig. 12b). Moreover, an inflammatory-like response was observed, with a clear mobilization of leucocytes from the lamina propia into the epithelium (not shown). LM was not able to reveal such morphological changes although it was possible to identify the mobilization of leucocytes such as macrophages and intraepithelial lymphocytes.

When Atlantic salmon gut was exposed to both *A. salmonicida* ($6x10^6$ cfu/ml) and *C. divergens* ($6x\ 10^6$ cfu/ml), foregut samples showed undamaged microvilli. Both LM and TEM showed abundant goblet cells and leucocytes were seen in between edemic epithelial cells close to the gut lumen. Moreover TEM examination revealed the presence of bacteria-like cells in between undamaged microvilli in the proximity to tight junctions. Other junctional complexes in the vicinity of those seen with the bacteria also seemed affected by previous or unobservable bacteria (Fig. 13).

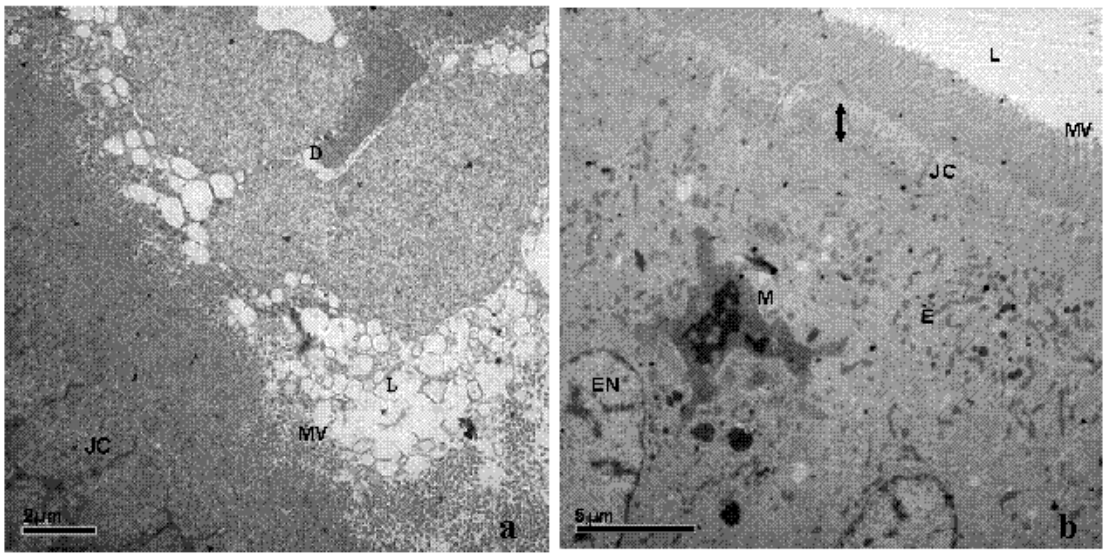

Figure 12: Transmission electron microscopy (TEM) of the foregut of Atlantic salmon exposed to $6x\ 10^6$ cfu/ml *Aeromonas salmonicida*. a) Note the cell debris in the gut lumen and disorganized microvilli. Bar: 2 µm. b) Note enterocytes showing a light density band (arrow). A macrophage (M) has migrated to the apical pole of the enterocytes (E). Cell debris (D), Enterocyte nucleus (EN), gut lumen (L), microvilli (MV), JC junctional complex. Bar: 5 µm.

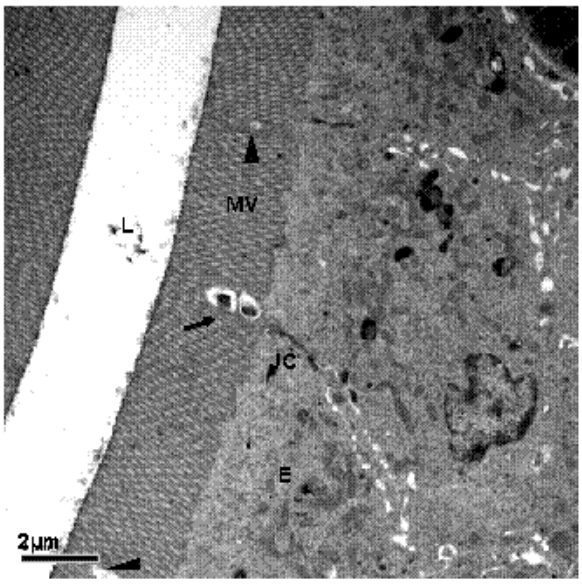

Figure 13: TEM micrograph of the foregut of Atlantic salmon exposed to 3x 10^6 cfu/ml *A. salmonicida* and 3x 10^6 cfu/ml *C. divergens* showing bacteria-like cells (arrow) between the microvilli of an enterocyte and signs of presence of other bacteria in contiguous enterocytes (arrow heads) close to the tight junctions. L Lumen, MV microvilli, JC junctional complex, E enterocytes. Bar: 2 µm.

Exposure of Atlantic salmon foregut to *Vibrio anguillarum* at both assayed concentrations (Fig. 14a and 14b) resulted in clear changes in the intestinal epithelium compared to control samples, only exposed to Ringer solution. At the highest dose, $6x10^6$ bacteria per ml, there was an inflammatory response of gut-associated lymphoid tissue (GALT) leucocytes migrating from the lamina propia towards the lumen (not shown). Whilst enterocyte microvilli themselves did not look altered, a light band between microvilli and the epithelial cell was observed in a similar way to that found in the *A. salmonicida* exposed tissue (Fig. 12b). Additionally, some signs of edema were seen between enterocytes (not shown). Surprisingly, the lower *V. anguillarum* dose also resulted in significant morphological changes in salmon gut (results not shown). Serious signs of tissue damage included cell debris in the lumen, shortening or absence of microvilli in some areas of the epithelium, protruding cells and disintegrated tight junctions. These protruding epithelial cells with patchy microvilli distribution were also seen under SEM (Fig. 14a). Moreover, phagolysosome-like vesicles containing degraded bacteria (about 2 µm) were seen in the enterocytes cytoplasm under TEM (Fig. 14b).

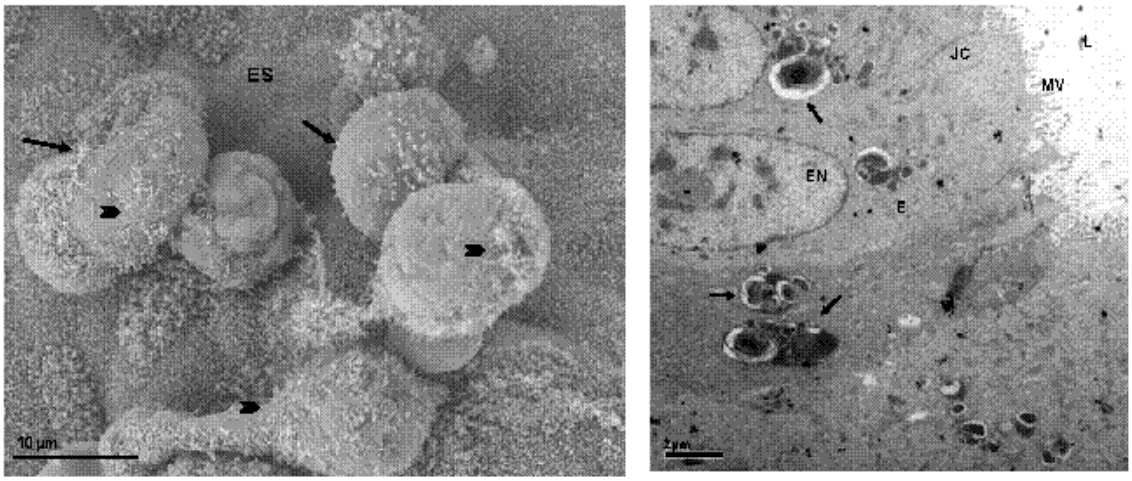

Fig. 14: a) Scanning electron microscopy micrograph of the foregut of Atlantic salmon exposed to $6x10^6$ *V. anguillarum*. Note several detached or detaching enterocytes (arrows) laying on the epithelial surface and lacking uniform microvilli (arrow heads). Bar 10 µm. b) TEM micrograph of the foregut of Atlantic salmon exposed to $6x10^4$ *V. anguillarum* showing the presence of phagolysosome-like structures containing degraded bacteria (arrows) and damaged microvilli. L lumen, MV microvilli, JC junctional complex, E enterocytes, EN enterocytes nucleus. Bar: 2µm.

When the foregut was simultaneously exposed to *V. anguillarum* and *C. divergens* either at higher or lower dosis, the damaging effect due to pathogenic bacteria were still apparent. When both bacteria were at the highest dose ($3x10^6$ cfu/ml), similar effects as those described for the *V. anguillarum* treatment were found by LM (Fig. 15a) characteristically showing the presence of elongated dark cell bodies with no visible nuclei that reached the lumen of the gut. However, phagolysosomes in the cytoplasm of the enterocytes were not observed by TEM. On the other hand, bacterium-like particles of about 0.5µm in size were seen under TEM close to the tight junctions between enterocytes (Fig. 15b). These putative bacteria seemed to be entering the epithelium not through the epithelial cells but in a paracellular manner by loosening tight junctions.

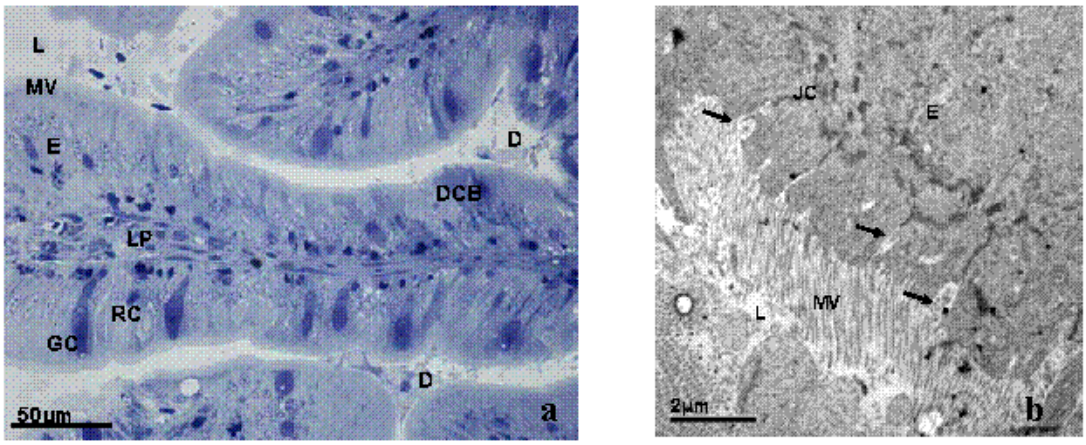

Figure 15: Foregut of Atlantic salmon (*Salmo salar* L.), incubated with *V. anguillarum* and *C. divergens* at $3x10^6$ cfu/ml. a) LM micrograph Bar: 50 µm. b) TEM micrograph showing bacterium-like particle close to tight junctions (arrows). Bar: 2 µm. L lumen, MV microvilli, D debris, DCB dark cellular bodies, JC junctional complex, E enterocytes, GC goblet cells, RC rodlet cell.

Co-exposure of salmon intestine to *V. anguillarum* and *C. divergens* at the lowest dose resulted in some areas with characteristic protruding cells and lack of healthy microvilli whereas other areas showed undamaged tight junctions and epithelial surface (data not shown). Finally, when *C. divergens* was used at a two fold higher concentration than *V. anguillarum*, the appearance of the samples under LM was normal and equivalent to that of controls (Fig. 16).

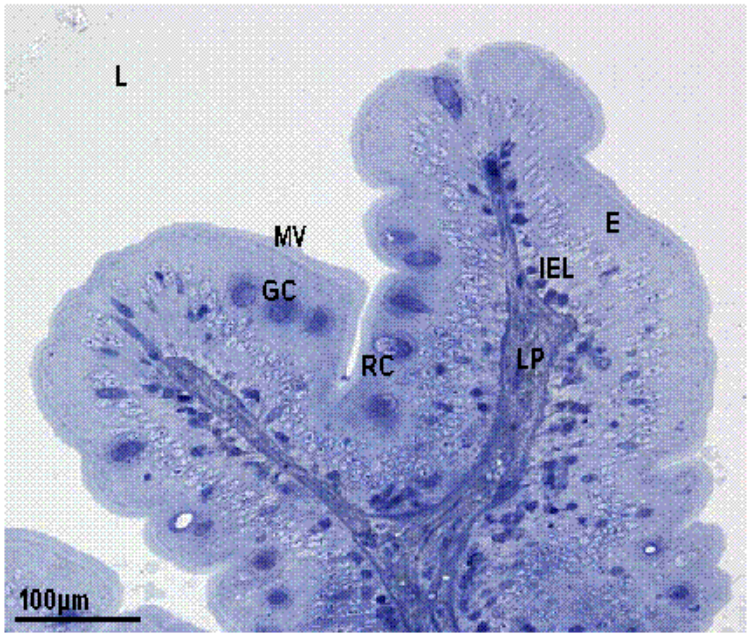

Figure 16: LM micrograph of the foregut of Atlantic salmon (*Salmo salar* L.), exposed to *V. anguillarum* at $3x10^4$ cfu/ml and *C. divergens* $3x10^6$ cfu/ml. L Lumen, LP lamina propia, MV microvilli, E enterocytes, GC goblet cells, RC rodlet cell. Bar: 100 µm.

TEM examination revealed nevertheless a reaction against the presence of bacteria with clear cell mobilization and activation within the intestinal epithelium (data not shown). Although the majority of the villi surface was not significantly damaged there were still few areas characterized by the presence of disorganized microvilli, few protruding cells and some dark cell bodies.

MORPHOLOGICAL OBSERVATIONS	1	2	3	4	5	6	7	8	9	10	11
Presence of cell debris in the lumen	0	C	C	C	0	0	0	B	0	B	B
Disorganised microvilli	0	0	C	C	A	A	0	A	A	C	C
Protruding epithelial cells	0	0	C	C	0	0	0	B	0	C	C
Edema	0	B	0	0	0	0	C	0	A	0	0
Low electron density band	0	C	0	C	0	0	B	0	A	0	0
Disintegrated tight junctions	0	B	C	C	0	0	A	B	0	C	C
Dark cellular bodies under LM	0	0	C	C	0	0	0	0	B	C	C
Loss of epithelial integrity	0	B	C	C	0	0	B	B	A	C	C
Leucocyte mobilization	0	C	C	C	0	0	B	A	B	B	B
Phagolysosome-like vesicles with bacteria	0	0	B	0	0	0	0	0	0	0	0
Bacterium-like particles close to tight junctions	0	0	0	0	0	0	C	0	0	0	C

Table 3: Morphological description of Atlantic salmon foregut incubated with different bacterial treatments 1-11 (see Table 2). Damage and tissular changes were assessed as follows: 0 not observed, A low frequency or intensity, B moderate frequency or intensity, C high frequency or intensity.

In a second experiment, we used *A. salmonicida* as a model for gut epithelial damage in salmon and we tested another probiotic strain, *L. delbrüeckii* subsp. *lactis*. Additionally, pre-treatment with the probiotic and then exposure to the pathogen was carried out. The fate of *Lactobacillus delbrueckii* ssp. *lactis* in Atlantic salmon intestine in vitro was studied by confocal microscopy. Samples incubated with Ringer solution only (not exposed to bacteria) had no red fluorescence at 596-700 nm examined under the confocal microscope. Incubation of Atlantic salmon foregut with TRITC labelled - *L. delbrueckii* ssp. *lactis,* revealed the presence of labelled bacteria in the gut lumen in association with the mucus. When bacteria were seen in the lumen, they were found in groups or clumps (Fig. 17a). Furthermore, labelled bacteria were also observed on the surface of villi, inside the mucosal epithelium and even in the lamina propia (Fig. 17b).

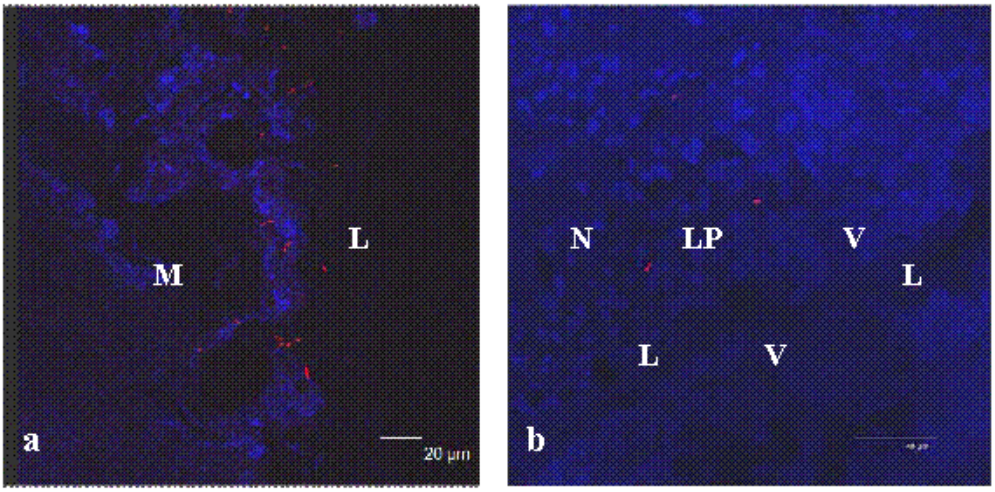

Figure 17: Confocal microscopy of the fate of TRITC-labelled *L. delbrüeckii* ssp. *lactis* in the salmon gut. a) Fluorescent image of salmon foregut cryosection after incubation for 30 min with *L. delbrüeckii* ssp. *lactis* (10^7 cfu/ml) labelled with TRITC. L lumen, M mucus. Note red fluorescence of labelled bacteria associated with mucus in the salmon gut lumen. Bar: 20 µm. b) Fluorescent image of salmon foregut cryosection after incubation for 30 min with *L. delbrüeckii* ssp. *lactis* (10^7 cfu/ml) labelled with TRITC. V villi, L lumen, LP lamina propria, N nuclei of enterocytes. Note red fluorescence of labelled bacteria located inside the gut villi (v) at the level of the lamina propria (LP). Bar: 40 µm.

Histological changes of Atlantic salmon intestine following incubation with *L. delbrueckii* ssp. *lactis* were studied. Atlantic salmon foregut of the control group resembled that of the foregut exposed to *L. delbrueckii* subsp. *lactis* for 30 min (Fig. 18a). No cell debris was observed in the lumen and microvilli were well organised and homogeneously distributed along the apical side of enterocytes. Enterocytes had intact junctional complexes and no signs of edema. Moreover, bacteria were seen in the lumen in association with microvilli and, in such areas, it was common to observe at least a

rodlet cell in close proximity to the apical pole of the epithelium (Fig. 18a). Additionally, bacterial particles were found paracellularly between enterocytes at the basal region of the epithelium (Fig 18b). With respect to GALT components, groups of leucocytes were abundantly found in the intestinal epithelium of these specimens (Fig. 18c). In this sense, hindgut samples revealed the presence of cells undergoing mitosis at the basal pole of the epithelium close to the lamina propria (Fig. 18d). These cells were leucocytes presumably transported from the connective tissue of the lamina propria into the intestinal epithelium.

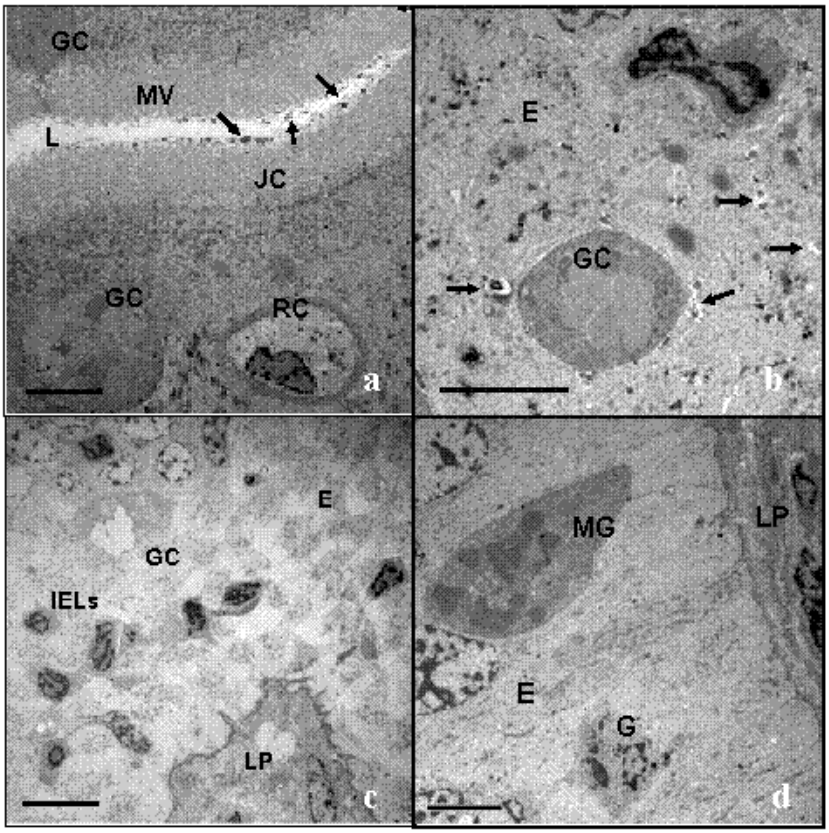

Figure 18: TEM micrographs of the tissue morphological responses in the salmon gut epithelium after exposure to *L. delbrüeckii* ssp. *lactis* (1.6x 10^5 cfu/ml) (30 min) and then sterile saline:TSB (1:1) (30 min). a) Bacterial cells (arrows) are seen in the lumen associated with microvilli. Bar: 5 µm. b) Note bacterial cells (arrows) in between enterocytes without causing oedema. Bar: 5 µm c) Note the abundant IELs in the epithelium. Bar: 10 µm. d) Undamaged epithelium and lamina propria showing a granulocyte and a mitotic leucocyte possibly a granulocyte. Bar: 5 µm. L lumen, MV microvilli, JC junctional complex, LP lamina propria, E enterocyte, GC goblet cell, IELs intraepithelial lymphocytes, G granulocyte, MG mitotic granulocyte, RC rodlet cell.

Finally *A. salmonicida* ssp. *salmonicida* results in disruption of the Atlantic salmon gut and pre-treatment with *L. delbrueckii* ssp. *lactis* protected against such damage. When Atlantic salmon gut was exposed to *A. salmonicida*, the integrity of the intestinal barrier

was damaged and cellular debris was frequently observed in the lumen. Edema, disorganization of the microvilli and the presence of dark, narrow and elongated cells (putative dying cells) indicate tissue damage produced by the pathogen (Fig. 19a). Rodlet cells were found in the proximity of grouped immune cells (Fig. 19b). These groups of immune cell effectors were present both in the foregut and in the hindgut with macrophages sometimes reaching the most apical part of the epithelium (not shown).

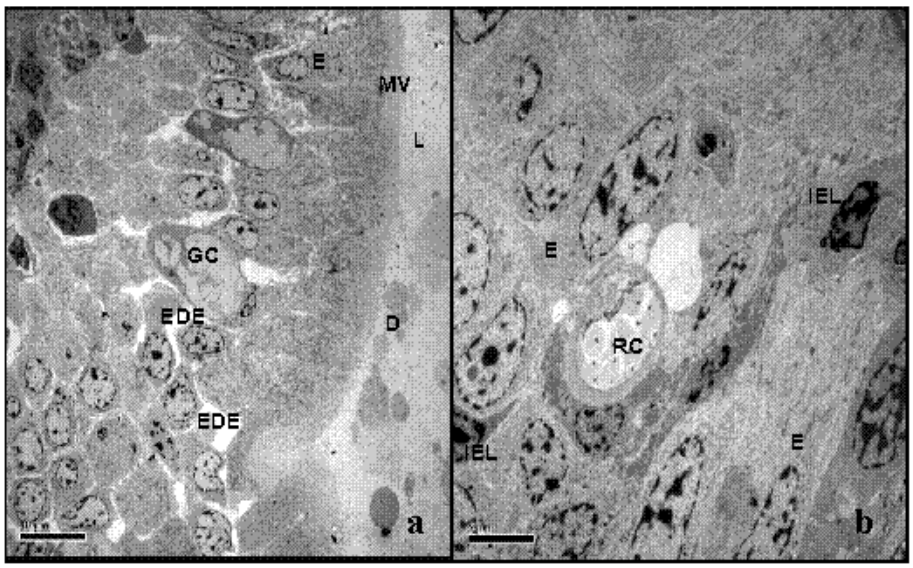

Figure 19: Epithelial damage in salmon gut caused by *Aeromonas salmonicida* ssp. *salmonicida*. a) TEM micrograph of the foregut of Atlantic salmon exposed to *A. salmonicida* ($7x10^7$ cfu/ml) (30 min) and then to sterile saline: TSB (1:1) (30min). L lumen, D debris, MV microvilli, E enterocytes, GC goblet cells, EDE oedema. Bar: 10 µm. b) TEM micrograph of the foregut of Atlantic salmon exposed to *A. salmonicida* ($7x10^7$ cfu/ml) (30 min) and then to sterile saline: TSB (1:1) (30 min). E enterocytes, IEL intraepithelial lymphocyte, RC rodlet cells. Bar: 5 µm.

Contrary to these observations, proximal intestines pre-incubated with *L. delbrueckii* ssp. *lactis* for 30 min and then exposed to *A. salmonicida*, had no signs of tissue disruption observable under TEM (Fig. 20a) or LM (Fig. 20b) except for some small areas where microvilli were slightly more disorganised. The histological appearance of these samples resembled that of controls or *L. delbrueckii* ssp. *lactis* only samples and redistribution of immune and abundance of rodlet cells were not observed.

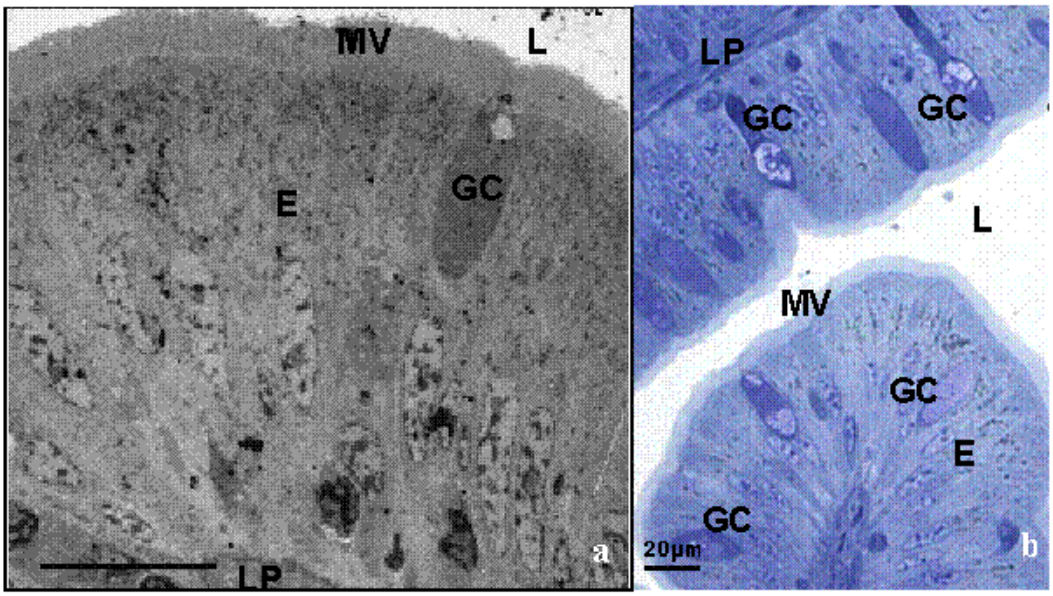

Figure 20: Morphological appearance of salmon gut incubated with the probiotic *L. delbrüeckii* ssp. *lactis* ($1.6x10^5$ cfu/ml) (30min) and then *A. salmonicida* ($7x10^7$ cfu/ml). a) TEM micrograph and b) LM micrograph. L lumen, MV microvilli, GC goblet cell, E enterocyte, LP lamina propria. Bar: 20 µm.

OBJECTIVE III: GALT ISOLATION AND MORPHOFUNCTIONAL CHARACTERIZATION IN THE SEABREAM.

Protocols for the isolation of seabream GALT cells, cell counts and viability

The total mean cell numbers per gram of tissue and their mean viability calculated for each of the tested protocols for the isolation of gut leucocytes are shown in Table 5.

Note that chemical treatment alone for 10 min (S_1) yielded significantly lower cell counts than other protocols, being the obtained cells also statistically less viable than any of the other cell collected suspensions. This lower viability could be due to the dissection procedure and consequent tissue damage during the initial stages of the protocol. However, the subsequent purification steps allowed refinement of the cell suspensions and the increase of the final cell viability.

When no collagenase was added to the washing medium, high numbers of cells were obtained, especially after gently shaking for 60 or 120 min. The viability of the cells was always higher than 75%. Intestines incubated for 30 min in washing media without collagenase released statistically fewer cells than when longer incubation times or higher enzyme concentrations were used.

Increasing the collagenase concentration from 0.15 mg/ml to 0.37 mg/ml and the digestion time from 30 to 120 min resulted in increasingly higher numbers of cells. These differences were statistically significant in all cases. Despite the absence of a significant decrease in viability, it was observed that cells obtained after longer enzymatic digestion tended to be slightly less viable than when shorter protocols were used.

Shortening collagenase digestion from 120 to 60 min yielded similar S_2 both in number and composition according to flow cytometry analysis. However, further reduction of the treatment (30 min) resulted in a different distribution of the dot plots obtained by flow cytometry.

The use of a purely mechanical method involving a cell scraper was the most time and cost-effective although the latter varied from fish to fish. Nonetheless, the viability of the cells obtained was high (88%) and similar to the viability using collagenase. Examination under LM revealed the presence of whole fragments of tissue where enterocytes were still attached to each other forming a simple cylindrical epithelium (data not shown). Therefore, the isolation of cells as a suspension was sometimes incomplete.

Treatment	Number of cells/g tissue x 10^7	Viability (%)
DTT 10 min	0.26 ± 0.14^{a}	65 ± 9.6^{a}
Collagenase 0 mg/ml		
30 min	$8.15\pm3,4^{c}$	$82.5\pm6,3^{b}$
60 min	42.8 ± 37.9^{e}	83.5 ± 9.1^{b}
120 min	48.2 ± 6.7^{e}	76.6 ± 6.5^{b}
Collagenase 0.15 mg/ml		
30 min	$19.4\pm0,9^{d}$	$82.6\pm4,7^{b}$
60 min	22.6 ± 2.4^{d}	80.5 ± 4.5^{b}
120 min	40.5 ± 3^{e}	74 ± 4.6^{b}
Collagenase 0.37 mg/ml		
30 min	$18.7\pm7,6^{d}$	$88\pm7,1^{b}$
60 min	48.75 ± 3^{e}	82.75 ± 7.1^{b}
120 min	75.6 ± 5.1^{f}	77 ± 2.8^{b}
Mechanical stripping	4.95 ± 1.5^{b}	82.2 ± 2.0^{b}

Table 4: Total number of cells and viability obtained from seabream intestine after different isolation protocols and their viability. Results are expressed as mean ± sd. One-way analysis of variance and Tukey test were conducted when necessary. Different letters stand for statistically significant differences ($p<0.05$) between isolation protocols.

Flow cytometry studies of seabream GALT cells obtained by different protocols

Identification of GALT cell subsets according to the FSC and SSC values obtained by flow cytometry was not easy because the dot plots showed no well defined subsets. Three cell subsets of variable relative abundance could be distinguished, such variations appearing not to be associated to a particular purification protocol but to differences between specimens. In general, the majority of cells corresponded to low FSC and low SSC values (R1). A second subpopulation, R2, consisted of medium FSC values but with relatively high spread and medium SSC values. Finally, a small population (R3) of low FSC and low to high SSC values was found (Fig. 21a).

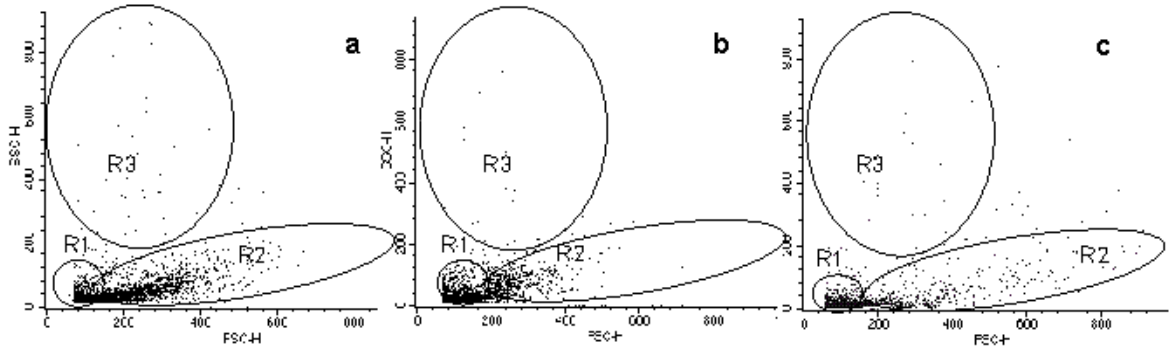

Figure 21: Flow cytometry SSC and FFC dot plots of seabream intestine cells showing three subpopulations (R1, R2 and R3). (a) Representative dot plot of a S_2 suspension obtained after isolation of cells using both chemical (DTT, 10 min) and enzymatic (collagenase 0.15 mg/ml, 60 min) treatments. (b) Representative dot plot obtained after the same suspension in figure 1a is passed through a nylon wool column (NW) for 60 min. (c) Representative dot plot of a total suspension obtained by mechanical stripping.

When mechanical stripping was conducted, cell suspensions showed a different picture according to their FSC and SSC values, with a R2 region that was typically more spread in the FSC axis compared to other treatments (Fig. 21c).

Use of nylon wool columns after isolation of seabream GALT

Nylon wool columns were an effective tool for eliminating mucus and yielded cleaner cell suspensions that did not form aggregates that were present prior to its use. No other methods to eliminate mucus were evaluated in the present study. The recovery of the loaded cells was over 90% and, after one hour in the nylon wool column, their viability was unaffected. Moreover, this step was necessary for the Percoll separation since the presence of mucus sometimes precluded adequate separation of cells according to their relative density. When NW cell pools were studied under flow cytometer, their viability was slightly higher that of S_2 suspensions and FSC scattering was reduced. All three subpopulations were still present although R2 showed less variability in the FSC axis compared to R2 from figure 21a (Fig. 21b).

Observation under SEM of nylon wool fibres from columns incubated for one hour with seabream gut cells, revealed considerable amounts of mucus attached to them (Fig. 22a). Additionally, some rounded small cells (diameter around 5μm) adhered to the fibres (Fig. 22b).

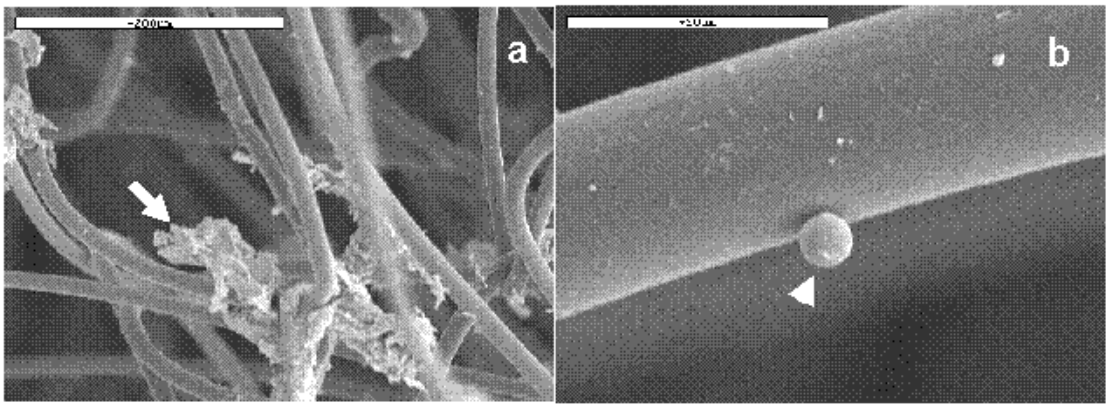

Figure 22: Scanning electron micrographs of nylon wool fibres from nylon wool columns incubated with seabream gut cells. (a) Mucus (arrow) from seabream gut trapped between nylon wool fibres. Bar: 200 µm (b) Small rounded cell (arrow head) adhered to a nylon wool fibre. Bar: 20 µm.

Purification by continuous Percoll gradients

Percoll gradients on cell suspensions that had not been passed through nylon wool columns sometimes yielded one sole layer at the interface between the Percoll and sRPMI medium, where almost all the cells were present due to the presence of mucous that caused cell aggregation.

When cell suspensions were previously purified in the nylon wool column and then layered over continuous Percoll gradients, three main bands were found: i) a low density band (LD) (density lower than 1.033 g/l) that remained in the supernatant; ii) a thicker intermediate band (ID) located between 1.055 and 1.060 g/l; iii) a higher density band (HD) with an approximate density of 1.075 g/l. The three bands were not consistently found in all the trials, sometimes we observed more bands and in few instances less. The LD, ID and HD pattern were nonetheless the most frequently observed.

Flow cytometry analysis of these bands revealed that the viability of ID and HD was higher than in the original cell suspension since dead cells stayed mainly in LD. Moreover, LD contained a cell subpopulation resembling those found in the S_2 and NW pools. The ID band was enriched in cells of intermediate size and low-medium complexity (Fig. 23a). Finally, HD was enriched in a cell populations characterised by both very low FSC and SSC (Fig. 23b).

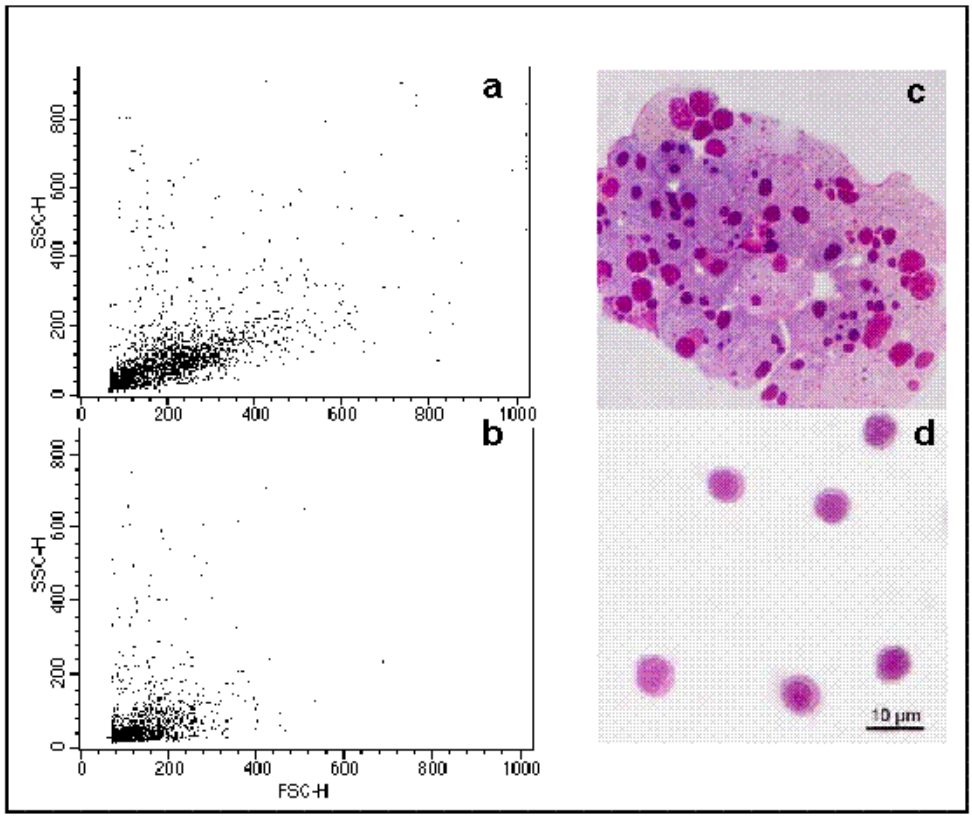

Figure 23: Purification of seabream GALT cells by Percoll density gradients. a) Representative flow cytometry dot plot of intermediate density band (ID) corresponding to 1.060 g/l. b) Representative flow cytometry dot plot of cells found at the highest density band (HD) (1.075 g/l). c) Light micrograph of a Giemsa stained cytocentrifugation of ID band cells. d) Light micrograph of a Giemsa stained cytocentrifugation of HD band cells.

Microscopy studies help to understand the chemical and enzymatic steps

Observation of semithin sections of the seabream gut tissue during the different steps of the isolation protocol permitted visualisation of the different degrees of digestion of the tissue as well as the progressive release of immune cells. Treatment with DTT loosened the spaces between mucosal epithelium enterocytes (Fig. 24b) compared to control fragments (Fig. 24a), whereas treatment with collagenase for 60 minutes sufficed to digest the connective tissue that forms the lamina propria of the intestinal epithelium (Fig. 24c).

The GALT extraction method here described led us to obtain cell pools that consisted of lymphocytes, granulocytes, macrophages, enterocytes, erythrocytes and caliciform cells. All cell types were seen in the cytocentrifugation samples after Giemsa staining. Caliciform cells were the largest of all the cell types, round and with a cytoplasm

mostly occupied by mucus vacuoles. Erythrocyte numbers varied from sample to sample but were always low. Enterocytes were smaller than caliciform cells but bigger than immune cells and contained features of picnocytosis- like vesicles that were intensely stained with Giemsa. Granulocytes and macrophages were scarce in all cases. Lymphocytes were abundant at all times, showing a typical round morphology, small size and big nucleus.

Cytocentrifugation of the three bands obtained by Percoll density separation pointed to the presence of all cell types in LD, along with cell debris (not shown). The ID band contained cell suspensions enriched in enterocytes and caliciform cells (Fig. 23c). Lymphocytes were also found. The HD band corresponding to low SSC and FSC values by flow cytometry was composed of highly homogeneous lymphocyte suspensions according to their morphological and staining properties, as shown in Fig. 23d. Some macrophages and granulocytes were observed but always in very low numbers.

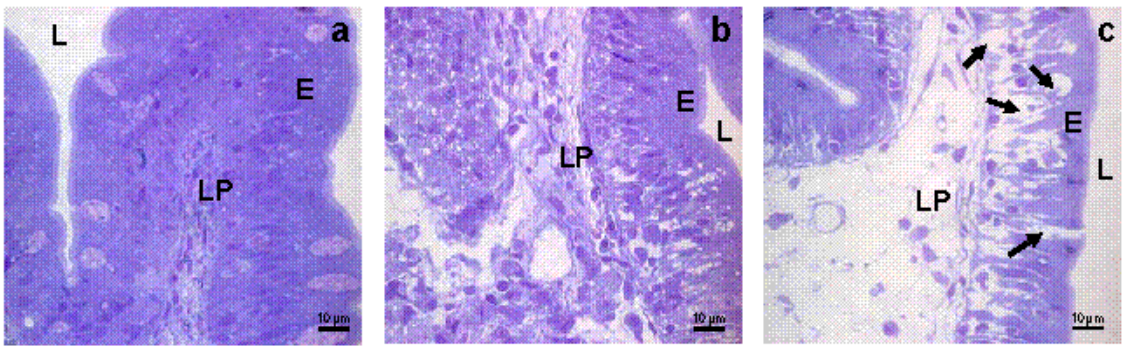

Figure 24: Light micrographs of semithin sections of seabream intestine at different stages of cell extraction. L (lumen), E (enterocytes), LP (lamina propria). (a) Gut semithin section at the beginning of the isolation procedure (control sample, time 0). Bar: 10 µm. (b) Gut semithin section after 10min in DTT. Bar: 10 µm (c) Gut semithin section after 10 min in DTT and 60 min in collagenase (0.15 mg/ml). Note the open interepithelial spaces (arrows) that allow leucocytes to be freed into the media. Bar: 10 µm.

Respiratory burst as an indicator of phagocyte release during isolation

NBT assays corroborated the low abundance of phagocyte (NBT-positive) cells present in the seabream GALT cells obtained by three different protocols (Fig. 25a). No significant differences were found between mechanical and enzymatic protocols. The use of 0.37 mg/ml collagenase for 120 min produced more NBT-positive cells, however the increase was not statistically significant compared to the other methods. The scarcity of NBT-positive cells was reflected in light micrographs (Fig. 25b). Positive controls

(head kidney suspensions) always contained high numbers of NBT-positive cells (not shown).

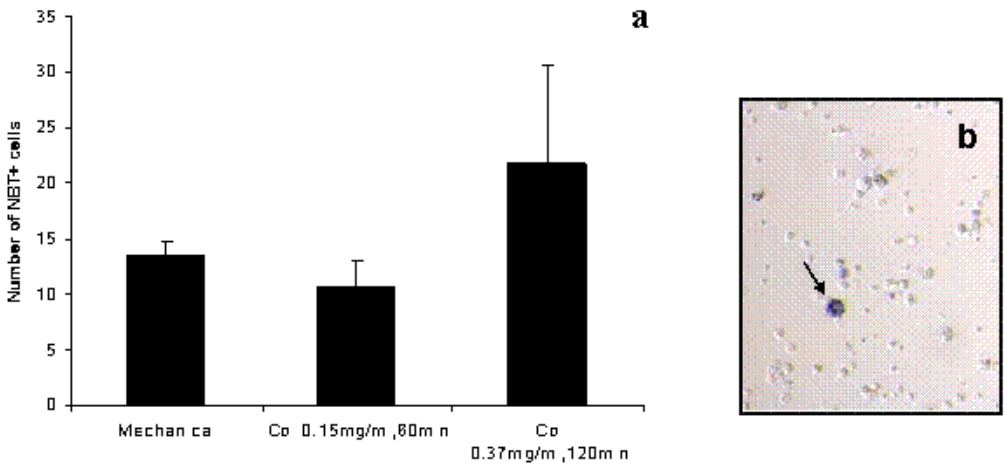

Figure 25: Number of NBT positive cells per well in total seabream GALT cells suspensions obtained after three different purification protocols. Data are expressed as mean ± se. Representative light micrograph (x400) after NBT assay showing a NBT-positive cell (arrow) containing formazan precipitates.

All results obtained in the present work are summarised in Table 5.

Activity	Gut leucocytes	HK leucocytes
Respiratory burst (total area under curve or sum)	14870 ± 3782	1677391 ± 42352
Peroxidase content (absorbance 450nm)	0.18 ± 0.015	0.397 ± 0.064
Natural cytotoxic activity (%)	33.5 ± 10.54	10.03 ± 2.94
Protein Alabelling (%)	20.6 ± 4.8	21.2 ± 3.8

Table 5: Different in vitro activities measured from seabream gut or head kidney leucocytes. Data are expressed as mean ± SE.

The respiratory burst response of seabream GALT leucocytes was a slow reaction (low maximum slope) and showed also a low maximum value. As a consequence, we took the mean area under the curve as the measure of this activity; the mean value recorded being 14870 ± 3782. The mean value obtained from HKLs was 1677391 ± 42352, which is about 113 times higher than in GALT leucocytes. When plates were incubated overnight and adherent and non-adherent GALT cells separated, only non-adherent cells showed respiratory burst activity. Adherent cells displayed similar values to those of the controls lacking PMA (not shown).

The total peroxidase content of seabream GALT leucocyte suspensions measured as the mean OD at 450nm was 0.18 ± 0.015, whereas the value recorded for HKLs was 0.397 ± 0.064 (2.2 times the peroxidase content of GALT leucocytes).

Mean natural cytotoxic activity of seabream GALT *in vitro* as measured by flow cytometry using DiO-labelled L1210 cells was 33.5% ± 10.54, over 3 times the recorded value for HKLs, which was 10.03 % ± 2.94 as shown in Table 5. The mean percentage of FITC-Protein A labelled cells from seabream GALT cells was 20.6 % ± 4.8, that of HKLs being very similar (21.2 % ± 3.8).

The phagocytic activity of fish head kidney leucocytes is routinely studied by flow cytometry. However, background values of green fluorescence and low percentage of phagocytosis (green labelled cells) rendered this technique invalid when applied to GALT leucocytes.

The study of phagocytosis by TEM further confirmed the scarcity of phagocytic cells in seabream GALT and explains the trouble faced when trying to quantify this activity by flow cytometry. Granulocytes and macrophages were the only two cell types observed engulfing target particles both in the case of *S. cerevisiae* and latex beads (Figs. 26 a-d). The maximum number of yeast or latex bead particles observed inside phagosomes was 4 and 7, respectively.

Lymphocytes, goblet cells, enterocytes and rodlet cells were sometimes contacting target particles but never ingesting or engulfing them. In the case of *L. del. lactis*, particle ingestion was lower (1 or 2 cells per leucocyte) and no signs of activation (e.g. cell processes or patent nucleoli) were observed in the cells from a morphological standpoint (Figs. 26 e-f).

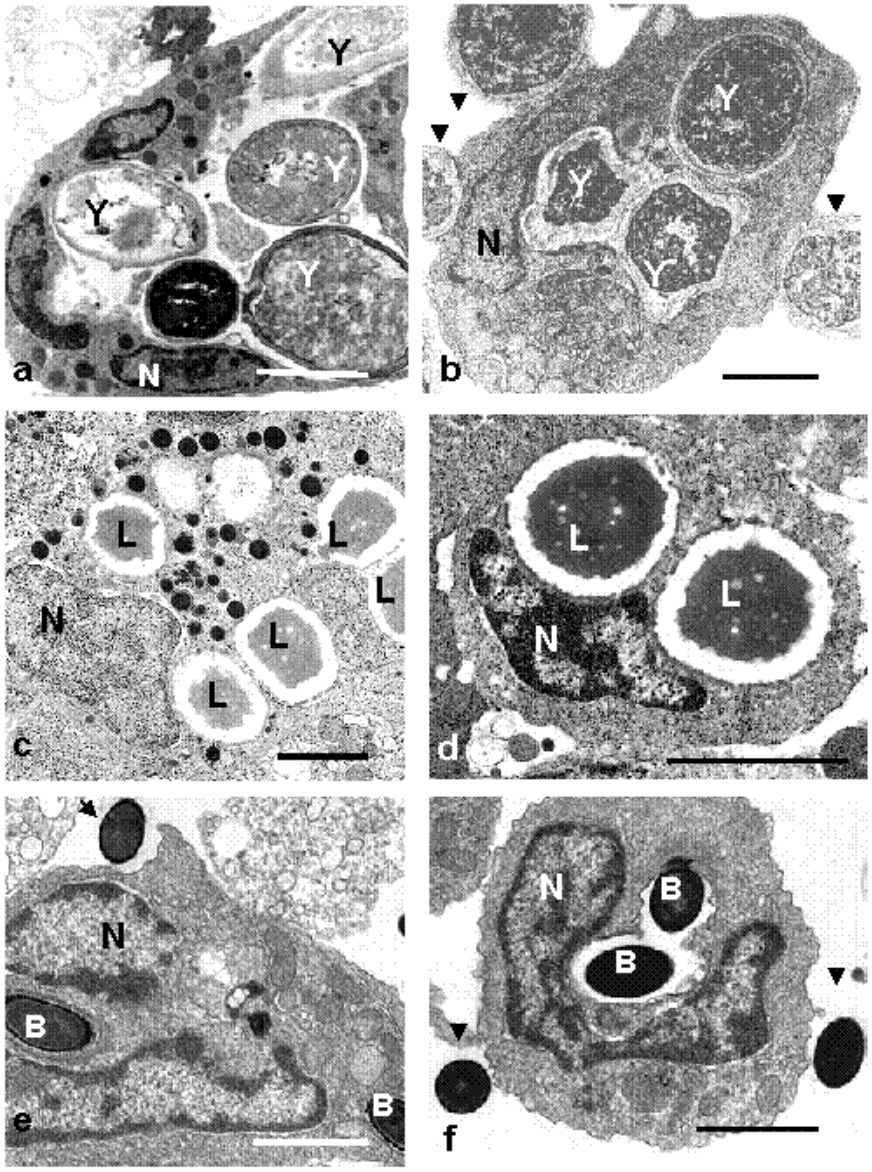

Fig. 26: TEM micrographs from phagocytosis studies of seabream gut leucocytes. a) Granulocyte showing four yeast particles (Y) engulfed in its cytoplasm. b) Macrophage with three yeast particles (Y) in its cytoplasm. Note the cytoplasmic membrane entering contact with other yeast cells (arrow heads). c) Granulocyte containing five latex beads (L) in its cytoplasm. d) Macrophage which engulfed latex beads (L). e) Granulocyte with two *Lactobacillus delbrüeckii* ssp. *lactis* particles (B) inside its cytoplasm and two more (arrow heads) in the proximity of the cell. f) Macrophage that ingested two *Lactobacillus delbrüeckii* ssp. *lactis* particles (B). Other bacterial cells which appear outside the cell are marked by arrow heads. N: nucleus. Scale bar = 2µm.

OBJECTIVE IV: ANTIPROLIFERATIVE EFFECTS OF PROBIOTIC BACTERIA ON FISH CELL LINES

The final purpose of this section required first setting up an inexpensive technique to measure apoptosis in fish leucocytes. The results corresponding to these experiments are presented prior to those obtained from the experiments related to antiproliferative effects of probiotic cytoplasmic extracts.

FDA labelling of seabream leucocytes

Different cell types required different FDA concentrations in order to acquire the green fluorescence. Green (FDA^+) cells correspond to viable cells whose esterases are capable of cleaving FDA into fluorescein and diacetate. Fig. 27 shows the percentage of each cell type that was stained in green after the addition of each FDA concentration. The optimal concentration of FDA to stain phagocytes was 50 µg FDA/ml, whereas lymphocytes required 75 µg FDA/ml. Higher concentrations did not result in greater staining percentages.

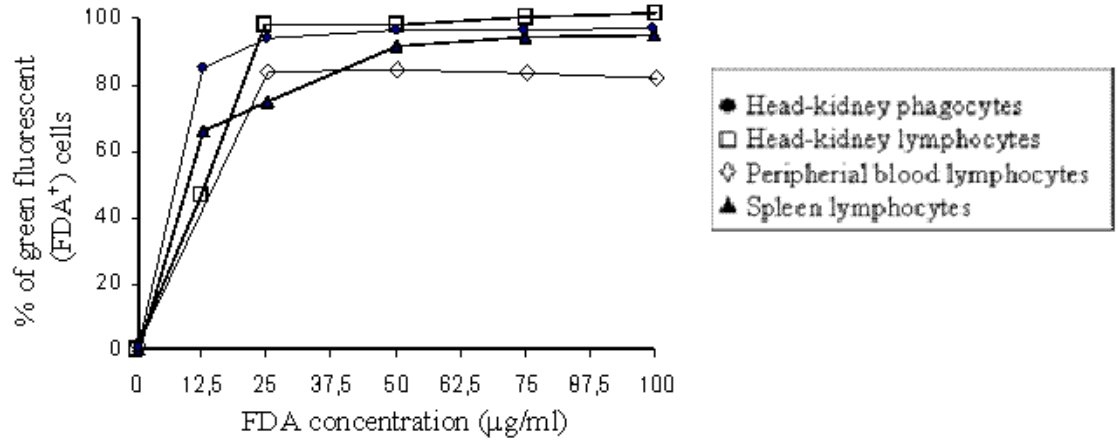

Figure 27: Percentage of fluorescent (FDA^+) seabream leucocytes after incubation with different concentrations of fluorescein diacetate (FDA). Note that maximum labelling occurs at 50 µg/ml FDA in the case of phagocytes whereas in the case of lymphocytes was 75 µg/ml.

Kinetics of the viability of seabream primary leucocyte cultures

Following double staining with FDA and PI, three subpopulations were identified and quantified by flow cytometry: i) viable (FDA^+/PI^-), ii) apoptotic (FDA^-/PI^-) and iii) necrotic (FDA^-/PI^+). Positive controls for necrosis consisting of permeabilised cells

fixed with formaldehyde were 100% PI^+ (data not shown). Fig. 28 (a-d) shows the dotplots and histograms recorded after staining the HKL suspensions.

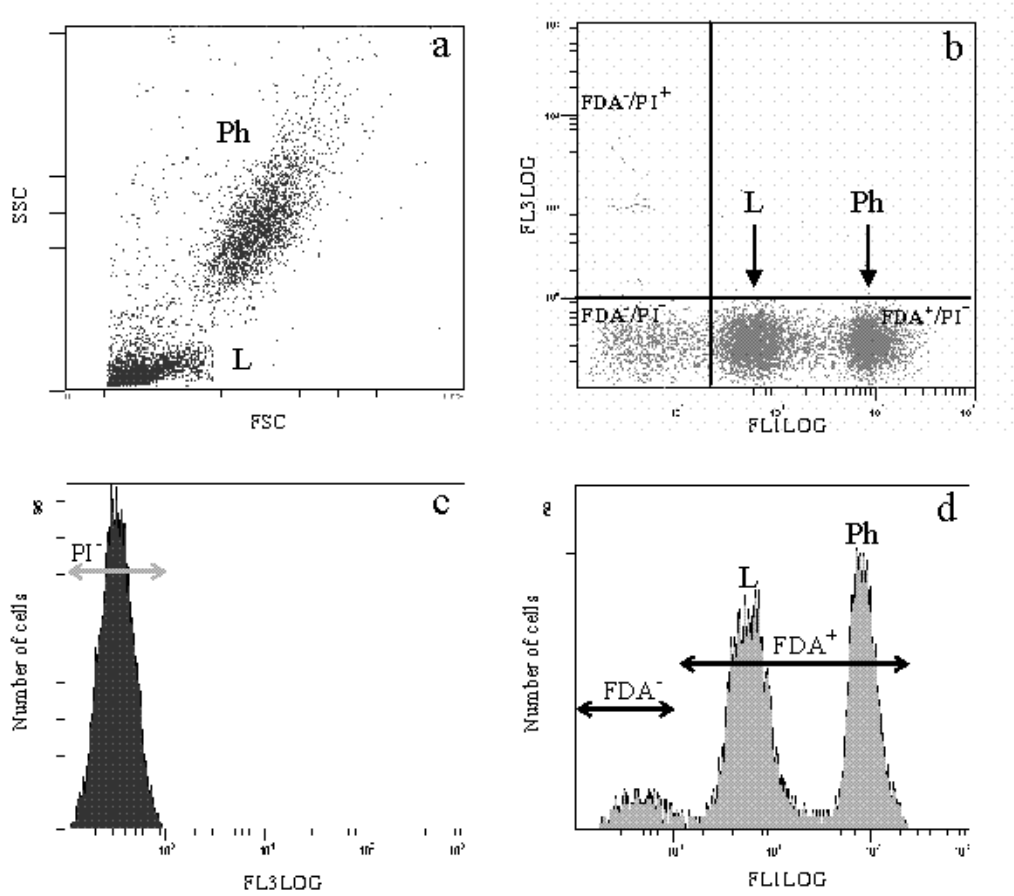

Figure 28: Flow cytometry analysis of a readily isolated head kidney leucocyte suspension after staining with FDA (50 µg/ml) and propidium iodide (PI) for 30 min. (a) Dotplot of showing two main populations: lymphocytes (L) and phagocytes (Ph) according to their SSC and FSC values. (b) Dotplot showing staining with FL1 (green fluorescence) and FL3 (red fluorescence). Note the presence of three populations according to the double staining pattern. The FDA^+/PI^- (viable) population contains two subpopulations, the phagocytes (Ph) that acquire greater green intensity and the lymphocytes (L) with a weaker fluorescence. (c) Histogram showing only FL3 (red fluorescence) after labelling of HKL´s with the double staining protocol. Note that almost all cells were not permeabilised and therefore are PI^- (d) Histogram showing the green labelled leucocytes (FL1) from a readily isolated HK suspension. Most cells were FDA^+ but lymphocytes (L) and phagocytes (Ph) clearly show different labelling with FDA.

The percentages of each cell leucocyte suspension analysed that were FDA^-/PI^+ at 0, 24 and 48 h in culture are shown in Table 6. HK lymphocytes and phagocytes displayed clearly different viabilities in the same culture conditions, the percentage of FDA^-/PI^- (apoptotic) lymphocytes being at least double that of phagocytes at all assayed times. Similarly, the percentage of FDA^-/PI^+ (necrotic) HK lymphocytes was higher than the percentage of FDA^-/PI^+ HK phagocytes and such difference increased with culture time.

		0 h		24 h		48 h	
Organ	**Cell Type**	**FDA^-PI^-**	**FDA^-PI^+**	**FDA^-PI^-**	**FDA^-PI^+**	**FDA^-PI^-**	**FDA^-PI^+**
HK	Phagocytes	7.6 ± 2.0^{a}	1.4 ± 0.7^{a}	18.2 ± 5.5^{ab}	2.0 ± 0.7^{a}	28.2 ± 4.3^{b}	4.3 ± 0.9^{a}
	Lymphocytes	25.2 ± 3.0^{a}	0.9 ± 0.2^{a}	44.7 ± 4.9^{b}	3.7 ± 0.5^{b}	56.4 ± 5.8^{b}	10.0 ± 0.8^{c}
PB	Lymphocytes	16.3 ± 0.7^{a}	0.3 ± 0.2^{a}	46.5 ± 1.7^{b}	7.6 ± 1.1^{a}	52.3 ± 1.5^{b}	14.1 ± 4.7^{b}
S	Lymphocytes	12.8 ± 2.7^{a}	1.8 ± 0.6^{a}	51.2 ± 2.4^{b}	10.2 ± 2.1^{b}	53.4 ± 3.1^{c}	29.7 ± 4.7^{c}

Table 6: Effect of culture time in viability of seabream leucocyte. Percentage of FDA^-/PI^- (apoptotic) FDA^-/PI^+ (necrotic) seabream leucocytes from head kidney (HK), peripheral blood (PB) and spleen (S) after 0, 24 or 48 h in culture. Results are expressed as mean ± se. Differences were statistically significant when $p<0.05$. One-way analysis of variance (ANOVA) and Tukey's comparison of means were conducted when necessary.

HK lymphocytes, nevertheless, seemed more sensitive to the initial isolation steps than S or PB lymphocytes, since at 0h, 25% of them were already apoptotic whereas only 12.8% of spleen lymphocytes and 16.3% of PB lymphocytes were FDA^-/PI^-. Lymphocytes isolated from all organs showed, in turn, a similar behaviour in culture, the FDA^-/PI^- population being around 47% or 54% of the total number of lymphocytes after 24 or 48 h of culture, respectively.

Finally, despite the fact that cells still cleaved FDA at 24 or 48 h, it was observed that the intensity or mean green fluorescence value decreased with culture time (data not shown).

Effects of apoptotic inducers on the viability of seabream primary leucocyte cultures

The effects of adding resveratrol or staurosporine to seabream primary leucocyte cultures at 0 h and 24 h are summarised in Table 7. At 0 h, there were no statistically significant differences between control cultures and cultures where apoptotic inducers had been added. HK phagocytes appeared more resistant to both treatments than the lymphocytes, since the percentage of FDA^-/PI^- only increased from 9.8% to 10.1% after 24 h incubation with resveratrol and from 8.8% to 14.6% in the case of staurosporine. On the contrary, the percentage of apoptotic lymphocytes after 24 h in resveratrol or

staurosporine almost doubled the percentage recorded immediately after their addition (0 h).

			Resveratrol		Staurosporine	
Organ	**Cell type**	**Incubation time**	**FDA$^-$PI$^-$**	**FDA$^-$PI$^+$**	**FDA$^-$PI$^-$**	**FDA$^-$PI$^+$**
HK	Phagocytes	0 h	9.8±2.7	3.5±1.3	8.8±3.1	2.3±0.6
		24 h	10.1±2.4	6.7±2.4	14.6±3.5	3.1±1.6
	Lymphocytes	0 h	35.3±4.3	2.9±0.9	29.8±5.1	1.5±0.3
		24 h	57.1±5.9*	20.0±4.9*	56.9±4.2*	13.6±2.7*
PB	Lymphocytes	0 h	19.1±4.2^a	0.3±0.1^a	19.7±4.7^a	0.3±0.2^a
		24 h	66.5±4.2*	7.5±2.8	77.5.4±1.5*	11.1±3.6
S	Lymphocytes	0 h	23.0±0.8	2.9±0.9	15.8±1.2	1.5±0.3
		24 h	53.9±4.3*	24.5±6.8*	61.4±3.1*	17.8±1.7*

Table 7: Apoptotic inducers on the viability of seabream leucocyte cultures. Effects of resveratrol and staurosporine in the percentage of FDA$^-$/PI$^-$ (apoptotic) FDA$^-$/PI$^+$ (necrotic) seabream leucocytes from head kidney (HK), peripheral blood (PB) and spleen (S) after 0 and 24 h in culture. Results are expressed as mean ± se. One-way analysis of variance (ANOVA) and Tukey's comparison of means were conducted when necessary. Differences were statistically significant when $p<0.05$. Asterisk denotes statistically significant differences between treated leucocytes at 24 h compared to treated leucocytes at 0 h. No statistically significant differences were found between control cultures and treated cultures at 0 h.

Viability of PB leucocytes was severely affected both by resveratrol and staurosporine treatments. There were significantly more apoptotic PB leucocytes after 24 h (more than 3 times in the case of resveratrol and almost 4 times in the case of staurosporine) than immediately after their addition. This population, however, was not significantly greater in treated cell cultures compared to non-treated (controls) cultures at 0 h. After 24 h in culture, the percentage of apoptotic PB leucocytes was significantly higher in the groups treated with the apoptotic inducers compared to control cultures.

Spleen lymphocytes showed a slightly different behaviour, with a lower percentage of apoptotic cells compared to PB leucocytes after 24 h in culture but greater values in the FDA^-/PI^+ subpopulation, indicating a more advanced stage of the apoptosis process with more cells already permeabilised at this time point. The addition of resveratrol or staurosporine left 22% of spleen lymphocytes viable after 24h in culture, which is indeed similar to what was found in the case of HKLs and PB leucocytes.

After 48 h in culture with either resveratrol or staurosporine, all the leucocytes were FDA^-/PI^+ (data not shown in Table 7).

Microscopy study

Observation of the leucocyte primary cultures from the different assays under the light or fluorescence microscope confirmed the presence of FDA^+/PI^- (viable), FDA^-/PI^- (apoptotic) and FDA^-/PI^+ (necrotic) cells. Figure 29 shows cells belonging to all three staining patterns from a suspension of HKLs after 24 h in culture.

TEM examination of leucocyte cultures revealed that control cultures (no staurosporine) consisted of cells of normal morphology at the beginning of the experiment.

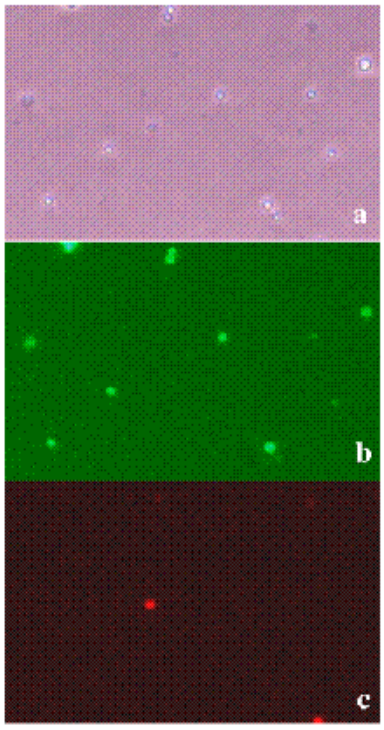

Figure 29: Fluorencence microscopy study after labelling a seabream head kidney leucocyte (HKL) suspension after 24 h in culture with the FDA/PI double staining technique. Cells belonging to all three staining patterns were observed. x400. (a) Phase contrast micrograph showing all cells present in the field. (b) Some of the cells in (a) but not all of them are viable and therefore stained in green (FDA^+). (c) Red (PI^+) fluorescent cells are already permeabilised and can be seen in (a) but not in (b).

The ultrastructure of seabream HKL cultures after 0, 24 or 48 h in the presence of staurosporine and the corresponding flow cytometry analysis after FDA/PI labelling are

shown in Fig. 30. Leucocytes cultured in the presence of staurosporine (dotplot Fig. 30a) were characterised by normal nucleus and cytoplasm morphology at the beginning of the experiment (Fig. 30d) and were no different from control leucocytes at the same time point. However, the number of cells with typical chromatin condensation and aberrantly shaped nuclei (apoptotic) dramatically increased after 24 h in staurosporine (Fig. 30e), which correlates with the flow cytometry analysis of this suspension (Fig. 30b). Apoptotic cells were also seen after 24 h in control cultures, although they were not as abundant as in the case of the staurosporine-incubated cultures. After 48 h of culture with staurosporine (dotplot Fig. 30c), the majority of the cells had lost their architecture, with their membrane and cytoplasm profoundly damaged, and therefore scored as necrotic (Fig. 30f).

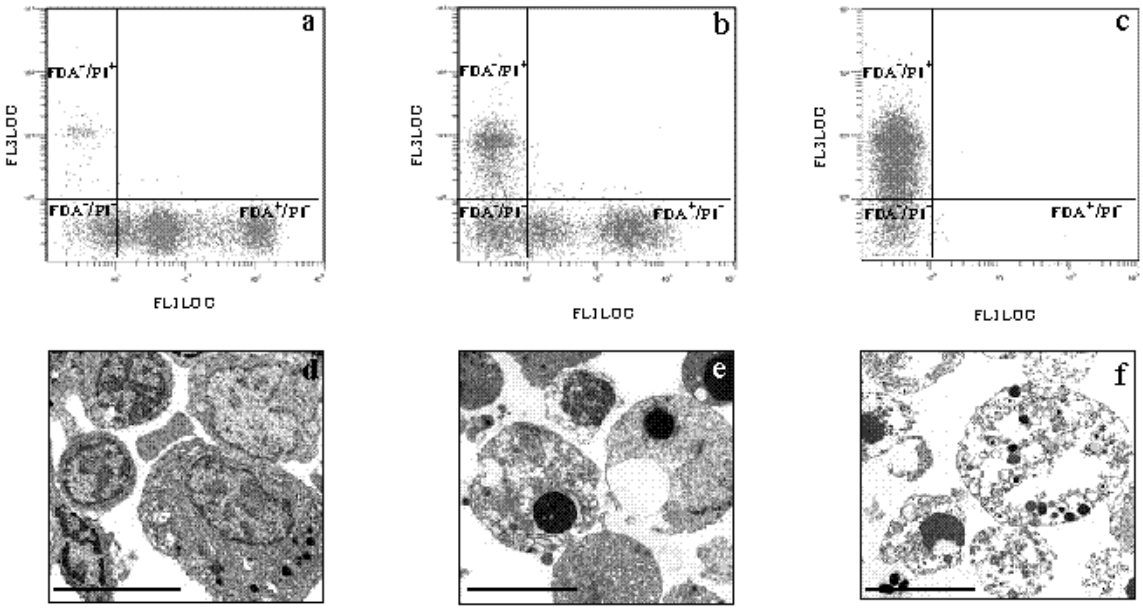

Figure 30: Flow cytometry (a-c) and transmission electron microscopy (d-f) study of a HKL suspension cultured for 48 h in the presence of staurosporine (1µg/ml). (a) Dotplot after labelling the suspension with FDA and PI immediately after isolation of the cells and addition of staurosporine (0h). (d) TEM micrograph of the same leucocyte suspension. (b) Dotplot after 24 h in culture with staurosporine. (e) TEM micrograph of the same leucocyte suspension. (c) Dotplot after 48 h in culture with staurosporine. (f) Transmission electron micrograph corresponding to (c). The FDA^+/PI^- population progressively moves towards the left throughout time. Concomitantly, the FDA^-/PI^- moves upwards entering the FDA^-/PI^+ quadrant. These shifts in the populations observed under flow cytometry were in agreement with the morphological changes seen under TEM with increasing numbers of apoptotic nuclei over time and membrane disruption at the end of the experiment.

Effects of probiotic bacteria cytoplasmic extracts on proliferation of fish cell lines

SAF-1 cells proliferation was significantly inhibited by *L. delbrüeckii* subsp. *lactis* cytoplasmic fraction in a dose dependent manner. The antiproliferative effect was significant at 4 and 24 h when the highest dose of protein cytoplasmic fraction was applied. Longer culture times (48 or 72 h) revealed that SAF proliferation was significantly inhibited by all *L. del. lactis* treatments (Fig. 31a).

Cytoplasmic fraction of 51M6 bacteria had a less marked effect on SAF-1 cell growth compared to those obtained from *L. del. lactis*. In fact, SAF-1 proliferation was inhibited only after 72 h in culture regardless of the dose. Shorter incubation times did not result in any effect on the numbers of cells compared to control samples (Fig. 31b).

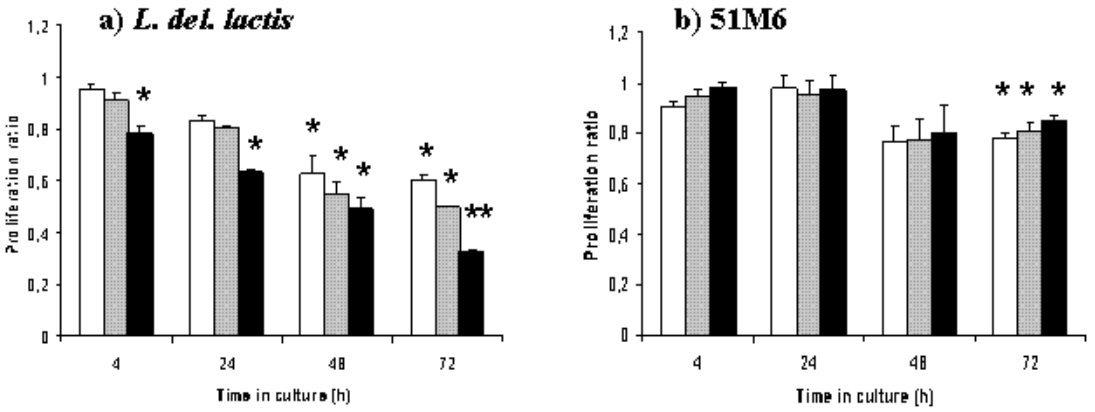

Figure 31: a) Effect of *Lactobacillus delbrüeckii* subsp. *lactis* cytoplasmic extract (µg protein/ml) on the proliferation of SAF-1 cells. b) Effect of 51M6 cytoplasmic extract (µg protein/ml) on the proliferation of SAF-1 cells. Data are expressed as mean proliferation ratio + se. Asterisks denote statistically significant differences ($p < 0.05$).

EPC proliferation was inhibited by *L. del. lactis* cytoplasmic fraction at the highest assayed dose (100 µg/ml) after 24 h or more in culture, the greatest inhibition occurring after 72 h, when the number of cells present was less than 50 % of the controls (mean ratio = 0.48). At this time, 25 and 50 µg/ ml of the cytoplasmic extract of *L. del. lactis* also significantly inhibited EPC proliferation, but proliferation ratios were about 0.8. A 20 % decrease in proliferation was also recorded after 4h in contact with both 50 and 100 µg/ml *L. del. lactis* cytoplasmic extracts, but only the former was statistically significant (Fig. 32a).

Cytoplasmic fraction of 51M6 only affected EPC proliferation at 4 and 72 h. The inhibition recorded at the first time point was only mild (proliferation ratios higher than 0.85) and not dose-dependent. It occurred at all the assayed protein concentrations to a

slightly greater degree (proliferation ratios around 0.75) after 72 h in culture compared to 4h. The observed effects were nevertheless less marked than those recorded for the *L. del. lactis* treatments (Fig. 32b).

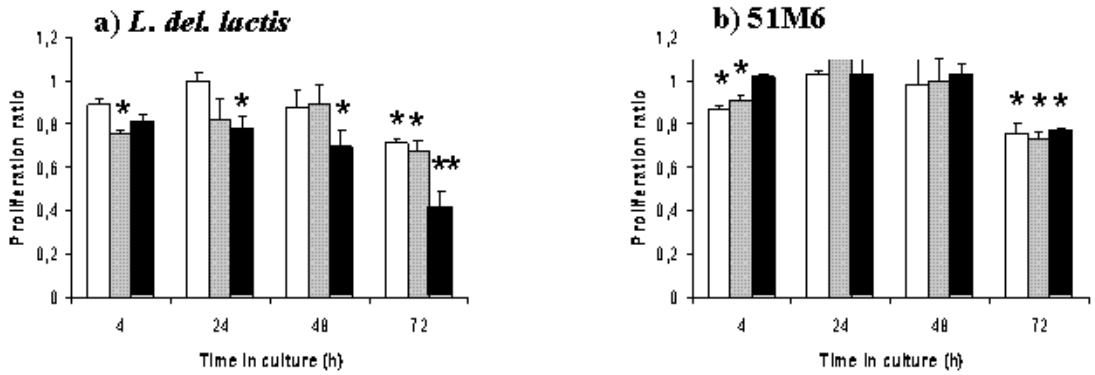

Figure 32: a) Effect of *Lactobacillus delbrüeckii* subsp. *lactis* cytoplasmic extract (µg protein/ml) on the proliferation of EPC cells. b) Effect of 51M6 cytoplasmic extract (µg protein/ml) on the proliferation of EPC cells. Data are expressed as mean proliferation ratio + se. Asterisks denote statistically significant differences ($p < 0.05$).

Induction of apoptosis in fish cell lines by cytoplasmic fraction of probiotic bacteria

SAF-1 cells entered apoptosis in a dose-dependent manner after the addition of *L. del. lactis* cytoplasmic fraction, although the lowest assayed dose had no effects on the apoptosis of SAF-1 cells at any assayed time (Fig. 33a). There were significantly more apoptotic cells in the samples incubated with the 100 µg/ml dose for 48 and 72 h. The intermediate dose (50 µg/ml) also resulted in apoptosis induction, but the result was

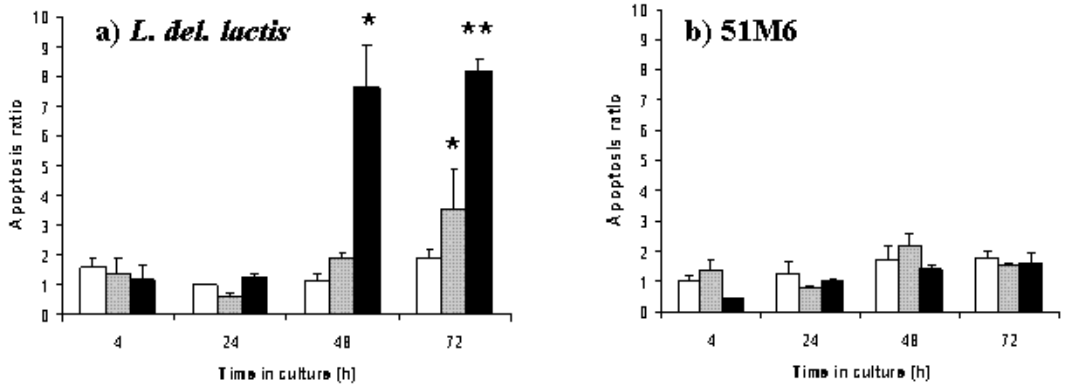

Figure 33: a) Effect of *Lactobacillus delbrüeckii* subsp. *lactis* cytoplasmic extract (µg protein/ml) on the apoptosis of SAF-1 cells. b) Effect of 51M6 cytoplasmic extract (µg protein/ml) on the apoptosis of SAF-1 cells. Data are expressed as mean apoptosis ratio + se. Asterisks denote statistically significant differences ($p < 0.05$).

only statistically significant after 72 h. At this time, the highest dose caused, approximately, 50% apoptosis, which was over 5 times higher than that present in control samples and 2.5 times the value recorded for the 50 µg/ml treatment.

The cytoplasmic extract of the 51M6 bacteria did not significantly induce apoptosis in SAF-1 cells. The greatest number of apoptotic SAF-1 cells occurred when they were incubated with the 51M6 cytoplasmic fractions at any concentration for 72 h; however, these increases were not statistically significant at any time of the experiment (Fig. 33b).

EPC entered apoptosis when 100 µg/ml *L. del. lactis* were added to the cultures. Whereas no effects were significant after 4 h, apoptosis ratios were 2, 4 and 9 after 24, 48 or 72 h, respectively (Fig. 34a). In absolute numbers, however, apoptosis was much lower than in SAF-1 cells; with a peak mean apoptosis value of 11.2 % after 72 h.

All three 51M6 treatments resulted in a 2-fold increase in EPC apoptosis after 48 h in culture but the effect was not significant at any other time of the experiment (Fig. 34b). In absolute numbers this effect was not very drastic since maximum necrosis values never represented more than 3.4 % of the total EPC cells at this time.

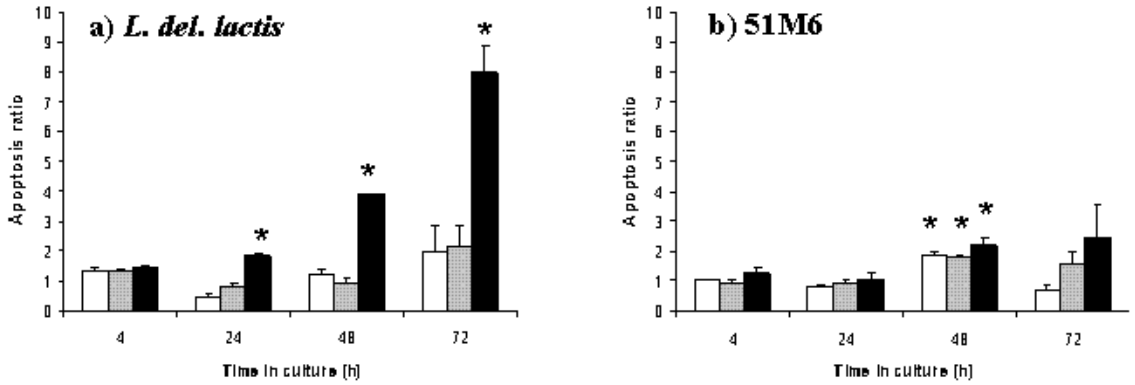

Figure 34: a) Effect of *Lactobacillus delbrüeckii* subsp. *lactis* cytoplasmic extract (µg protein/ml) on the apoptosis of EPC cells. b) Effect of 51M6 cytoplasmic extract (µg protein/ml) on the apoptosis of EPC cells. Data are expressed as mean apoptosis ratio + se. Asterisks denote statistically significant differences ($p < 0.05$).

Induction of necrosis in fish cell lines by cytoplasmic fraction of probiotic bacteria

In SAF-1 cultures, necrosis also took place although total numbers of necrotic cells never exceeded those of apoptotic cells. Neither 25 nor 50 µg/ml *L. del. lactis* resulted in significant necrosis at any time. 100 µg/ml *L. del. lactis*, in turn, produced a 5-fold

increase and 6-fold increase in necrosis after 48 or 72 h in culture, respectively (Fig. 35a). In total numbers this meant that over 32% of SAF-1 cells were necrotic at the end of the experiment when 100 µg/ml *L. del. lactis* was applied.

51M6 protein cytoplasmic fraction, at all the assayed doses, induced necrosis in SAF-1 cells after 48 and 72 h, when it was between 3 and 4 times higher than in the controls (Fig. 35b). This represented between 13 % and 18 % of the total cell numbers, a significantly lower value compared with the necrosis rate induced by *L del. lactis*.

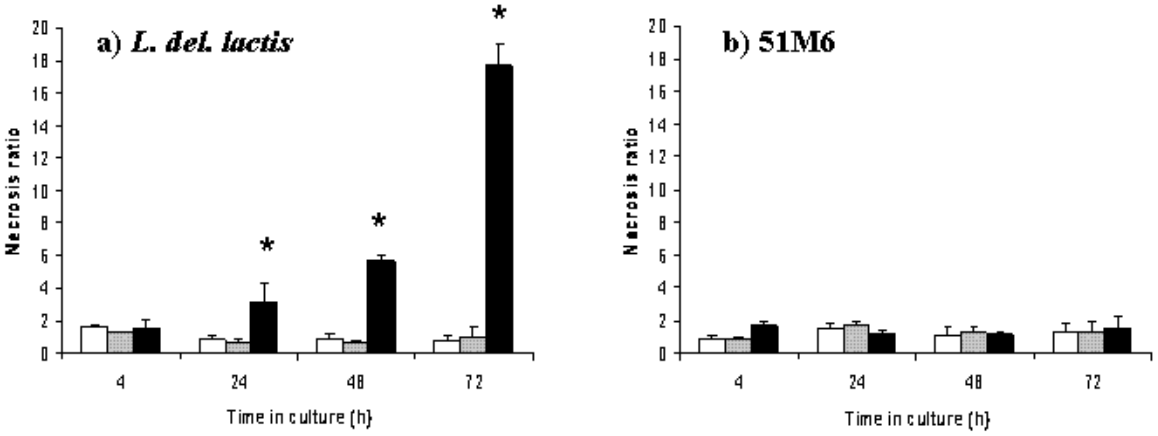

Figure 35: a) Effect of *Lactobacillus delbrüeckii* subsp. *lactis* cytoplasmic extract (µg protein/ml) on the necrosis of SAF-1 cells. b) Effect of 51M6 cytoplasmic extract (µg protein/ml) on the necrosis of SAF-1 cells. Data are expressed as mean necrosis ratio + se. Asterisks denote statistically significant differences ($p < 0.05$).

There were 4, 7 and 18 times more necrotic EPC cells after 24, 48 or 72 h, in culture with the cytoplasmic fraction of *L. del. lactis* (100 µg/ml), respectively (Fig. 36a). In absolute numbers, however, necrosis was always low; reaching a peak value of 6.7 % of the total cells at 72 h. Lower doses had no effect and did not differ between each other. 51M6 treatments had no effect on necrosis of EPC cells at any of the assayed doses or times (Fig. 36b).

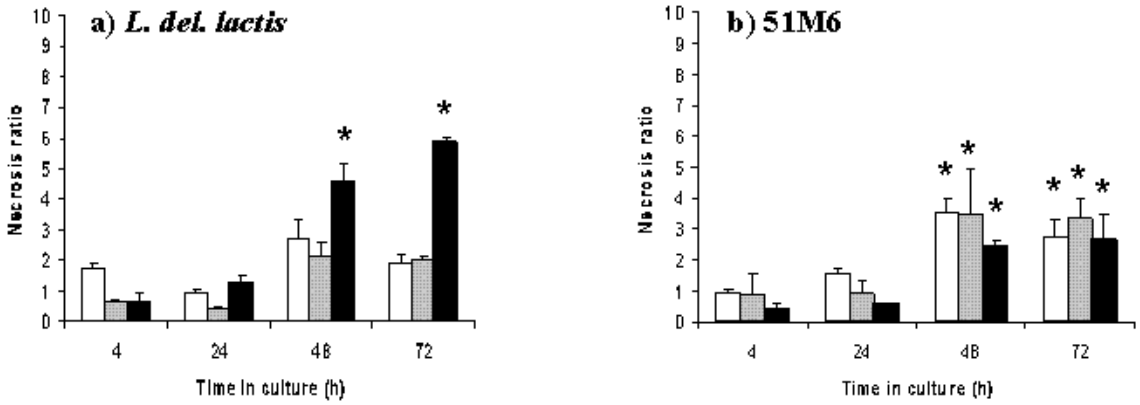

Figure 36: a) Effect of *Lactobacillus delbrüeckii* subsp. *lactis* cytoplasmic extract (µg protein/ml) on the necrosis of EPC cells. b) Effect of 51M6 cytoplasmic extract (µg protein/ml) on the necrosis of EPC cells. Data is expressed as mean necrosis ratio + se. Asterisks denote statistically significant differences ($p < 0.05$).

VII. DISCUSSION

OBJECTIVE I: MODULATION OF THE SEABREAM SYSTEMIC INNATE IMMUNE RESPONSES BY PROBIOTIC BACTERIA

The gastrointestinal tract of animals is in permanent contact with nutrients and commensal bacteria present in its surface. Both are vital for the adequate functioning and wellbeing of the host, having important regulatory effects on the host's local and systemic immune responses (Cunningham-Rundles and Lin; 1998). Amongst the several modes of action through which probiotic bacteria exert their beneficial effects, there is increasing evidence that such bacteria can also effectively improve the host innate and adaptive immune responses (Mihal et al., 1990; Isolauri et al., 2002; Heyman and Ménard, 2002). The bacterial species used in the present work were chosen on the basis of previous microbiological studies that pointed to them as good potential probiotics (Jacobsen et al., 1999; Nikoskelainen et al.; 2001) as well as the lack of studies assessing the effects of their administration to farmed fish species and, more particularly, to the seabream. Fish were fed live *Lactobacillus delbrüeckii* ssp. *lactis*, *Bacillus subtilis* or a mixture of the two bacteria for 3 weeks. During this time, changes in the cellular innate immune response of fish fed bacteria-supplemented diets occurred.
LABs have been thoroughly studied in human nutrition due to their outstanding properties as desirable bacteria, including a clear immune modulation potential (Mihal et al., 1990). *L. delbrüeckii* was identified as one of the five species with better probiotic properties amongst the 47 lactobacilli screened by Jacobsen et al. (1999). Rainbow trout fed *L. rhamnosus* (8×10^4 cfu/g) for 2 weeks showed a significant increase in the respiratory burst activity compared to the control group (Nikoskelainen, 2003). Such activity was the only cellular immune response measured in the study despite the fact that previous work conducted in humans indicated that the immunomodulatory effects of *L. helveticus* more specifically refer to other cellular immune parameters (Matar et al., 2001).
In our study, supplementation with 10^7 cfu/g *L. delbrüeckii* ssp. *lactis* did not affect the respiratory burst activity of seabream HKLs. Differences between our results and those of Nikoskelainen et al. (2003) could be attributed to the higher dose of bacteria used here or to the chosen bacteria species. We found, nevertheless, that the phagocytic activity of seabream that had received the *L. delbrüeckii* ssp. *lactis* supplemented diet for 2 weeks was significantly higher than that of control fish. This increase did not

correlate with any increase in other assayed cellular innate immune activity. However, at week 3 of our feeding trial, phagocytosis stimulation was no longer noticeable and a significant decrease in the peroxidase content of HKLs occurred. It seems that modulation of phagocytic activity is one of the most immediate and key effects produced by probiotic bacteria on the host immune system, both in higher vertebrates (Schiffrin et al., 1997) and fish (Irianto and Austin, 2002).

Bacillus spores are being used as probiotics for human and animal consumption due to their immunostimulatory properties on the gastrointestinal immune system (Casula and Cutting, 2002). Different *Bacillus* species have been isolated from fish, crustacean and bivalves (Gatesoupe, 1999). Additionally, *B. subtilis* possesses antibiotic activity against pathogenic *Vibrio* spp. and effectively improves pond water quality and the survival of the black tiger prawn (Vaseeharan and Ramasamy, 2003). Research concerning oral administration of *Bacillus* spp. to cultured fish species has merely consisted of challenge experiments. *B. toyoi* was evaluated for its capacity to reduce edwardsiellosis in the European eel (Chang and Liu, 2002) whereas a mixture of *Bacillus* spp. added to the diet of 120-day-old rainbow trout for 42 days increased the survival of fish against *Yersinia ruckeri* infection (Raida et al., 2003). However, possible changes of the host immune response elicited by the oral administration of *Bacillus* strains were not evaluated. The present work represents the first report of the immunomodulatory effects caused by dietary administration of live *B. subtilis* alone or in conjunction with a second bacterial strain to a fish host. It resulted in similar changes to the cellular innate immune parameters to those described for the *Lactobacillus* group. In this group, phagocytic capacity of seabream leucocytes was already significantly higher at week 1 of the experiment, whereas phagocytic ability became significantly greater than that of the control group after 2 weeks of feeding the *Bacillus* supplemented diet. By week 3, stimulation of phagocytosis ceased in both single bacteria groups. The rest of the parameters studied in the first in vivo study were not significantly affected, except for leucocyte cellular peroxidase content, which was significantly lower in the *Bacillus* supplemented group at week 3 of the experiment, as observed in the *Lactobacillus* fed group. Phagocytosis was again the most sensitive of all assayed cellular activities.

Very few studies have contemplated the use of two or more bacteria as a probiotic mixture in fish. However, combination of bacterial strains that complement each other and occupy different niches within the gut microflora environment could result in an

enhancement or prolongation of the desirable effects on the host immune response and health. Our findings point towards a possible interaction between the two bacteria chosen for this study. When the increased phagocytic activity of the leucocytes from fish fed the single bacteria groups disappeared at week 3, fish that received the two bacteria-supplemented diets maintained their greater leucocyte phagocytic ability. More surprisingly, cytotoxic activity, which had not varied during the first 2 weeks of feeding in any experimental group, was significantly higher in the two bacteria-supplemented groups at week 3. These data suggest a longer lasting and long-term effect of the probiotic mixture on the systemic cellular innate immune response of the seabream.

When experimental groups were fed the control diet during the last week of the experiment the values of all assayed activities tended to recover. Thus, differences observed during the previous weeks of the experiment between groups became no longer apparent. However, phagocytic capacity of the two groups fed the single bacteria-supplemented diets significantly decreased at the end of the experiment. It is unknown if such a decrease was due to the lack of adaptation to the control diet and whether or not prolonging the period during which fish were fed the control diet after having been fed the bacteria-supplemented diet would have masked this effect. The general trend followed by all cellular innate immune parameters when all groups were fed the control diet points to a transient state of the fed bacteria in the gastrointestinal tract of the seabream, which is in agreement with previous available results (Robertson et al., 2000; Irianto and Austin, 2002; Panigrahi et al., 2005). This finding led us not to use this strategy in the second *in vivo* trial, where groups were fed the experimental diets from the beginning to the end of the experiment. Additionally, we tested non-viable forms of probiotic bacteria.

First definitions of probiotics described them as live microbial preparations that benefit the host by improving its health status and well-being (Fuller, 1989). In aquatic animals they show a transient state in the host gut, and are rapidly expelled out when the dietary supply ceases (Irianto and Austin, 2002). Hence, new definitions of ‘‘probiotics’’ in aquaculture have emerged, which encompass the use of inactivated bacteria despite the fact that they are not able to colonise intestinal surfaces. Probiotic formulations consisting of inactive bacteria avoid the risk of releasing foreign bacteria into an open environment.

We evaluated both the humoral and cellular innate immune responses of seabream fed for 1, 2, 3 or 4 weeks with 51M6 (10^8 cfu/g feed), Pdp11 (10^8 cfu/g feed) or 51M6 and Pdp11 (0.5×10^8 cfu/g feed of each bacterium).

Despite the existence of previous reports revealing the significant positive effect of different probiotic bacteria in the level of complement (Panigrahi et al., 2004; 2007), all the experimental diets in this study led to increased complement activity although differences were not statistically significant compared with the control group. The finding that the highest values were reached at the end of the experiment agrees with results found by Panigrahi et al. (2004) in rainbow trout fed live *Lactobacillus rhamnosus*.

The other humoral immune parameter that was assayed, the peroxidase content in serum, was not significantly affected by any experimental diet at any time of the trial. The highest levels of peroxidase were observed during the third week, especially in seabream that received the diet containing Pdp11. On the other hand, the peroxidase content of leucocytes responded in different ways, with values that peaked in the second week in the groups fed the single bacterial diets, although the differences with respect to control group were not statistically significant. It is worth noting the significant decrease in the leucocyte peroxidase content of fish fed live *Lactobacillus delbrüeckii* ssp. *lactis* and *Bacillus subtilis*-supplemented diets recorded at the third week of our first *in vivo* study.

Phagocytic activity was significantly enhanced in seabream fed Pdp11 at week 2 but the enhancement occurred a week later in the groups fed 51M6 or the mixture of bacteria. These results suggest that in order to enhance phagocytic activity by using dietary 51M6, longer administration periods are required than when Pdp11 supplemented diets are provided. Increased phagocytic activity in the HKLs of fish fed live probiotic bacteria-supplemented diets (Irianto and Austin; 2002; Nikoskelainen et al., 2003; Panigrahi et al., 2004) and killed bacteria-supplemented diets has been reported by other authors (Irianto and Austin, 2003; Irianto, 2003). However, *Lactococcus lactis* supplementation did not affect the phagocytic activity of turbot head kidney macrophages after 1 week of daily administration (Villamil et al., 2002). As shown in our first *in vivo* study, seabream fed live *L. delbrüeckii* ssp. *lactis*, *B. subtilis*, or both in equal amounts, showed a more sustained stimulatory effect in their phagocytic activity of HKLs, the mixture of both bacteria again having the greatest stimulatory effect. Although phagocytic activity showed a significant increase, no statistically significant

differences were found in the respiratory burst activity of seabream HKLs between control and treated fish at any time of the experiment. Supplementation with Pdp11 provoked the greatest stimulation after 4 weeks of administration.

It has been reported that immunostimulants, such as glucans, are able to increase macrophage cytotoxic activity against tumour cells of mammals (Bogwald et al., 1982). The present work studied the cytotoxic activity of HKLs in individuals fed three different killed bacteria-supplemented diets. This parameter reached its highest level after week 3 in the group fed with 51M6. These results agree with our results using live bacteria. We can conclude that the bacterium with the greater stimulatory effect on the seabream innate immune system is Pdp11, since specimens fed this bacteria possessed higher levels of serum peroxidase and their HKLs showed higher levels of phagocytic and respiratory burst activities. Despite the fact that Pdp11 and 51M6 belong to the same family (*Vibrionaceae*), their immunostimulant effects are rather different, with 51M6 mainly affecting cytotoxic activity. On the other hand, the immunomodulatory effects caused by Pdp11 administration were faster than those prompted by 51M6. This time-course difference was well illustrated by the changes recorded in the phagocytic activity of HKLs from each of these experimental groups. While, phagocytic ability was significantly greater with respect to the control group after the second week of being fed Pdp11 and the mixture of the two bacteria, it was not until week 3 that a stimulatory response was observable in the group fed 51M6. More importantly, the combination of both bacteria resulted in higher phagocytic activities at week 3, probably due to the presence of 51M6. The present study investigated the effects of two monostrain probiotics and one multistrain formulation. Timmerman et al. (2004) concluded that multistrain probiotics (more than one strain of the same species or closely related species) and/or multispecies probiotics could be more effective and more consistent than that of a monostrain probiotic, since mixed cultures may contain bacteria that complement each other's health effect and thus have synergistic probiotic properties. The two bacteria assayed in the second *in vivo* experimental trial had no synergistic immunostimulatory effects, which could be either due to the close systematic relationship between them or to their inactivated form. On the contrary, synergistic effects between *Lactobacillus* ssp. and *Bacillus* ssp., which do not belong to the same family and were alive, were clearly demonstrated in the first trial. Differences in immunostimulatory properties between viable and inactive forms of the probionts have been found in rainbow trout (Panigrahi et al., 2005)

It is worth pointing out that most of the innate immune parameters assessed were enhanced in the third week of the trial, and so longer periods of feeding are not necessary. This agrees with our first findings concerning the live bacteria and could be a consequence of the way of administration. It is known that the most effective method for administering immunostimulants to fish is by injection, whereas the efficacy of oral and immersion methods decreases with long term administration (Timmerman et al., 2004).

Furthermore, we carried out an *in vitro* evaluation of the modulatory properties of the four probiotic bacteria on the seabream HKL innate immune activities. The four species could be divided into two different groups; 51M6 and Pdp11 are *Vibrio*-like bacteria of the *Shewanella* genus present naturally on seabream skin (Chabrillón et al., 2005), while the *Lactobacillus* and *Bacillus* genera are well-known bacteria intended for human- or terrestrial livestock use (Jacobsen et al., 1999). Therefore, this study throws light on whether or not the bacterial strain or its origin is related to the *in vitro* immunomodulatory properties of a probiotic.

Characterization of bacterial species that are suitable probiotic candidates by *in vitro* studies has obvious advantages over *in vivo* experiments since the time and number of experimental animals can be greatly reduced. In this sense, new assays should evaluate not only microbiological properties (adhesion and antimicrobial potential) but also other important benefits, such as nutritional and immunological benefits (Pathmakanthan et al., 2000; Koenen et al., 2004). Regarding the last one, stimulation of the host innate immune response is a key aspect of a probiotic, especially when aiming to control infectious diseases in production systems. Taking into account the lack of studies in this field, we investigated the *in vitro* capacity of the four bacteria previously studied *in vivo* to modulate the seabream cellular innate immune parameter.

Our investigations show that heat-killed probiotic bacteria possess a great potential for modulating the cellular innate immune parameters of a teleost fish following short *in vitro* incubation. 51M6 and Pdp11 have recently been isolated from fish surfaces and *L. del. lactis* and *B. subtilis* are probiotics intended for human and livestock use. All bacterial species were inactivated by heat and incubated *in vitro* with seabream leucocytes for 30 min. The observed stimulatory effects were strongly affected by the bacterial strain and concentration, which emphasises the importance of optimising the effective dose at which a probiotic should be administered. Previous studies have helped enlighten the latter factor. Peripheral blood leucocytes from humans who had ingested

$5x10^5$ cfu/ml *L. johnsonii* La1 did not show enhanced respiratory burst or phagocytic activities, whereas a 10-fold increase in the ingested dose stimulated both parameters (Donnet-Hughes et al., 1999). Overall, our results show that the highest dose here assayed was the most effective in terms of immune stimulation.

Among the studied parameters, leucocyte peroxidase content was statistically unaffected while cytotoxic and respiratory burst activities were increased by leucocyte incubation with any of the bacterial strains assayed herein. Although we did not quantify the phagocytosis, microscopical study revealed that *L. delbrüeckii* subsp. *lactis* and *B. subtilis* were ingested in a greater number. The effects observed *in vitro* on the peroxidase content and cytotoxic activity correlate well with *in vivo* data though do not fit in the case of the respiratory burst activity. Unfortunately, little information is available about the role of probiotics in fish immune modulation. Only one study screened several lactic acid bacteria for their *in vitro* potential to stimulate turbot macrophage immune functions (Villamil et al., 2002). Out of the six LABs investigated only heat-killed *Lactococcus lactis* produced a significant enhancement of the macrophage chemiluminiscent response after 24 h of incubation. The lower bacteria concentrations assayed (10^3 and 10^6 cfu/ml) may account for the lack of effects recorded for the three *Lactobacillus* subsp. (*L. lactis*, *L. helveticus*, *L. brevis*) on turbot macrophage immune functions.

The individual analysis of the present results obtained *in vitro* for each bacterial species reveals that all four assayed strains show a similar *in vitro* immune stimulatory potential, which agrees with the results obtained *in vivo*. However, phagocytosis assays reveal that autochthonous bacteria were less actively ingested by seabream phagocytes than foreign strain. It may be hypothesised that host immune cells do not react against bacteria that naturally occur on their surfaces as strongly as they do against bacteria that they do not normally encounter in their environment. Differences in the effects achieved by different bacteria species suggest that other properties may be decisive: e. g. adhesion to intestinal surfaces, production of antimicrobial substances, enzymes or others. Any of these could make an important contribution to the positive immune stimulatory effects of a probiotic strain.

Although this was not the initial purpose of this thesis, the results obtained in the objective I are currently being completed in our research group with challenge experiments with *Photobacterium damselae* subsp. *damselae*, causative agent of pasteurellosis in seabream.

OBJECTIVE II: STUDIES ON THE INTEGRITY OF THE GUT EPITHELIAL BARRIER

Bacterial translocation in animal models is promoted, amongst other factors, by the disruption of the ecologic GI equilibrium allowing intestinal bacterial overgrowth. The integrity of the intestinal epithelium seems to determine whether translocation takes place by an intracellular route or intercellularly between epithelial cells (Berg, 1999).

We investigated the changes in Atlantic salmon gut histology following *in vitro* exposure to three different bacterial strains, one probiotic (*Carnobacterium divergens*) and two pathogens, *Aeromonas salmonicida* and *Vibrio anguillarum*, at different doses. Both pathogens have been previously shown to enter fish through the gastrointestinal barrier (O'Toole, 2004; Ringø et al., 2004), however morphological studies on how dose or presence of other bacterial strains affect the integrity of the intestinal barrier are not available.

It is generally accepted that probiotic bacteria provide beneficial health effects to the host (Ouwehand et al., 2002). However, there is no available data on the effects of probiotics on the fish epithelial intestinal barrier.

Exposure of salmon foregut to bacteria strains revealed clear differences between pathogenic (*A. salmonicida* and *V. anguillarum*) and non-pathogenic (*C. divergens*) strains. Whilst exposure of the foregut to pathogens at both assayed concentrations, resulted in different damaging effects, these were not found following exposure to *C. divergens*. Morphological changes were seen both by light and electron microscopy and consisted of epithelial cells with altered microvilli and damaged tight junctions, protruding epithelial cells sloughing into the lumen and presence of cell debris in the gut lumen. However, *A. salmonicida* and *V. anguillarum* resulted overall in different histological and morphological changes, which suggests the use of different invasive and pathogenesis mechanisms in each bacterial strain. This is in agreement with previous work conducted in Atlantic salmon intestine exposed to *A. salmonicida* (Ringø et al., 2004); whereas no previous work had assessed *V. anguillarum*-induced effects on salmon gut histology. Additionally, changes in number of immune cells and goblet cells were observed with typically higher numbers of these cell types in the bacteria-exposed samples. Foregut exposed to *C. divergens* were histological similar to control samples showing an integrated epithelial barrier, which agrees with Ringø et al. (2004) who suggested that the indigenous bacteria do not affect gut cellular integrity.

Surprisingly, exposure to *V. anguillarum* at the lower concentration resulted in the presence of phagolysosomes in the cytoplasm of enterocytes. The latter would mean that enterocytes were processing the bacteria and acting as phagocytic cells or as antigen presenting cells in the way M-cells do in higher vertebrates. This is in agreement with Hansen and Olafsen (1999) who observed endocytosis of bacteria by enterocytes in herring (*Clupea harengus* L.) larvae. However, it is not possible to ascertain that such structures were unequivocally associated to engulfed *Vibrio* particles since indigenous strains are always present in fish GI tract. Similarly, we cannot rule out the possibility of a similar process taking place when the bacteria were present at the higher concentration since microscopy is not a quantitative nor a holistic tool.

In order to unravel a possible protective role of probiotic bacteria against tissue damage by pathogenic bacteria we exposed salmon intestines to combinations of pathogenic and probiotic bacteria at different concentrations. An epithelial tissue reaction against pathogenic bacteria was still visible when the probiotic was simultaneously administered. Histological changes described above were still present in most samples thus *C. divergens* was not able to completely prevent tissue damage by *A. salmonicida* or *V. anguillarum* in Atlantic salmon when the pathogens were present at the highest concentration. However, mitigation of tissue damage was observed when the probiotic was present at a higher concentration than the pathogen in the case of *V. anguillarum*, indicating a possible protective role of *C. divergens* when administered at an optimal dose. This is in agreement with previous reports conducted with human cell lines (Otte and Podolsky, 2004). Cells coexposed to the pathogen *Salmonella dublin* and a mixture of probiotic strains at different concentrations showed significantly attenuated damaging effects caused by the pathogen. Due to differences in incubation times, bacterial strains and studied cells, it is not possible to conclude that *C. divergens* in not capable of attenuating *V. anguillarum* caused damage on intestinal epithelium.

In foregut samples coincubated with *A. salmonicida* and *C. divergens* or *V. anguillarum* and *C. divergens* both at the highest concentration, bacterium-like particles of small size (around 0.5 µm) gaining access into the epithelial layer through the tight junctions were seen under TEM. Whereas the first treatment led to undamaged microvilli but edema in between the epithelial cells, the second still presented typical signs of damaged, especially protruding cells, seen after the exposure to *V. anguillarum* only. Similar small bacterium-like particles could not be observed in any other assayed treatment. However, translocation of small bacteria-like particles through the GI tract enterocytes

has previously been reported in larvae, fry and adult fish (Ringø, 2001;2004;2006). It remains unclear if these particles are the pathogenic or the probiotic bacterial cells but it opens a question concerning the possible use of different invasive routes by pathogenic bacteria depending on the presence of other bacteria such as *C. divergens*. Identification of the unknown bacteria-like particles by immunogold labeling techniques would give light to some of these questions and it is subject of further work.

When *C. divergens* was present at the highest dose and the pathogen at the lowest, less damage was seen. The latter could be due to some extent to the antagonistic activity of the probiotic bacteria against the pathogens resulting in less live pathogens to colonize the foregut; and therefore to less tissue damage. Based on this hypothesis, the administration of appropriate level of probiotic bacteria in the farmed fish pelleted diets may inhibit colonization of pathogenic bacteria in the digestive tract.

Alternatively, not simultaneous but prophylactic use of probiotic bacteria may achieve greater protective effects. The latter possibility was not studied in this first study but preincubation with probiotics has been was proven to mitigate S. *dublin* induced effects (Otte and Podolsky, 2004). As a consequence, we aimed to test so in a second investigation.

For beneficial health effects such as competitive exclusion of pathogens or immune regulation, an effective probiotic has to colonize gut mucosa at least temporarily (Salminen t al., 1998; Saarela et al., 2000). In the present thesis, a non-indigenous strain, *Lactobacillus delbrueckii* spp. *lactis* was labelled with TRITC and its fate in the Atlantic salmon gut was studied after *in vitro* exposure. Since intestines were thoroughly washed before samples were taken, only those bacteria able to adhere to the mucus or to the epithelial cells and those bacteria that had passed into the GI tract should be found. Confocal micrographs showed that not only labelled - *L. delbrueckii* ssp. *lactis* adhere in big lumps to salmon intestinal mucus but also that some bacteria contacted epithelial cells and gained access to the lamina propia. Although some authors postulate a host-specific effect of probiotic bacteria (Dogi and Perdigon, 2006) that would restrain their benefits to those hosts where it is commonly found, others have reported absence of specificity in lactic acid probiotic bacteria when binding host intestinal mucus (Rinkinen et al., 2003). Actually, Nikoskelainen et al. (2001) studied the adhesion capacity of different LAB strains to the rainbow trout (*Oncorhynchus mykiss*) mucus.

L. delbrueckii ssp. *lactis* was proven to stimulate systemic immune defence in another teleost fish, the gilthead seabream (*Sparus aurata* L.) in the objective 1 of this thesis. The findings in the objective 2 could explain one of the mechanisms by which this probiotic strain can have effects on the systemic immune response.

The second purpose of the present study was to evaluate the effects of the probiotic strain, *L. delbrueckii* ssp. *lactis*, on the morphology and integrity of Atlantic salmon gut when incubated *in vitro*.

The concentration of immune cells in the gut epithelium observed following incubation with the probiotic strain resembled the response that occurs when the intestine is exposed to a pathogen like *A. salmonicida*. Moreover, rodlet cells were in close contact with immune cells suggesting a cross-talk between both cell types and a role of rodlet cells in the cellular immune mechanisms of the teleost fish gut. The role of rodlet cells as leucocytes in the fish immune system has been recently reviewed by other authors (Reite and Evensen, 2006) even though it still remains controversial.

We used a model of intestinal epithelial damage in salmon to investigate the possible protection against tissue damage by probiotic bacteria since we used this same model in the first experiment within this objective. The ability of probiotics as gut integrity preservers has been reported in murine and human models before (Madsen et al., 2001). *A. salmonicida* caused epithelial damage was almost completely counteracted by pre-incubation with *L. delbrueckii* ssp. *lactis*. The fact that single incubation with the probiotic strain induced a focalisation of the immune cells present in the salmon GI tract leads to hypothesise that protection against pathogen tissue damage is achieved by the action of local immune defences. If probiotic bacteria were able to stimulate or pre-activate such defence mechanisms, the host intestinal barrier would be more competent at impeding pathogen translocation and pathogen-derived damage. Further studies should address the possible greater capacity of probiotic-stimulated fish GALT cells to mount an immune response against gastric pathogens.

It is now widely accepted that although cell-surface characteristics can play a role in the pathogenesis of furunculosis they are not the sole virulent determinants of *A. salmonicida* (Toranzo et al., 2005). In fact, some of the typical signs of the disease are due to the combined effect of two enzymes, a serine protease and a phospholipase complex with LPS. Some information is available demonstrating that *A. salmonicida* causes intestinal cell damage in the foregut of Atlantic salmon (Ringø et al., 2004; Ringø et al., 2007). Because these studies and the present investigation use the

pathogen without washing the culture supernatant the effects observed might be due either to the toxins, to the bacteria themselves or to both toxins and bacterial cells. However, as a blocking effect was observed when the intestine was exposed to the lactobacilli it can be speculated that the lactobacilli compete with the pathogen in adherence to the mucus layer, by producing bacteriocins which would inhibit pathogenic colonisation. Due to the lack of a conclusive demonstration further studies should be undertaken on this topic.

In a recent review on bacterial translocation and pathogenesis in the GI tract of larvae and fry (Ringø et al., 2007), the authors stated that paracellular translocation has only been reported in one fish study (Ringø et al., 2006). However, in this study with spotted wolf fish (*Anarhichas minor* Olafsen) the authors suggested that the paracellular bacterial translocation is a secondary effect of detachment and loss of enterocytes, creating large intercellular space as a result of *V. anguillarum* infection. In contrast to these results we noted paracellular bacteria between the enterocytes when the proximal intestine was exposed to *L. delbrueckii* ssp. *lactis*, but this translocation occurred in virtually intact gut epithelia. In other words, translocation of the probiotic bacteria was not associated to the creation of oedemic spaces, a characteristic of pathogenic bacteria.

OBJECTIVE III: GALT ISOLATION AND MORPHOFNUCTIONAL CHARACTERIZATION IN THE SEABREAM.

Traditionally, primary lymphoid organs have received most attention but, at present, there is growing interest in understanding the immunity of mucosal surfaces, since they are strategically placed in areas constantly exposed to external pathogens (Schley and Field, 2002). The study of GALT cellular components, their specific location and their morphological and functional characteristics are all key aspects when trying to understand the role of GALT within the whole fish immune system.

In general, two main immune cell populations can be identified in association with gastrointestinal surfaces: the intraepithelial leucocytes (IEL) and the lamina propria leucocytes (LPL). In higher vertebrates, IEL is exclusively represented by T-cells, whilst the lamina propria hosts T and B lymphocytes, plasma cells, mast cells and macrophages (Schley and Field, 2002). In fish, the lamina propria of gut folds is known to contain the largest population of GALT leucocytes (Press and Evensen, 1999). As a consequence, studies based on purification protocols that only free immune cells within the epithelium give a partial view of the total immune defences associated to a particular intestinal surface.

It was assumed that lymphoid cells from gut tissue belong to two fractions: the DTT/EDTA-fraction, containing IELs, and the collagenase-fraction, containing the lamina propria lymphocytes (LPL) (Schwarz et al., 2005; Di Sabatino et al., 2007). Apart from the theoretical basis stated before and the available literature, we further confirmed this assumption by microscopic observation. Whilst we found no degradation of the lamina propria during the chemical treatment, the connective tissue was clearly loosing its integrity due to the action of the collagenase enzyme.

The present study has addressed the enrichment of total cell pools obtained from the seabream digestive tract in immune cells. The heterogeneity of the cell types that make up this tissue and its nature as a secondary lymphoid organ make this a complex task. It is noteworthy that goblet cells are known to vary in number and mucus content in animal intestine mucosa depending on the diet, health status or age (Brown et al., 2006; Faure et al., 2006). When high mucus content is present GALT isolation becomes even more troublesome. In this context, we found essential that fish were starved for at least

48 h. When some food content was still present in the digestive tract the protocol yielded variable results.

The use of a purely mechanical procedure was the most time and cost effective, providing cell pools of high viability. Previous data on carp GALT (Rombout et al., 1993) found no clear differences between mechanical and enzymatic extraction protocols although no information on the quality of the cell pools obtained in each case was given. In our case, flow cytometry revealed some differences in the FSC and SSC values of the isolated cells. The greater scattering of the R2 region in cell suspensions obtained by this method suggests that not single cells but groups of them, typically enterocytes, were present, as corroborated in the semithin sections. Thus, care should be taken to avoid the presence of small tissue fragments which hinder the use of flow cytometry analysis.

Microscopic observation of fragments during the different isolation steps revealed that DTT and collagenase treatment released immune cell allocated both within the epithelium and in the lamina propria. Viability of the isolated cells was not significantly affected by the collagenase step duration or the collagenase concentration.

Nylon wool columns have been used to enrich T cells based on the differential adherence properties of T-cells, B-cells and other cells to nylon (Hathcock, 1992). When IELs from rainbow trout were isolated after 1 h incubation in DTT and then filtered through nylon wool columns (McMillan and Secombes, 1997), this step reduced the epithelial cell content although the cells were not examined by microscopy or flow cytometry. We found both mucus and some rounded cells not further characterised attached to nylon wool fibres under scanning electron microscopy. Their morphological features resembled that of lymphocytes but whether fish B lymphocytes display adherence properties to nylon wool, as their mammalian counterparts, remains to be investigated. The use of these columns eased, nonetheless, subsequent handling and study of the isolated cells without affecting their viability, and is therefore to be recommended.

Density gradients helped us to purify a population of putative lymphocytes, according to flow cytometry and light microscopy features. Similarly, IELs from rainbow trout were purified using the same technique (Bernard et al., 2006). We reveal consistently high numbers of lymphocytes in the seabream gut tissue, which is in agreement with previous studies that pointed to a preponderance of T cells in the gut immune system of the sea bass (Picchieti et al., 1997) or carp (Rombout et al., 1998). Lymphocytes were

abundant in both the ID and HD band although the latter had greater levels of purity, containing almost only lymphocytes. It is possible that the lymphocytes found in each band display differential properties, although this was not morphologically obvious under light microscopy. Rainbow trout IELs also appeared in two Percoll density bands, high (HD) and intermediate (LD), the former being the most abundant (McMillan and Secombes, 1997).

The absence of a density band containing phagocytes alone led us to conduct respiratory burst assays using NBT as substrate. Very low percentages of NBT^+ cells were found, confirming the data obtained by flow cytometry and cytocentrifugation slides. Moreover, interfish variation was greater than variations due to different isolation protocols, underlining the fact that these cells are present in low numbers in the seabream gut. Low numbers of granulocytes have also been reported in carp GALT (Hébert et al., 2002). Alternatively, seabream phagocytes could have been present in our GALT cell suspensions but not capable of bursting without the presence of a previous stimulus to prime them. Further studies should address the possibility of phagocytic cells migrating into the gut compartment in case of infection.

Although fish gut is known to be an antigen uptake site (Hart et al., 1988) and an increase of gut leucocytes following oral immunostimulation has been described (Picchieti et al., 2007), little is known about the potential immune activities of gut leucocytes. Studying the capacity of different leucocyte cells to respond to danger stimuli is essential if strategies like oral immunostimulation are to be developed in order to strengthen such capacities.

In this study, we measured the main innate immune parameters from seabream GALT cells under non-stimulated conditions. Phagocytosis, respiratory burst and peroxidase activities were low and seem to be linked to the scarcity of phagocytes present in our cell suspensions. This is in agreement with the results form the first study of this objective and also with data from other teleost species (Davidson et al., 1991; Rombout et al., 1993). The phagocytic activity of fish head kidney leucocytes is routinely studied by flow cytometry (Esteban et al., 1998). However, background values of green fluorescence and low percentage of phagocytosis (green labelled cells) rendered this technique invalid. Thus, the use of microscopy technique was required. Transmission electron microscopy revealed that only granulocytes and macrophages were able to engulf foreign particles such as the yeast *S. cerevisiae,* as previously demonstrated in

head kidney leucocytes (Rodríguez et al., 2003) or latex beads, the latter being ingested in greater numbers perhaps due to the lower particle size.

Other previously described phagocytic cells in teleost fish, such as thrombocytes (Meseguer et al., 2002) and melanomacrophages (Meseguer et al., 1994), were not found in our samples. Moreover, in the conditions here assayed neither lymphocytes nor goblet cells, enterocytes or rodlet cells were seen with foreign particles in their cytoplasm, which contrasts with recent data in rainbow trout which shows that B lymphocytes are active phagocytes (Li et al., 2006a). However, that study took B lymphocyte from primary lymphoid organs and the target particles used were smaller or opsonised. Further studies using other target particles should address whether seabream gut B lymphocytes have preserved phagocytic abilities in the same way as trout B lymphocytes isolated from primary lymphoid organs.

Finally, very low ingestion of the probiotic bacteria *L. del. lactis* was observed. Macrophages and granulocytes only ingested one or two bacteria particles and no morphological signs of activation accompanied bacterial engulfment. Additionally, bacteria were not seen inside a phagolysosome and did not seem to suffer enzymatic degradation by the ingesting cell. These observations are in contrast with the results obtained from seabream head kidney phagocytes, which avidly incorporated abundant *L. del. lactis* in their cytoplasm as presented in the results section page 50. Thus, the process here observed may not be truly a phagocytosis like the process observed in head kidney cells. This could be due to the fact that probiotic bacteria lack danger signals and gut leucocytes have been educated or become tolerant to their presence since early life stages. Similar processes involving the education of gut lymphoid cells have been described in higher vertebrates and appear vital for the homeostasis of gut mucosal immunity (Magalhaes et al., 2007). However, lymphoid cells in seabream gut must received signals from probiotic bacteria since systemic stimulation of innate immune parameters takes place after oral administration of *L. del. lactis* as shown consistently in the results from objective I.

In the case of the respiratory burst, low activity was recorded, as was found in the case of rainbow trout gut leucocytes (Davidson et al., 1991). Besides, we found no activity in the adherent cells of our suspensions, which could also be due to the low numbers of phagocytic cells present or to the lack of adherence capacity in gut phagocytes. The loss of adherent cells during the isolation process can be ruled out since the plastic tubes used are not suitable for seabream macrophage adhesion as previously observed by

members of our group. More studies are needed to further investigate such capacities. The literature remains controversial on this aspect since carp intestine macrophages show poor or no adherence to glass or plastic (Rombout et al., 1993), while the respiratory burst activity of trout gut leucocytes was measured by using adherence to plates (Hébert et al., 2002).

Natural cytotoxic activity was an important measurable innate immune parameter in our seabream GALT cells, and was even higher than in suspensions from head kidney, a primary lymphoid organ in fish. McMillan and Secombes (1999) also detected this activity in intraepithelial lymphocytes (IELs) from rainbow trout gut. In the seabream, lymphocytes, monocyte-macrophages and granulocytes display non-specific cytotoxic activity (Cuesta et al., 1999). Additionally, when isolated from main lymphoid tissues, these leucocytes expressed the receptor NCCRP-1, both at gene and protein level (Cuesta et al., 2005). However, such information is at present lacking for gut leucocytes although we present here some data pointing to the fact that GALT cells include a well-represented population characterised by low SSC and FSC values. This population corresponded to lymphocytes according to their light microscopy morphological features as shown in page 67. Our transmission electron micrographs confirmed the abundance of lymphocytes and the scarcity of macrophages and granulocytes. As a consequence, it can be concluded that lymphocytes are the main NCC effectors in the seabream gut. Interestingly, cell-mediated cytotoxicity has been pinpointed as the main innate immune mechanism involved in the cellular defence of gilthead seabream against *Enteromyxum leei* (Myxozoa) (Cuesta et al., 2006).

Protein A from *Staphylococcus aureus* possesses five domains that specifically bind to the Fc region of immunoglobulin molecules, especially IgG, and is routinely used to isolate immunoglobulin from several mammalian species (Moks et al., 1986). In fish, Fc domains should theoretically be present on the surface of B lymphocytes and macrophages. The use of an anti-IgM monoclonal antibody in trout IELs revealed that only 1% of the total cells were IgM^+ (Bernard et al., 2006). Other Ig isotypes, however, also contain Fc domains and they are not measured when specific antibodies are employed. Complementary studies and the use of multiple specific markers will help to fully characterise the cell types that account for the percentage of protein A-labelled cells here reported, which was similar in GALT and HK.

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OBJECTIVE IV: ANTIPROLIFERATIVE EFFECTS OF PROBIOTIC BACTERIA ON FISH CELL LINES

Among the properties attributed to probiotics, especially those from the lactic acid bacteria (LAB) group, is that of cancer reduction, mainly in the colorectal portion of the digestive tract (Rafter, 2002). How this effect is achieved is still unknown. However, mechanisms may include: alteration of the metabolic activities of intestinal microflora; alteration of physico-chemical conditions in the colon; binding and degrading potential carcinogens; quantitative and/or qualitative alterations in the intestinal microflora incriminated in producing putative carcinogen(s) and promoters (e.g. bile acid-metabolising bacteria); production of antitumourigenic or antimutagenic compounds; enhancing the host's immune response and effects on physiology of the host (Rafter, 2002). No studies have until now described similar effects in fish cells.

We chose two cell lines, one of tumoral origin (EPC) the other (SAF-1) lacking this feature. SAF-1 cells were for the first time characterised over a decade ago showing normal karyotype and DNA content (Béjar et al., 1997). Further studies have identified telomerase activity, a typical feature in immortalised cells, in SAF-1 cell line and a correlation with cell proliferation rates (Béjar et al., 2005). EPC cell line was first discovered in the eighties, thus more studies are available than in the case of SAF-1 cells. The epitheliome originated from a hyperplastic lesion in carp skin consequence of a rhadbovirus infection. However, virus could not be isolated after serial passage of the cell line *in vitro* (Fijan et al., 1983). Both SAF-1 and EPC cell lines have been proven useful as models for pathogen infection research in fish (Pérez-Prieto et al., 1999; López-Dóriga et al., 2000; Wang and Leung, 2000).

Additionally, we assayed cytoplasmic extracts from two bacterial strains of different origin, a LAB strain, *L. del. lactis,* and 51M6, a strain from the *Vibrioneaceae* family present on seabream skin. Both bacteria had been studied for their immunomodulatory properties in seabream in the objective I. This choice was made in order to test whether host specific effects can be attributed to probiotic bacteria as suggested by some authors (Saarela et al., 2000) but refuted by others (Rinkinen et al., 2003; Chabrillón et al., 2005).

Our results show that growth of the two cell lines was differentially inhibited by the probiotic cytoplasmic extracts. Although *L. del. lactis* decreased proliferation and induced apoptosis both in SAF-1 and EPC cells, apoptosis induction was over 5 times

higher in SAF-1 cells than in EPC cells. Also, low dose and short incubation times produced a significant effect, which was not observed in EPC cells until 72 h in culture. Thus, it appears that tumour cells are more resistant to the action of the cytoplasmic extracts of probiotic bacteria than non-tumoral cells according to the assayed parameters in this study.

On the other hand, it is clear that the lactic acid strain, *L. del. lactis*, has stronger antiproliferative properties than 51M6 on both cells lines, since shorter times and lower doses sufficed to produce a significant inhibitory effect. This is in agreement with available data from other LAB strains, like *L. casei* and *Bifidobacterium longum*, tested in mammalian cell lines, which produced a dose-dependent decrease in proliferation rates (Lee et al., 2004). Since 51M6 is present on the fish natural environment, it is likely that a higher dose is needed due to ecological adaptation or tolerance compared with an allochtonous probiotic bacteria. The low prevalence of skin tumours in fish could be due to the homeostatic effects exerted by probiotic bacteria found on fish external surfaces, but this hypothesis has not yet been investigated. This probiotic, found in the fish skin surface may play a role in controlling the appearance of epithelial tumours, although an *in vivo* model should be used in order to confirm this hypothesis. The existence of a fish model of malignant melanoma in *Xiphophorus* spp. (Meierjohann and Schartl, 2006) represents a perfect scenario to elucidate the role of probiotics in controlling fish neoplasia both in experimental models and in the environment.

We aimed to determine whether apoptosis of fish cell lines was triggered by the cytoplasmic extracts of probiotic bacteria. Apoptosis is a programmed cell death that occurs during many physiological processes and during all developmental stages of living organisms. When primary leucocyte cultures are used to investigate different aspects of their biology, cells may die either by apoptosis or necrosis during culture procedures. At present, there is a wide array of techniques that can estimate the degree of apoptosis of cells in a more or less quantitative way. These techniques include visualisation of DNA fragmentation by electrophoresis, microscopical observation, caspase activation or TUNEL. They are nonetheless semiquantitative and tedious in some cases. Flow cytometry appears as a good alternative since it enables high numbers of cells to be counted and the apoptosis state of each individual cell to be identified. In this context, the Annexin V/PI double fluorescent labelling has been widely used both in mammals and lower vertebrates (Bacsó et al., 2000). A less expensive double

fluorescence labelling technique using FDA and PI was demonstrated to be comparable to Annexin V/PI staining (Sändstrom et al., 2000) in human eosinophil cultures. This technique is based on the fact that apoptotic cells lack esterases are therefore unable to metabolize FDA. Viable cells that do have active esterases are able to cleave FDA into diacetate and fluorescein, which has a green fluorescence. As is known from viability tests, PI cannot penetrate membranes of apoptotic cells, which makes it possible to distinguish these from necrotic-permeabilised cells (Pullen et al., 1981). The technique here developed for seabream was based on the same principle and involves a double staining protocol using FDA and PI to measure apoptosis and necrosis in primary leucocyte cultures. The results show that FDA staining differed between head-kidney phagocytes and lymphocytes, the intensity of green fluorescence being markedly higher in phagocytes. This indicates that phagocytes contain more esterases or more active ones than in lymphocytes, probably due to the presence of cytoplasmic granules which contain lysosomal enzymes such as esterases in macrophages and granulocytes (Bainton and Farquhar, 1968). Moreover, there was always a low percentage (around 10%) of cells that remained unstained and therefore appeared apoptotic probably due to the leucocyte isolation procedure. Readily isolated carp leucocytes with FDA acquired green fluorescence in 90% of the cases (Sata et al., 2003). The FDA/PI staining procedure was seen to be inexpensive and required little time. After 30 min of staining, flow cytometry enabled individual cells to be analysed in only few minutes.

Kinetic studies of apoptosis in seabream leucocyte cultures from head kidney, spleen and blood reveal that culture time is a vital factor when immunological or biological studies of fish leucocytes involve cell culture techniques. The number of apoptotic HK phagocytes increased linearly with culture time (28% at 48 h) and necrosis was only 4% at the same time. Thus, it can be concluded that *in vitro* culture of seabream HK phagocytes results in cell death via apoptosis and the low necrotic numbers may represent advanced stages of apoptosis. Similarly, Lund et al. (2001) observed that the first 24 h in culture were also the most critical for cryopreserved human monocytes, 21% of the total cells being apoptotic and 10% necrotic at this time, as measured by Annexin V/PI and flow cytometry. An analysis of carp total leucocyte suspensions after 24 h in culture showed that around 75% of the cells were still viable, as measured both by FDA/PI and Annexin/PI procedures (Sata et al., 2003).

A different view was offered by seabream lymphocytes and, after 48 h, around 55% (from head kidney, spleen or blood) had entered apoptosis. However, this value was

already over 45% after the first 24 h, indicating that the first 24 h in culture were the most critical in terms of triggering apoptosis. Necrosis also occurred at a higher rate than in the case of phagocytes, especially in spleen lymphocytes which showed almost 30% of necrosis at 48 h. It is possible that this subpopulation was originated both indirectly via secondary necrosis and/or directly via necrosis.

Finally, mean green fluorescence decreased over time within the population stained as FDA^+/PI^-. This is in agreement with previous data and suggests that the cell metabolic rate decreases with culture time (Sandström et al., 2000).

Culture media, serum content, antibiotics, culture temperature and other variables should be tested in order to optimise and standardise culture conditions for primary cultures. From this study, we can conclude that the culture conditions assayed were more favourable for the phagocytic subpopulation than for the lymphocytes and more effort should be put into fish primary lymphocyte cultures.

The combination of FDA and PI made it possible to identify viable, apoptotic and necrotic cells. The use of proapoptotic agents like resveratrol and staurosporine served as a good indicator of cell death in seabream leucocytes. Resveratrol, a stilbene derived from plants, has been shown to inhibit proliferation and trigger apoptosis in a number of human cancer cell lines (Aggarwal et al., 2004). Staurosporine is a protein kinase inhibitor commonly used for inducing apoptosis. In the present study, the assayed resveratrol dose produced significant differences in the viability of both phagocytes and lymphocytes compared to control treatments after only 30 min (time 0). Staurosporine, in turn, had no effects at this sampling point and only spleen lymphocytes were significantly less viable after staurosporine treatment than controls. However, due to the different doses of each apoptosis inducer assayed, the results between the two cannot be compared. Both compounds, nevertheless, managed to induce apoptosis in seabream lymphocytes from all tissues at a significantly greater rate than controls after 24h in culture.

Transmission electron microscopy is a highly informative method for studying of apoptotic processes (Guejes et al., 2003). The semiquantitative information obtained from the study of seabream leucocyte cultures under TEM correlated with quantitative data form the flow cytometry analysis. Apoptotic cells showed signs of shrinkage, chromatin condensation and rounded electron dense nuclei. Few cells with these characteristics were observed in readily isolated leucocyte cultures (time 0), indicating

that isolation procedure does not yield necrotic cells (PI^+) but produces some apoptosis, mainly in the lymphocyte population.

Apoptosis occurs spontaneously in malignant tumors, often markedly retarding their growth, and it is increased in tumors responding to irradiation, cytotoxic chemotherapy, heating and hormone ablation (Kerr et al., 1994). Conclusive studies concerning the mechanisms involved in cancer control by probiotics are scare. Only recently, the induction of apoptosis by conjugated linoleic acid produced by different probiotic strains was demonstrated in HT-29 and Caco-2 mammalian cell lines (Ewaschuk et al., 2006).

It is known, nevertheless, that probiotic bacteria can interact with the cytoskeleton of gut epithelial cells (Ait-Belgnaoui et al., 2005). Recently, 2 novel proteins from LGG-s have been purified and they activated Akt, an effector of cell survival, inhibited cytokine-induced epithelial cell apoptosis, and promoted cell growth in human and mouse colon epithelial cells and cultured mouse colon explants (Yan et al., 2007). In a similar way, other proteins may be responsible for the opposite effects when certain undesired cells may attempt to proliferate.

Our study shows that both *L. del. lactis* and 51M6 cytoplasmic extracts have antiproliferative effects on SAF-1 and EPC cells lines, generally in a dose-dependent manner. However, EPC cells were more resistant and required higher doses and longer incubation times than SAF-1 cells. Therefore, malignancy as opposed to immortality may be a key factor determining downregulatory effects of probiotic bacteria on cells. Nevertheless, proteins present in the cytoplasm of probiotic bacteria may have a therapeutic use in animal cancer and fish probiotics naturally occurring on fish surfaces may indeed be doing so in the wild. Finally, apoptosis and necrosis were induced following the inhibition of growth, which points them as modes of action by which probiotics reduce cancer incidence in animal hosts.

VIII. CONCLUSIONS

1.- Dietary administration of live *Lactobacillus delbrüeckii* subsp. *lactis*, *Bacillus subtilis* or both modulates the systemic cellular innate immune responses of seabream leucocytes. The duration of the observed effects is always restricted to the period of administration.

2.- Oral administration of fish-derived potential probiotics (Pdp11 or 51M6) has stimulatory effects on both humoral and cellular innate immune parameters of the gilthead seabream.

3.- Heat-inactivated *L. delbrüeckii* subsp. *lactis*, *B. subtilis*, Pdp11 and 51M6 stimulated *in vitro* the cellular innate immune parameters of the seabream, generally in a dose dependent manner.

4.- Exposure to *V. anguillarum* or *A. salmonicida* produces damaging changes in the epithelial barrier of salmon foregut that can be summarised as the loss of the epithelial integrity, while exposure to probiotic bacteria *C. divergens or L. delbrüeckii* subsp. *lactis* causes no damaging effects on the gut epithelial integrity.

5.- Some alleviation of *in vitro* intestinal damage was observed when concomitant exposure of *Vibrio anguillarum* (pathogen) and *C. divergens* (probiotic) was conducted if the pathogen concentration was lower than that of the probiotic.

6.- *L. delbrueckii* subsp. *lactis* is able to adhere to Atlantic salmon intestinal mucus and reach the lamina propria of the intestine.

7.- Incubation of the salmon foregut with *L. delbrueckii* subsp. *lactis* prior to exposure to *A. salmonicida* was able to prevent tissue damage caused by *A. salmonicida* pointing probiotic bacteria as useful prophylactic tools against fish gastrointestinal tract aggressors.

8.- Seabream GALT cells, characterised by low numbers of phagocytic cells (granulocytes and macrophages) and abundant lymphocytes, were isolated by using relatively short and low cost protocols. Nylon wool columns and Percoll gradients enabled us to purify abundant gut lymphocytes with high viability.

9.- *In vitro* studies of the innate immune parameters of the seabream gut leucocytes show that respiratory burst, peroxidase content and phagocytic activity are low compared to the same activity in head kidney leucocytes whereas natural cytotoxic activity of gut leucocytes was higher than that of head kidney leucocytes.

10.- The combination of FDA and PI can be used in seabream leucocyte cultures to quantify apoptosis by flow cytometry. The kinetics of apoptosis events was different in lymphocytes and phagocytes, the latter showing a greater resistance to *in vitro* culture conditions.

11.- *L. delbrüeckii* subsp. *lactis* and 51M6 cytoplasmic extracts have antiproliferative effects on SAF-1 and EPC cells lines, generally in a dose-dependent manner. EPC cells were more resistant to the probiotic extracts effects than SAF-1 cells, perhaps as a consequence of the malignancy of the studied cells and/or due to differences in the fish species (carp vs. seabream).

12.- *L. delbrüeckii* subsp. *lactis* posses stronger antiproliferative effects than 51M6 on both studied cell lines. Autochthonous bacteria derived from fish mucosal surfaces are therefore not as effective as lactic acid bacteria.

13.- Apoptosis and necrosis were induced in SAF and EPC cell lines following the inhibition of growth, which points them as modes of action by which probiotics act as cancer controlling agents in animal hosts.

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XI. LIST OF SPECIES

LIST OF FISH SPECIES

Scientific name	Spanish name	English name
Amphiprion percuda	Pez payaso	Clown fish
Anguilla anguilla	Anguila	Eel
Anarhichas minor	Perro chico	Spotted wolffish
Carassius auratus	Carpín	Goldfish
Centropomus undecimalis	Róbalo blanco	Common snook
Clupea harengus	Arenque	Herring
Cyprinus carpio	Carpa común	Carp
Dicentrarchus labrax	Lubina	Seabass
Gadus morhua	Bacalao	Cod
Hippoglossus hippoglossus	Fletán del Atlántico	Halibut
Ictalurus punctatus	Pez gato	Channel catfish
Labeo rohita	Labeo roho	Indian major carp, rohu
Miichthys miiuy	--	Mi-iuy croaker
Oncorhynchus mykiss	Trucha arcoiris	Rainbow trout
Oreochromis niloticus	Tilapia	Tilapia
Salmo salar	Salmón atlántico	Atlantic salmon
Salmo trutta	Trucha de mar	Brown trout
Salvelinus alpinus	Salvelino o trucha alpina	Artic charr
Scophtalmus maximus	Rodaballo	Turbot
Solea solea	Lenguado	Lemon sole
Sparus aurata	Dorada	Gilthead seabream
Xiphophorus spp.	Espada sureña	Platyfish

XII. SUMMARY IN SPANISH

Los peces de cultivo pueden llegar a ser bastante susceptibles frente a determinadas enfermedades infecciosas debido en gran medida a las condiciones intensivas que impone la producción animal. Dentro de las medidas profilácticas utilizadas para evitar pérdidas económicas en la industria de la acuicultura, se ha contemplado en los últimos años el uso de probióticos. Los probióticos son preparados microbianos que mejoran la salud y el bienestar animal. A pesar de sus características prometedoras como agentes terapéuticos y profilácticos, los efectos que los probióticos producen sobre el sistema inmunitario de los peces, se desconocen en gran medida. El objetivo global de la presente Tesis Doctoral es conocer las interacciones que ocurren entre las bacterias probióticas y las células de los peces teleósteos de interés comercial con un enfoque inmunológico.

La administración en dieta de cuatro especies de bacterias probióticas en la dorada (*Sparus aurata* L.) reveló que las principales respuestas celulares y humorales innatas de esta especie son moduladas por dichas bacteria probióticas. Destacaron, por alcanzar los mayores valores de estimulación *in vivo*, la fagocitosis y la respuesta natural citotóxica. Se ensayaron tanto especies bacterianas no aisladas de teleósteos (*Lactobacillus delbrüeckii* subsp. *lactis* y *Bacillus subtilis*) como otras aisladas de piel de dorada (51M6 y Pdp11) así como formulaciones consistentes en células bacterianas viables o inactivadas por calor. *In vitro*, las cuatro especies de probióticos ya nombradas tras su inactivación por calor, estimularon los parámetros celulares innatos de los leucocitos de riñón cefálico de la dorada de un modo dosis dependiente.

Se evaluaron los cambios morfológicos que tiene lugar en la barrera epitelial intestinal del salmón atlántico (*Salmo salar* L.) tras la exposición frente a bacterias patógenas (*Vibrio anguillarum* o *Aeromonas salmonicida* subsp. *salmonicida*) o bacterias probióticas (*Carnobacterium divergens* y *Lactobacillus delbrüeckii* subsp. *lactis)*. Las especies patógenas produjeron claros signos de desintegración de la barrera epitelial intestinal los cuales fueron aliviados por el tratamiento previo con las bacterias probióticas.

Dado que la administración de los probióticos en dieta hace que estos interaccionen en primer lugar con las células linfoides asociadas al epitelio intestinal, dichas células fueron aisladas y estudiadas. Tras evaluar los protocolos disponibles en la literatura para

otras especies de peces y para mamíferos, se describieron morfológica y funcionalmente los leucocitos del intestino de la dorada. Tanto la actividad fagocítica como el estallido respiratorio resultaron ser muy bajos comparados con los leucocitos de riñón cefálico. Sin embargo, la respuesta natural citotóxica de los leucocitos de intestino de dorada resultó elevada. El empleo de distintas partículas diana para estudiar la fagocitosis desveló diferencias entre los fagocitos intestinales y los presentes en el riñón. Estos resultados contribuyen al conocimiento de la inmunología de la mucosa intestinal de peces teleósteos y a saber la influencia que ejercen sobre ella estímulos tales como los probióticos.

Además, se han investigado los posibles efectos antiproliferativos de los extractos citoplasmáticos de dos bacterias probióticas (*Lactobacillus delbrüeckii* subsp. *lactis* y 51M6) sobre dos líneas celulares epiteliales de peces (SAF-1 de dorada y EPC de carpa (*Cyprinus carpio* L.). Los extractos ensayados inhibieron la proliferación de ambas líneas celulares siendo los efectos de la *L. delbrüeckii* subsp. *lactis* mayores que los de 51M6 y resultando la línea EPC más resistente que la línea SAF-1 a dichos efectos. La inhibición de la proliferación fue seguida por una inducción de apoptosis y posterior necrosis de dichas células.

Los resultados aquí presentados contribuyen a dilucidar el papel biológico de las bacterias probióticas en el sistema inmunitario de teleósteos de interés comercial.

Probióticos

Ya en la era de la Microbiología, Metchkinoff y otros atribuyeron efectos positivos sobre la salud a cambios en el equilibrio de la microflora intestinal. Por ello, recomendaban tomar yogures que contuvieran bacterias, de los grupos lactobacilli o bifidobacteria, las cuales reducirían el número de bacterias productoras de toxinas en el intestino aumentando así la esperanza de vida del hospedador.

Fuller (1985) definió los probióticos como "suplementos microbianos vivos de la dieta que benefician al hospedador animal al mejorar el equilibrio de su flora intestinal", limitando el ámbito de acción de estos microorganismos al intestino e implicando la administración del probiótico en la dieta. Más recientemente, se amplió el concepto a otros sitios de acción incluyéndose todas las superficies mucosas de modo que se reconocen otras rutas de administración.

En el campo de la medicina, hay evidencia más o menos contundente del uso de probióticos como agentes terapéuticos para enfermedades intestinales de tipo inflamatorio como la diarrea infantil aguda (Billoo y col., 2006), la enfermedad de Bowel (Marteau, 2006), la diverticulitis (White, 2006) o el cólera (Focareta y col., 2006). Por otro lado, las enfermedades alérgicas como la rinitis alérgica o la polinosis (Xiao y col., 2006) parecen aliviarse con el uso de probióticos. También se ha comprobado que los efectos positivos de las fórmulas probióticas llegan a ser incluso de carácter general o sistémico, habiéndose relacionado su consumo con una mejoría del estado de humor de personas deprimidas (Benton y col., 2006).

Además, se ha experimentado en el tratamiento de colitis en ratones, de metritis en ganado (Otero y col, 2006), de síndromes virales (Kritas y Morrison, 2007) y de infecciones entéricas en aves (Dalloul y col., 2005).

Los probióticos tienen efectos interesantes en animales sanos como a nivel de producción y crecimiento del ganado (Guo y col., 2006) a través de su papel en la digestibilidad de nutrientes, en la reducción del colesterol del suero, en la producción de ácidos grasos volátiles, en la modulación de la motilidad gastrointestinal (Massi y col., 2006) y, como no, en la composición de la microbiota intestinal (Li y col., 2006b).

A pesar de la larga lista de efectos asociados a la administración de probióticos en animales, los mecanismos celulares y moleculares a través de los cuales los probióticos ejercen sus acciones positivas no siempre se conocen (Ouwehand y col., 2002).

Hasta la fecha, los modos de acción de los probióticos se podrían resumir de la manera siguiente:

- Efectos metabólicos o nutricionales, como por ejemplo la bioproducción de ácido linoléico en el intestino (Ewaschuk y col., 2006).
- Inhibición del crecimiento o capacidad de adhesión de microorganismos patógenos (Ruas-Madiedo y col., 2006). Ello se debe a la producción de exopolisacáridos o enzimas en la mayoría de los casos. Por ejemplo, *Lactobacillus* delb. *lactis* inhibe la actividad nitrato reductasa de *Escherichia coli* (Hugo y col., 2006).
- Interacción con la barrera epitelial de la mucosa del intestino (Otte y Podolsky, 2004; Parasol y col., 2005; Qin y col., 2005; White y col., 2006). Dentro de esta categoría se ha demostrado que los probióticos son capaces de interaccionar con el citoesqueleto de los enterocitos (Ait-Belgnaoui y col., 2006), de regular el transporte transepitelial de iones de calcio (Gilman and Cashman, 2006), de alterar la producción de mucinas (Otte y Podolsky, 2004) y de prevenir la traslocación bacteriana desde el lumen hasta el tejido conectivo gracias a la protección de las uniones estrechas (Qin y col, 2005; Zareie y col., 2006).
- Modulación de la respuesta inmune local (Galdeano, 2006; Duncker, 2006; Vinderola, 2006) y sistémica (Isolauri, 2001). Parece claro que los probióticos son capaces de modular las respuestas innatas y específicas a través de diferentes tipos de mecanismos. Se ha demostrado que la producción de citoquinas como interleucina 12 (IL-12) (Takeday col., 2006) y TNF (Kim y col., 2006) así como señales de transducción de tipo innato como NF-kβ (Ruiz, 2005) puede ser modulada por las bacterias probióticas.

Los factores humorales específicos también son susceptibles de la acción de los probióticos como se ha demostrado en el caso de la IgA humana (Rautava y col., 2006), de los anticuerpos naturales del pollo (Haghighi y col., 2006) o de la respuesta ante vacunas en la que se atribuye una acción adyuvante a los probióticos (Tanasienko y col., 2005; Goldman y col., 2006).

- Efectos antiproliferativos y antitumorales: distintas cepas probióticas, sobre todo las bacterias del grupo de los lactobacilli, han sido estudiadas por su capacidad de inhibir la proliferación de líneas celulares tumorales (Pessi y col., 1999; Lee, 2004). Los probióticos se han contemplado como estrategia de prevención de cáncer colorectal y

cáncer de piel tanto en modelos experimentales en ratones como en pacientes (Commane y col., 2005; Malkov y col., 2005).

Probióticos y acuicultura

Los animales acuáticos y sus comunidades bacterianas simbióticas son altamente dependientes de la microbiología del agua en la que se encuentran. Por ese motivo, la flora intestinal de los peces posee un carácter mucho más temporal e inestable que la de los vertebrados terrestres, reflejando en gran medida la del ambiente y sobre todo la de la comida (Al-Harbi y Naim Uddin, 2004). Así, la definición inicial de probiótico fue modificada de manera que contemplara estas diferencias: "probióticos son preparaciones microbianas que mejoran el bienestar y la salud del hospedador"

Dado el constante contacto de los hospedadores con el medio acuático que les rodea, los probióticos pueden, al contrario que en animales terrestres, ser adicionados directamente al agua en el caso de sistemas cerrados, y no necesariamente en dieta (Verschuere y col., 2000).

En cuanto a los preparados microbianos ensayados, se pueden clasificar de la siguiente forma:

i) Según su origen:

- aislados del medio acuático o del propio hospedador, también llamados autóctonos.
- cepas con propiedades probióticas ya conocidas en medicina o en vertebrados superiores, por tanto son alóctonas. Suelen ser bacterias del grupo del ácido láctico.
-

ii) Según el número de cepas administradas en una preparación:

- Monoespecíficas: una sola cepa bacteriana es administrada
- Multiespecíficas: varias cepas se combinan en un preparado o cóctel microbiano.
-

iii) Según la viabilidad de los microorganismos:

- Viables
- Inactivados

Los efectos beneficiosos de los probióticos en el campo de la acuicultura pueden resumirse en:

1. Mejora de la calidad del agua. Sobre todo se ha aplicado en sistemas de cultivo de crustáceos a través de la adición de bacterias Gram^{+} como las del género *Bacillus* (Vaseeharan y Ramasamy, 2003).

2. Modificación de la microflora intestinal (Balcázar y col., 2007) y competición con bacterias causantes de enfermedad por sitios de adhesión, fuentes de energía y nutrientes. Esta afirmación posee una base teórica más que empírica ya que las demostraciones *in vivo* sobre la misma son de escasa solidez científica.

3. Inhibición de microorganismos patógenos *in vitro* (Nikoskelainen y col., 1999; Chabrillón y col., 2005, Balcázar, 2006a).

4. Estimulación de la respuesta inmune sistémica y local, celular y humoral. La influencia que la administración de probióticos ejerce sobre el sistema inmunitario de peces ha sido evaluada con mayor profundidad sólo en el último lustro. Sin embargo, no se conocía el potencial inmunomodulador de ningún probiótico en la dorada (*Sparus aurata* L.), la especie de mayor importancia comercial en el sector de la acuicultura del área mediterránea. Los descubrimientos relacionados con este apartado serán desarrollados a continuación, dada su relevancia para la presente Tesis Doctoral.

5. Aumento de la resistencia a infecciones. Hasta la fecha se ha demostrado una mayor resistencia de distintas especies de peces teleósteos frente a la lactococosis y streptocococis (Brunt y Austin, 1995), saprolegniosis (Bly y col., 1997), vibriosis (Spaangaard y col., 2001), furunculosis (Irianto y Austin, 2002; 2003), yersiniosis (Raida y col., 2003) y edwardselliosis (Chang y Liu, 2002; Pirarat y col., 2006).

En cuanto a la relación entre probióticos y el sistema inmunitario de animales acuáticos de cultivo podemos resaltar los siguientes puntos:

i. Respuesta celular sistémica: varios estudios han demostrado recientemente que las actividades innatas de los leucocitos de riñón cefálico de distintas especies de peces son estimuladas tras la administración de probióticos. Estas incluyen la explosión respiratoria, la actividad fagocítica, la citotoxicidad tumoral o la

producción de peroxidasa (Irianto y Austin, 2002; Nikoskelainen; 2003; Pirarat; 2006; Panigrahi, 2007).

ii. Respuesta humoral sistémica: tanto el sistema del complemento como la actividad lisozima del suero (Kim y Austin, 2006; Panigrahi; 2007) aumenta en peces alimentados con probióticos. Asimismo los niveles totales de Ig en suero son mayores en peces que reciben dieta probióticas (Irianto y Austin, 2002).

iii. Expresión de citoquinas: sólo en el último año se ha estudiado este aspecto en trucha arco iris tras administrársele dos cepas de *Carnobacterium* spp. Sin embargo, las conclusiones del trabajo no son claras aunque los autores revelen un aumento de la expresión de IL-1 y TNFα en el intestino de ejemplares que recibieron la dieta probiótica (Kim y Austin, 2006; Panigrahi y col., 2007).

iv. Respuesta local de las células del sistema inmunitario asociado a la mucosa intestinal: la actividad fagocítica de los leucocitos intestinales (Balcázar y col., 2006b) y el número de granulocitos acidófilos en el tejido del intestino (Picchieti, y col. 2007) parece aumentar con la administración de bacterias probióticas.

El sistema linfoide asociado a la mucosa intestinal (GALT) en peces teleósteos

Cronológicamente, los descubrimientos en el campo de la inmunología de la mucosa intestinal de peces sucedieron de la siguiente forma. En primer lugar se detectó la presencia de inmunoglobulina en moco intestinal, en moco de la piel y en bilis tras inmunizar oralmente a los ejemplares (Hawai y col., 1981). Poco después, durante la misma década, se desveló la existencia de un sistema linfoide secundario asociado a la mucosa intestinal (GALT) en peces. Estos experimentos consistieron en la administración por vía oral o anal antígenos particulados pudiéndose observar que ciertas células presentes en el epitelio que tapiza el intestino eran capaces de incorporarlos en su interior (Rombout y col., 1985; Malean y col., 1990). La importancia de dichos descubrimientos estaba en directa relación con el desarrollo de vacunas orales en el campo de la acuicultura, un reto que todavía está por ser completamente alcanzado.

Las diferencias morfológicas y funcionales entre el sistema inmunitario de mamíferos y de vertebrados inferiores como los peces teleósteos, no atañen únicamente a los órganos linfoides primarios (Press y Evensen, 1999). Pronto se apreciaron obvias

diferencias a nivel de la organización, morfología y distribución de estas células linfoides intestinales de peces al compararlas con el GALT de mamíferos. Tanto elasmobranquios como teleósteos carecen de centros linfoides donde se concentran las células linfoides encargadas de hacer frente a cualquier antígeno que supere la barrera epitelial intestinal. Así, las placas de Peyer y los nódulos linfáticos, ambos de estructura y organización específica, no han sido observados en ninguna especie de pez (Hart, 1988) ver Figura 1 en la página 16. Además, no se conoce la existencia de células M, de células dendríticas intestinales, ni de un isotipo de inmunoglobulina equivalente a la Ig A de mamíferos que sea preferentemente secretada por los linfocitos B en superficies mucosas o en respuestas locales (Suzuki y col., 2006; McGhee y col., 2007).

Sin embargo, sí se reconocen dos tipos de poblaciones de células linfoides: los linfocitos intraepiteliales (IELs) y los leucocitos que se encuentran en la lámina propia (LPLs). Ambas poblaciones se distribuyen a lo largo de toda la longitud del tracto digestivo y de una manera difusa (Hart y col., 1988).

Parece ser que la población de IELs en peces consiste principalmente de linfocitos T citotóxicos (McMillan y Secombes, 1999). Estudios recientes realizados en trucha han puesto de manifiesto que el repertorio de TCR $\alpha\beta$ de estos linfocitos está altamente diversificado debido a la falta de placas de Peyer y de nódulos linfáticos mesentéricos (Bernard y col., 2006). En algunas especies como la carpa, se observan también algunos macrófagos de gran tamaño que podrían realizar una función análoga a la de las células dendríticas intestinales de mamíferos. En trucha, el marcaje de células IgM^+ con un anticuerpo monoclonal reveló que sólo el 1% de los IELs presentan IgM en su superficie (Bernard y col., 2006). Además, existe un tipo celular único en peces conocido como células rodlet presentes de manera variable en las distintas superficies mucosas y ocasionalmente en sangre. En el intestino de teleósteos, las células rodlet se sitúan en el epitelio desde su zona basal hasta su zona apical según el grado de madurez de las mismas. Existe cierta controversia sobre la función de estas células las cuales han sido descritas tanto como parásitos del pez o como leucocitos del propio hospedador (Reite y Evensen, 2005). Parece ser que esta última hipótesis es la más aceptada en los últimos años aunque no haya, hasta la fecha, una demostración clara de su función.

La lámina propia, en cambio, alberga tanto linfocitos como granulocitos, macrófagos y células plasmáticas. Estudios en trucha y carpa a finales de los años 80 e inicios de los 90 ya determinaron la escasa presencia de fagocitos capaces de ingerir partículas de látex y producir radicales libres de oxígeno en el intestino de estas especies (Davidson y

col., 1991). Se puede pues afirmar que, en general, el volumen de literatura dedicado a estudiar las capacidades funcionales de las células del GALT en peces así como su papel en la respuesta inmunitaria es casi inexistente.

El aislamiento de las células pertenecientes al GALT de teleósteos se ha basado desde sus inicios en técnicas ya probadas en humanos u otros mamíferos. En general, se trata de métodos mecánicos, químicos, enzimáticos o combinaciones de los mismos. Se asume que la población de los IELs se extrae mediante agitación constante en un medio que contiene EDTA y DTT durante un periodo de incubación que oscila entre 10 y 60 min. Los LPLs, por su parte, requieren la digestión enzimática del tejido conectivo de la lámina propia, por ello se recurre al uso de la colagenasa entre 30 min y 3 h (Davidson y col., 1991). Por último, otros autores utilizan un método puramente mecánico que consiste en desprender el epitelio intestinal a través del uso de un rascador de células o del lado romo de un escalpelo. Dicho raspado es filtrado posteriormente a través de un tamiz de nylon y la fase de los leucocitos purificada como en los casos anteriores (Rombout y col., 1998).

Efectos antiproliferativos de las bacterias comensales o probióticas

En vertebrados superiores se atribuye a las bacterias comensales del intestino un papel supresor en la inflamación intestinal (Riedel y col., 2006). Se cree que el control de la inflamación en la barrera intestinal, la cual es bombardeada por millones de antígenos alimentarios cada día, se debe a las microbiota intestinal (Isolauri y col., 2001; Braat y col., 2004). Más aún, se ha demostrado que las bacterias probióticas son capaces de inhibir la proliferación de monocitos (Pessi y col., 1999) y líneas celulares (Lee y col., 2004). Por tanto, se ha sugerido que los probióticos puedan ser agentes terapéuticos anticancerígenos (Rafter, 2002). La esperanza de vida de animales de laboratorio con tumores se ve alargada con el consumo de probióticos (Lee y col., 2004) y la eficacia de vacunas antitumorales también aumenta gracias a estos (Tanasienko y col., 2006).

En peces se desconoce por completo si la actividad biológica de la microbiota intestinal incluye el control de la inflamación o si los probióticos son capaces de inhibir la proliferación de sus células.

El objetivo principal de la presente Tesis Doctoral es el estudio de las interacciones entre bacterias probióticas y el sistema inmunitario de los peces teleósteos de cultivo a nivel celular y tisular principalmente.

Como objetivos específicos se plantearon:

I. El estudio de los efectos inmunomoduladores de bacterias probióticas sobre el sistema inmunitario innato de la dorada (*Sparus aurata* L.), tanto *in vivo* como *in vitro*.

II. Los cambios morfológicos de la barrera intestinal de salmón atlántico (*Salmo salar* L.) tras ser expuesta a bacterias patógenas y/o bacterias probióticas.

III. El estudio morfofuncional del tejido linfoide asociado a la mucosa intestinal de la dorada.

IV. Evaluar los efectos antiproliferativos de bacterias probióticas sobre líneas celulares de teleósteos.

OBJETIVO I: EFECTOS INMUNOMODULADORES DE LAS BACTERIA PROBIÓTICAS EN EL SISTEMA INMUNITARIO INNATO DE LA DORADA

Para los dos experimentos *in vivo* el probiótico se administró en dieta a las doradas mientras que el experimento *in vitro* se utilizaron leucocitos de riñón cefálico de dorada y se incubaron con las bacterias probióticas en el laboratorio.

La preparación de piensos suplementados con bacterias probióticas se hizo a partir de una dieta comercial de pellets secos de ProAqua, Palencia. Para ello, el pienso fue molido, humedecido con agua del grifo y se le añadieron las suspensiones bacterianas correspondientes: *Lactobacillus delbrüeckii* subsp. *lactis* (10^7 unidades formadoras de colonias (ufc)/g), *Bacillus subtillis* (10^7 ufc/g) o la mezcla de ambas ($0.5x10^7$ ufc/g de cada una). A continuación los pellets fueron hechos de nuevo y se secaron en una estufa a 37°C. Estas dietas se administraron al 1% del peso medio de las doradas por día durante 3 semanas y se continuó durante una semana más el experimento administrando a todos los peces el pienso no suplementado con bacterias.

En un segundo experimento se ensayaron las bacterias Pdp11 inactivada por calor (10^8 ufc/g), 51M6 inactivada por calor (10^8 ufc/g) o la mezcla de ambas a $0.5x10^8$ ufc/g de cada una. Estas bacterias fueron aisladas por Chabrillón (2003) y cedidas generosamente por el Dr. Moriñigo, Departamento de Microbiología, Universidad de Málaga. Las dietas experimentales se realizaron tal y como se ha descrito y los peces fueron alimentados al 1% de su peso medio por día durante 4 semanas ininterrumpidamente.

Dichas dosis bacterianas fueron elegidas, en primer lugar, en base a la bibliografía disponible hasta la fecha. En el segundo experimento *in vivo*, al tratarse de bacterias autóctonas aisladas de la propia dorada, se especuló que sería necesaria una dosis más elevada para conseguir un efecto. Esta teoría fue confirmada *a posteriori* en los estudios *in vitro* como se verá más tarde.

Las doradas no mostraron en ningún momento rechazo al nuevo alimento de manera que la totalidad de la dieta administrada cada día a los ejemplares era ingerida y se muestrearon 6 ejemplares de cada grupo a las 1, 2, 3 y 4 semanas de ambos experimentos *in vivo*.

En el primer experimento se estudiaron las respuestas inmunitarias innatas de los leucocitos de riñón cefálico: peroxidasa intracelular (Quade y Roth, 1997), fagocitosis

(Rodríguez y col., 2003), estallido respiratorio (Bayne y Levy, 1991) y la actividad citotóxica natural (Cuesta y col., 1999).

En el segundo experimento se estudiaron las mismas respuestas celulares que en experimento anterior y además se estudiaron parámetros de la inmunidad humoral en suero, en concreto, los niveles de peroxidasas y la actividad hemolítica del complemento.

Por último, se realizó un estudio *in vitro* con la cuatro cepas bacterianas (*Lactobacillus delbrüeckii* subsp. *lactis, Bacillus subtillis,* Pdp11 y 51M6) pero inactivadas por calor. Para ello, los leucocitos de riñón cefálico de dorada fueron incubados durante 30 min con cada una de dichas bacterias a tres dosis diferentes (concentración final por pocillo: $5x10^5$, $5x10^6$ y $5x10^7$ ufc/ml. Transcurrido el tiempo de incubación, se cuantificaron siguiendo la metodología anteriormente especificada, la peroxidasa intracelular, el estallido respiratorio y la actividad natural citotóxica. En cuanto a la fagocitosis, se pretendió investigar la respuesta de los leucocitos de riñón cefálico de dorada al enfrentarse a las células bacterianas probióticas, lo que se realizó siguiendo la metodología de Esteban y col. (1998) y posterior procesado de las muestras para su estudio microscópico electrónico de transmisión.

Los datos se presentaron como la media del índice de estimulación (calculado al dividir cada valor por la media de los valores del grupo control a cada tiempo) más menos el error estándar. Los datos se analizaron estadísticamente realizando una comparación de las varianzas de un factor. Las diferencias se consideraron significativas cuando $p < 0.05$ y, en el caso de haberlas, se realizó un test de Tukey. EL mismo procedimiento se llevó a cabo en el resto de los apartados de esta Tesis Doctoral.

OBJETIVO II: CAMBIOS MORFOLÓGICOS EN EL EPITELIO INTESTINAL DEL SALMÓN ATLÁNTICO CAUSADOS POR BACTERIAS PATÓGENAS Y PROBIÓTICAS

Para conseguir este objetivo se evaluaron los cambios morfológicos que suceden en el epitelio intestinal de salmón atlántico (*Salmo salar* L.) en dos experimentos distintos. La metodología seguida en los dos experimentos fue similar incubándose *in vitro* los intestinos completos con distintas concentraciones de bacterias patógenas, bacterias

probióticas o combinaciones de ambos grupos. Estos experimentos se realizaron en el Institute of Marine Research, Bergen, Noruega y en la plataforma MIC (Molecular Imaging Center) de la Universidad de Bergen.

En el experimento inicial, se eligieron como bacterias patógenas *Aeromonas salmonicida* subsp. *salmonicida* VI-88/09/03175 (Central Veterinary Laboratory, Oslo, Noruega) y *Vibrio anguillarum* LFI317 serotipo O1, agentes causales de la furunculosis y la vibriosis respectivamente. Como probiótico empleamos *Carnobacterium divergens* 6251, una cepa aislada de trucha alpina (*Salvelinus alpinus*) con propiedades probióticas en salmónidos. La lista de todos los tratamientos aplicados en el experimento I aparece en la Tabla 2. Es importante destacar que en este primer trabajo, el tratamiento combinado de bacteria probiótica y bacteria patógena siempre fue de manera simultánea, es decir, las incubaciones fueron siempre de 60 min y las bacterias se mezclaban antes de ser adicionadas al intestino.

Tratamiento	Bacteria y dosis (ufc/ml)
1	Solución Ringer (control)
2	*A. salmonicida* 6×10^6
3	*V. anguillarum* 6×10^4
4	*V. anguillarum* 6×10^6
5	*C. divergens* 6×10^4
6	*C. divergens* 6×10^6
7	*A. salmonicida* 3×10^6 y *C. divergens* 3×10^6
8	*V. anguillarum* 3×10^4 y *C. divergens* 3×10^4
9	*V. anguillarum* 3×10^4 y *C. divergens* 3×10^6
10	*V. anguillarum* 3×10^6 y *C. divergens* 3×10^4
11	*V. anguillarum* 3×10^6 y *C. divergens* 3×10^6

Tabla 2: Tratamientos experimentales a los que se sometieron los intestinos de salmón *in vitro*

Para llevar a cabo los tratamientos se diseccionaron los ejemplares de salmón, se extrajeron los intestinos completos cuidadosamente y se lavaron tres veces antes de atar

con un hilo uno de los extremos del tubo digestivo. Entonces se adicionó 1ml de la suspensión bacteriana correspondiente. Se cerró el intestino por el otro extremo y se colocó la muestra en un tubo de cristal que contenía una solución salina estéril al 0.9%. Los tubos se incubaron durante 60 min en un baño mantenido a 10 °C. Tras la incubación se cortaron ambos extremos y se lavaron las muestras tres veces con PBS recogiéndose una muestra de 0.5 cm del intestino anterior que se colocó en fijador McDowell. Dicha muestra se procesaría para microscopía óptica, micoscopía electrónica de barrido y microscopía elecrónica de transmisión.
A fin de evaluar los cambios morfológicos observados en el intestino realizamos una lista con los cambios estimados que fueron:

1. la presencia de restos celulares en el lumen intestinal
2. la desorganización de las microvellosidades de los enterocitos
3. la presencia de células epiteliales que hacen protrusión hacia el lumen
4. el edema
5. la desintegración de las uniones estrechas entre enterocitos
6. la presencia de cuerpos celulares oscuros en la base del epitelio
7. la pérdida de la integridad epitelial
8. la presencia de estructuras parecidas a fagolisosomas
9. la presencia de partículas de tipo bacteriano cerca de las uniones estrechas.

A continuación, elegimos una escala de 0 a 3 en la que estimamos si el cambio era 0= no observado, 1 = observado entre 1 y 3 imágenes de 10; 2 = observado entre 4 y 6 imágenes de 10 y 3 = observado en 7 o más imágenes de 10.

Este primer experimento nos permitió probar que *A. salmonicida* es un buen modelo de daño tisular en el intestino anterior del salmón. A continuación se llevó a cabo el experimento II en el que por primera vez estudiamos los cambios morfológicos que suceden en éste como consecuencia del tratamiento con *Lactobacillus delbrüeckii* subsp. *lactis* y su posible función protectora de la integridad del epitelio intestinal frente *A. salmonicida*.
En este caso, y valiéndonos de los resultados del experimento anterior, el tratamiento con el probiótico ($1.6x10^5$ cfu/ml) se realizó durante los primeros 30 min. Tras ese tiempo, se cortaron los extremos del tubo intestinal el cual se lavó 3 veces para eliminar

las bacterias no adheridas, se volvió a atar un extremo, se adicionó 1ml de la suspensión de *A. salmonicida* ($7x10^7$ cfu/ml), se cerró el otro extremo y se procedió a incubar tal y como se ha explicado durante 30 min más. Los tratamientos control consistieron en incubaciones con *Lactobacillus delbrüeckii* subsp. *lactis* ($1.6x10^5$ cfu/ml) o *A. salmonicida* ($7x10^7$ cfu/ml). Al final de ambas incubaciones se recogió una muestra de 0.5 cm en McDowell para ser estudiada por métodos convencionales de microscopía óptica y electrónica de transmisión.

Además se investigó el destino de *L. delbrüeckii* subsp. *lactis* en el microambiente del intestino de salmón gracias a un marcaje fluorescente con TRITC y usando criosecciones y microscopía confocal. Para ello, se incubaron intestinos con o sin *L. del. lactis* marcado con fluorescencia roja. Tras 60 min se lavaron los intestinos con PBS y se tomó una muestra de 1 cm de longitud que fue congelada inmediatamente en un baño de nitrógeno líquido. La muestra fue montada en Tissue Tech a fin de poder realizar criosecciones (5µm) las cuales fueron observadas en un microscopio confocal Leica TCS SP2 AOBS.

OBJETIVO III: AISLAMIENTO Y CARACTERIZACIÓN MORFOFUNCIONAL DEL GALT DE DORADA

El sistema linfoide asociado a la mucosa intestinal de la dorada (*Sparus aurata* L.) fue aislado adecuando los protocolos disponibles en la literatura para distintas especies tanto de peces como de vertebrados superiores. Se evaluaron métodos puramente mecánicos y otros que combinan tratamientos mecánicos, químicos y enzimáticos (Davidson y col, 1991; Rombout y col., 1993;1998; McMillan y Secombes, 1997; Hébert y col., 2002; Bernard y col, 2006). Se evaluaron distintos tiempos de incubación en presencia de la enzima colagenasa tipo IV (30, 60 y 120 min) y distintas concentraciones de dicha enzima (0, 0.15 y 0.37 mg/ml) fueron ensayadas. Las células obtenidas se contaron a fin de obtener el número medio de células que cada protocolo proporcionaba. Asimismo, se cuantificó la viabilidad de las suspensiones celulares cuyas poblaciones se analizaron por citometría de flujo. A continuación, se utilizaron columnas de algodón y nylon y gradientes continuos de Percoll para eliminar el moco y separar las distintas poblaciones celulares por densidad, las cuales fueron estudiadas por citometría y microscopia óptica tras ser citocentrifugadas y teñidas con Giemsa.

Una vez que fueron optimizados los protocolos de aislamiento se investigaron *in vitro* las principales respuestas inmunitarias innatas de la suspensión celular obtenida comparándolas con las de los leucocitos de riñón cefálico y utilizando las mismas técnicas rutinarias de nuestro laboratorio ya nombradas.

El protocolo de aislamiento utilizado para el estudio de las actividades funcionales fue el siguiente:

Se extrajo el intestino de cada uno de los ejemplares desde los ciegos pilóricos hasta el ano y se lavaron en solución salina en frío (PBS) eliminando así posibles restos de la digestión. El intestino se seccionó en trozos de aproximadamente 1 cm que fueron cortados longitudinalmente y colocados en 15 ml de solución I (PBS con ácido etilendiaminacético (EDTA) (0.37 mg/ml) y dithiothreitol (DTT) (0.145 mg/ml, Sigma) durante 10 minutos en agitación suave. Los respectivos sobrenadantes se recogieron y filtraron a través de un tamiz de 100 µm y, a continuación, los fragmentos de tejido se colocaron en 20 ml de solución II (Solución salina de Hanks, suero bovino fetal al 5 %, 100 I.U./ml de penicilina (Flow), 100 µg/ml de estreptomicina (Flow), DNAse I (0.05 mg/ml; Sigma) y colagenasa (0.15 mg/ml, Sigma) durante una hora a temperatura ambiente y en agitación suave.

Tanto los sobrenadantes como los fragmentos se filtraron a través del mismo tamiz donde se había recogido el primer sobrenadante y toda la suspensión se centrifugó a 462 g durante 10 min a 24 °C. Tras realizar dos lavados con medio sRPMI con 5 % de suero bovino fetal. Se contabilizó el número de células presentes por ml de la suspensión final en una cámara de Neubauer, así como su viabilidad.

Dada la imposibilidad de cuantificar la fagocitosis llevada a cabo por los leucocitos de intestino de dorada mediante citometría de flujo, se procedió al estudio por microscopía electrónica de transmisión. Para ello se utilizaron como partículas diana: *Saccharomyces cerevisiae*, bolas de látex (diámetro 2 µm, Sigma) o la bacteria probiótica *Lactobacillus delbrüeckii* subsp. *lactis* inactivada por calor. En el caso de la levadura y la bacteria, los leucocitos se incubaron con la partícula durante 30 min. Para las bolas de látex la incubación fue de 3 h. Bacteria y bolas de látex se adicionaron a un ratio de 10: 1 con respecto a los leucocitos. Las levaduras se utilizaron, como en el caso de citometría, a un ratio 6:1.

OBJETIVO IV: EFECTOS ANTIPROLIFERATIVOS DE LAS BACTERIAS PROBIÓTICAS EN CÉLULAS DE PECES TELEÓSTEOS

Para el estudio de la capacidad antiproliferativa de las bacterias probióticas *L. delbrüeckii* subsp. *lactis* y 51M6 en líneas celulares de peces, se extrajeron los extractos citoplasmáticos de estas bacterias por sonicación y ultracentrifugación. La concentración de proteína presente en dichos extractos se cuantificó a fin de evaluar los efectos de tres concentraciones diferentes de proteína (0, 12.5, 25 y 50 µg/ml en PBS). Dos líneas celulares epiteliales de peces teleósteos fueron elegidas para este experimento. SAF-1 de aleta dorsal de dorada y EPC, original de un papiloma de carpa (*Cyprinus carpio* L.). Las líneas celulares fueron mantenidas tal y como se explica en la página 43. Las células se sembraron en placas de 96 pocillos a una densidad de 10^4 células por pocillo en el caso de la línea SAF-1 y de $2x10^4$ células por pocillo en el caso de la línea EPC en presencia o ausencia de los extractos citoplasmáticos de las bacterias *L. delbrüeckii* subsp. *lactis* y 51M6.

La proliferación de las células en cultivo se midió tras 4, 24, 48 o 72 h mediante la técnica del cristal violeta. Además, se cuantificó por citometría de flujo la presencia de células viables y no viables tanto apoptóticas como necróticas a través de una doble tinción fluorescente. Esta técnica fue previamente puesta a punto en cultivos primarios de leucocitos de dorada y consiste en teñir las células con diacetato de fluoresceína (FDA) y yoduro de propidio (PI) (Bartkowiak y col., 1999; Sandström y col., 2000; Saha y col., 2003). Las células apoptóticas no poseen esterasas citoplasmáticas viables de modo que no rompen la molécula de FDA y no producen fluorescencia verde, a diferencia de las células viables. Además, ya que sus membranas no están permeabilizadas, las células apoptóticas son PI negativas, al contrario de lo que sucede con las necróticas. Esta base teórica sirvió para optimizar el marcaje con FDA para células de dorada, lo que se consiguió con 50 µg/ml de una solución madre compuesta por 0.05g de FDA en 4 ml de DMSO. Las células en cultivo se marcaron durante 30 min con FDA y PI y posteriormente fueron analizadas por citometría de flujo en un citómetro Coulter Epics XL.

OBJETIVO I: EFECTOS INMUNOMODULADORES DE LAS BACTERIA PROBIÓTICAS EN EL SISTEMA INMUNITARIO INNATO DE LA DORADA

Los experimentos *in vivo* en los que *L. delbrüeckii* subsp. *lactis* o *B. subtilis* se administraron oralmente a doradas durante tres semanas más una semana de alimento control, revelaron la capacidad de dichas bacterias de modular las respuestas celulares innatas de los leucocitos de riñón cefálico de la dorada. Mientras que la peroxidasa intracelular resultó disminuida, la capacidad y la habilidad fagocítica de los leucocitos de riñón se vio aumentada en los grupos alimentados con la dieta probiótica durante las tres semanas. La actividad citotóxica natural sólo fue significativamente mayor tras tres semanas de tratamiento. En general, los efectos fueron más duraderos y marcados cuando fue administrada la dieta con ambas bacterias. Al cesar la administración del probiótico en la dieta los efectos remitieron.

En el segundo experimento *in vivo*, las bacterias Pdp11 y 51M6, solas o combinadas, se administraron oralmente durante 4 semanas tras ser inactivadas por calor. En este caso se evaluaron las respuestas innatas tanto humorales como celulares. En lo que respecta a las humorales, se observó un aumento progresivo de la peroxidasa del suero y de la actividad del complemento en los ejemplares alimentados con probióticos durante tres y cuatro semanas. Con respecto a las respuestas celulares, la actividad fagocítica fue de nuevo la respuesta más estimulada, tanto por las bacterias individuales como por la dieta multiespecífica. El estallido respiratorio no fue afectado por la dieta mientras que la respuesta natural citotóxica sólo fue estimulada por la dieta con 51M6 después de tres semanas de tratamiento. En este experimento, la combinación de ambas bacterias no fue tan eficaz como en el primero. Además, la prolongación de la dieta de 3 a 4 semanas no contribuyó a una mejora en los parámetros estudiados.

Por último, se estudiaron los efectos *in vitro* de las cuatro especies bacterianas utilizadas *in vivo* tras ser inactivadas por calor, sobre las respuestas celulares de los leucocitos de riñón cefálico de dorada. Este estudio reveló efectos inmunoestimuladores dosis-dependientes en los que destaca la mayor eficacia de las bacterias no autóctonas (*L. delbrüeckii* subsp. *lactis* y *B. subtilis*) con respecto a aquellas originales del mismo hospedador (Pdp11 y 51M6). Este mismo resultado quedó reflejado en el estudio por microscopía electrónica de la actividad fagocítica de los leucocitos de riñón cefálico frente a estas cuatro bacterias. Tanto macrófagos como granulocitos interiorizaron

activamente las cuatro bacterias pero las bacterias no autóctonas parecían despertar una mayor avidez en los fagocitos de la dorada.

Por tanto, en dorada, como en otras especies de peces teleósteos cultivados (Irianto y Austin, 2002; Pirarat y col., 2006; Panigrahi y col., 2007), las bacterias probióticas son capaces de estimular las principales respuestas inmunitarias innatas. De nuestros resultados se puede observar que las bacterias vivas administradas en la dieta producen efectos mientras que los animales reciben la dieta, pero que dichos efectos remiten cuando se alimentan de nuevo con la dieta control. Este resultado apunta a que, en peces, la microbiota intestinal está altamente influida por factores ambientales, sobre todo el alimento y el agua, y que la adición de la bacteria al pienso no logra que las cepas administradas colonicen de manera permanente el tracto gastrointestinal de la dorada.

Por otro lado, se ha comprobado que, como se sugirió en humanos (Timmerman y col., 2004), la combinación de dos especies bacterianas, en este caso *L. del. lactis* y *B. subtilis*, produce efectos sobre el sistema inmunitario innato del hospedador distintos y más intensos tanto en magnitud como en duración, que los de las dietas monoespecíficas. Dicha afirmación no fue corroborada, sin embargo, en nuestro segundo experimento, aunque esto puede ser debido a que Pdp11 y 51M6 fueron administradas tras su inactivación, o alternativamente, a que ambas especies pertenecen al mismo género y proceden de la misma fuente (piel de dorada).

Nuestros resultados *in vitro* revelan la gran importancia que las dosis y las cepas tienen a la hora de provocar o no efectos inmunomoduladores en las células del sistema inmunitario innato del pez. Mayores dosis indujeron mayor estimulación en los parámetros estudiados excepto la peroxidasa que se mantuvo inalterada. La bacteria Pdp11 resultó poseer menor potencial de estimulación de las respuestas celulares innatas de la dorada. Por tanto, los resultados *in vitro* e *in vivo* no siempre coinciden siendo la validez de los primeros dependiente de la cepa probiótica estudiada. El estudio de la fagocitosis *in vitro* nos permitió ampliar nuestro conocimiento sobre el comportamiento de los fagocitos en presencia de bacterias no patógenas. Queda claro que los fagocitos de riñón cefálico de dorada reconocen, ingieren y degradan las bacterias probióticas incluso cuando éstas son aisladas de la propia piel del hospedador. Dicho comportamiento puede deberse a la falta de contacto en una situación real de estas células localizadas normalmente en un órgano interno y las bacterias presentes en superficies mucosas, ya sea la piel o el intestino.

OBJETIVO II: CAMBIOS MORFOLÓGICOS EN EL EPITELIO INTESTINAL DEL SALMÓN ATLÁNTICO CAUSADOS POR BACTERIAS PATÓGENAS Y PROBIÓTICAS

Tanto *A. salmonicida* como *V. anguillarum* son capaces de producir daños morfológicos en el epitelio intestinal del salmón atlántico. Este resultado corrobora lo encontrado por Ringø y col. (2004) en cuanto a *A. salmonicida* mientras que *V. anguillarum* no había sido estudiado hasta la fecha. Los cambios observados como resultado de la incubación con estos dos patógenos no fueron iguales, lo cual sugiere el uso de mecanismos de patogénesis diferentes. En el caso de muestras control y los tratamientos con *C. divergens* no se observaron cambios morfológicos ni daños titulares en el intestino anterior.

La incubación con *A. salmonicida* resultó en la aparición de debris celulares en el lumen, la pérdida de contacto entre las microvellosidades y el epitelio y la movilización de leucocitos de la lámina propia al epitelio. La coincubación del patógeno con el probiótico no produjo daños en las microvellosidades de los enterocitos pero abundantes leucocitos y células caliciformes aparecieron en los espacios resultantes del edema celular entre enterocitos. *V. anguillarum*, a ambas dosis ensayadas, causó daños importantes en la estructura de la barrera intestinal. Además de debris celular en el lumen, edema y movilización leucocitaria, las microvellosidades aparecieron acortadas o ausentes en algunas regiones. Por otro lado, las uniones estrechas se mostraron afectadas, y células muertas en protrusión se asomaban hacia el lumen intestinal. Algunos enterocitos mostraron estructuras de tipo fagolisosoma con bacterias en degradación tras incubarse los intestinos con *V. anguillarum* a $3x10^4$ ufc/ml. Esta observación sugiere que las células epiteliales intestinales de peces teleósteos son capaces de interiorizar bacterias patógenas. En el caso de presentar el antígeno tras interiorizar el patógeno, significaría que los enterocitos de teleósteos o algunos de ellos, poseen características análogas a las células M de vertebrados superiores. Hansen y Olafsen (1999) también observaron endocitosis de bacterias en larvas de arenque.

La coincubación con una dosis de *C. divergens* menor a la de *V. aguillarum* no consiguió eliminar los signos de daño tisular, sin embargo dosis mayores lograron paliar los efectos nocivos de este patógeno. Esto puede ser debido a que el probiótico *C. divergens* realiza un papel protector de la barrera intestinal en el salmón, sin embargo,

las dosis del probiótico y del patógeno requeridas para obtener dicho efecto necesitan ser investigadas con mayor detalle. El hecho de que esta bacteria inhiba *in vitro* el crecimiento de patógenos de peces teleósteos (Ringø y col., 2005) apoya nuestros resultados ya que el probiótico podría inhibir o disminuir el número de células patógenas viables que colonizarían el intestino del salmón.

Cuando *A. salmonicida* fue coincubada con *C. divergens*, pequeñas partículas de 0.5 μm de diámetro fueron vistas entre las microvellosidades y penetrando en el epitelio a través de las uniones estrechas. Estas partículas, ya fueran el patógeno o el probionte, no se hallaron en ningún otro tratamiento, y despiertan la hipótesis de cómo un patógeno entra a través de la barrera intestinal del hospedador según la presencia de otras bacterias como en este caso *C. divergens*.

En cuanto a *L. delbrüeckii* subsp. *lactis*, la incubación del intestino de salmón con este probiótico marcado con fluorescencia reveló, por microscopía confocal, la adhesión de la bacteria en grandes números al moco intestinal. Además, unas pocas bacterias fueron capaces de translocar al interior del intestino observándose tanto a nivel epitelial entre enterocitos como a nivel del tejido conectivo. Este probiótico no produjo daño alguno sobre la morfología intestinal y pudo ser observado por microscopía electrónica de transmisión tanto en la microvellosidades como paracelularmente entre los enterocitos pero sin causar edema alguno. Además, los intestinos de salmón incubados con este probiótico mostraron leucocitos agrupados en la zona basal del epitelio así como mayor número de células rodlet que los controles. Cuando se incubaron los intestinos con *L. del. lactis* y luego *A. salmonicida*, ésta no logró causar el daño tisular anteriormente descrito. Así, *L. del. lactis* es efectivo como tratamiento profiláctico a la hora de proteger frente al daño causado por *A. salmonicida* y explica cómo la administración de bacterias probióticas resulta en una mayor resistencia frente a infecciones experimentales, incluyendo la furunculosis (Irianto y Austin, 2003; Irianto y col. 2003).

OBJETIVO III: AISLAMIENTO Y CARACTERIZACIÓN MORFOFUNCIONAL DEL GALT DE DORADA

Fueron evaluados distintos protocolos para el aislamiento del GALT de dorada. En general, la viabilidad no fue afectada por el tratamiento empleado pero sí el número de células obtenidas y sus características en el citómetro de flujo. En general, los tratamientos puramente químicos o mecánicos liberaron menos número de células que aquellos que usaron la enzima colagenasa. La degradación progresiva del intestino quedó patente a través del estudio microscópico de las distintas fases del protocolo enzimático.

El uso de columnas de nylon y algodón resultó útil porque elimina el moco y es sabido que su presencia dificulta la separación de los tipos celulares por métodos como el de centrifugación en gradiente de Percoll, basados en la diferente densidad de las células. Por tanto, una incubación de las suspensiones de leucocitos de intestino de 60 min de duración en dichas columnas permitió la subsiguiente separación en tres bandas de densidad según el gradiente continuo de Percoll establecido. En la banda de mayor densidad (mayor de 1,080 g/l) se identificó una población homogénea y de alta viabilidad constituida por células pequeñas, redondeadas, con núcleo grande y poco citoplasma según los estudios de microscopía y citometría. Ninguna banda contenía sólo células linfoides granulares tipo macrófago o granulocito. Estas aparecieron en la banda de densidad media entremezcladas con enterocitos, células caliciformes y algún linfocito.

La presencia de células NBT positivas aumentó de manera no significativa al alargarse el periodo de incubación y aumentar la concentración de colagenasa. En cualquier caso, el número de células NBT positivas siempre fue muy inferior al observado en suspensiones de riñón cefálico. Este dato se corroboró al estudiarse el estallido respiratorio por una técnica de luminiscencia. Sólo las células no adherentes liberaron radicales libres de oxígeno de manera muy débil. Se descarta una posible pérdida de células adherentes responsables del estallido respiratorio durante el proceso de aislamiento ya que los tubos de plástico utilizados durante todo el proceso no son un sustrato al que los macrófagos de dorada se puedan adherir.

Igualmente, el contenido en peroxidasas de las suspensiones de leucocitos intestinales de dorada fue reducido y la actividad fagocítica tan baja que no fue posible cuantificarla

por citometría de flujo. Por esto último tuvo que realizarse el estudio por microscopía electrónica de transmisión, viéndose que los macrófagos y los granulocitos son los únicos tipos celulares presentes en el intestino de dorada con capacidad fagocítica. Es de destacar que la avidez de estas células frente a bolas de látex pareció superior a aquella frente a la levadura *S. cerevisiae* aunque hay que resaltar que nuestro estudio no fue cuantitativo. Por último, el probiótico *L. delbrüeckii* subsp. *lactis* despertó una muy leve respuesta en los fagocitos intestinales de la dorada. Ya que la misma bacteria fue interiorizada de manera activa y notable por los fagocitos de riñón de esta misma especie, es de destacar que el compartimiento linfoide intestinal presenta características y comportamientos que se alejan del compartimiento linfoide sistémico localizado en el riñón cefálico. Las bacterias probióticas, por tanto, al no emitir señales de peligro tal y como se demostró en el apartado anterior, no estimulan a los fagocitos con los que están en continuo contacto. Lanzamos por ello la hipótesis de que los fagocitos intestinales de peces también pudieran ser "educados por los estímulos locales" tal y como sucede en mamíferos (Magalhaes y col., 2007).

Por último, las células aisladas del intestino de dorada, tienen una alta actividad natural citotóxica, lo cual concuerda con lo hallado por McMillan y Secombes (1997) en trucha arco iris y que explica la baja presencia de células IgM^+ en suspensiones de IELs de trucha y la alta expresión de marcadores de células T (Bernard y col., 2006). Parece que este patrón se cumple también en la dorada. Sin embargo, el marcaje con proteina A y FITC fue mucho mayor que el 1% encontrado por Bernard y col. (2006) al usar un anticuerpo monoclonal en IELs de trucha. Por un lado, esta discordancia se puede deber a una liberación de células B cuando la lámina propia se digiere en el proceso de aislamiento (y por tanto no sólo IELs sino también LPLs son estudiados). Alternativamente, nuestra cuantificación fue indirecta y no tan específica como el uso de un anticuerpo monoclonal como el de trucha. Así, todos los fragmentos Fc presentes en la superficie de las células de intestino fueron cuantificados. Es probable que otros isotipos de inmunoglobulina, no únicamente IgM, contribuyan al valor obtenido usando nuestra técnica indirecta.

OBJETIVO IV: EFECTOS ANTIPROLIFERATIVOS DE LAS BACTERIAS PROBIÓTICAS EN CÉLULAS DE PECES TELEÓSTEOS

En este objetivo logramos poner a punto una técnica para la cuantificación de células viables, apoptóticas y necróticas de dorada. El uso combinado de FDA y PI permitió diferenciar entre células viables, apoptóticas y necróticas tanto de cultivos primarios de leucocitos de teleósteos como de cultivos de líneas celulares establecidas. Los linfocitos mostraron una menor intensidad de tinción con FDA que los fagocitos y mueren en mayor porcentaje que los fagocitos bajo condiciones de cultivo *in vitro*. La técnica de doble marcaje con FDA y PI por citometría de flujo fue contrastada de manera semicuantitativa a través de microscopía de fluorescencia y de transmisión. En general, el tiempo de cultivo disminuyó la viabilidad de los cultivos primarios de leucocitos de dorada siendo los fagocitos más resistentes que los linfocitos de los tres órganos estudiados. La estaurosporina y el resveratrol, utilizados como controles primarios por ser inductores de la apoptosis resultaron en valores significativamente mayores de apoptosis comparados con los controles no tratados con dichas sustancias.

El cultivo de las líneas celulares de peces SAF-1 y EPC y el estudio de su proliferación demostró que los extractos citoplasmáticos de *L. delbrüeckii* subsp. *lactis* y 51M6 tienen importantes efectos antiproliferativos al igual que sucede en vertebrados superiores con otras cepas estudiadas (Lee y col., 2004). Mientras que *L. delbrüeckii* subsp. *lactis* inhibe la proliferación de SAF-1 de manera dosis dependiente y a tiempos muy cortos de incubación, 51M6 produce efectos más leves y requiere mayores concentraciones y tiempos para reducir la proliferación de esta línea celular. En cuanto a las células EPC, también epiteliales pero de origen tumoral, los extractos citoplasmáticos ensayados lograron frenar su crecimiento pero de manera menos marcada que en el caso de la línea SAF-1. La inhibición de la proliferación se vio acompañada de la inducción de la muerte por apoptosis de las células estudiadas seguida por la necrosis de las mismas.

Por tanto, aunque hasta la fecha no se hubiera estudiado el potencial antiproliferativo de los probióticos en peces teleósteos, queda claro que tanto especies de bacterias probióticas alóctonas como autóctonas, presentan en su citoplasma compuestos capaces

de inhibir la división de células epiteliales. Esta propiedad ha hecho que los probióticos sean considerados como agentes terapéuticos antitumorales en vertebrados superiores (Rafter, 2002; Lee y col., 2004). Se desconoce en la actualidad si esta actividad antiproliferativa es ejercida en el epitelio intestinal de teleósteos ante episodios de inflamación del mismo.

1.- La administración en dieta de *Lactobacillus delbrüeckii* subsp. *lactis*, *B. subtilis* o la combinación de ambas bacterias vivas modula a nivel sistémico las respuestas celulares inmunitarias innatas de leucocitos de dorada. La duración de los efectos inmunomoduladores se restringe al periodo de su administración.

2.- La administración en dieta de candidatos probióticos (Pdp11 ó 51M6), estimula el sistema inmunitario innato, tanto humoral como celular, de la dorada.

3.- Las bacterias *L. delbrüeckii* subsp. *lactis*, *B. subtilis*, Pdp11 y 51M6, inactivadas por calor, estimulan *in vitro* las respuestas celulares innatas de la dorada, generalmente de una manera dosis dependiente.

4.- La exposición a *V. anguillarum* o *A. salmonicida* produce daños tisulares en el epitelio intestinal del salmón atlántico que pueden resumirse en la pérdida de la integridad epitelial, mientras que las bacterias probióticas *C. divergens* o *L. delbrüeckii* subsp. *lactis* no causan ningún efecto negativo en la morfología e integridad del epitelio intestinal de esta especie.

5.- El daño causado en el epitelio del salmón por incubación con *V. anguillarum* se ve parcialmente mitigado a través de la incubación simultánea de *V. aniguillarum* con *C. divergens* si la dosis del patógeno es menor que la del probiótico.

6.- *L. delbrueckii* subsp. *lactis* es capaz de adherirse al moco intestinal del salmón atlántico y llegar hasta la lámina propia del intestino.

7.- La incubación con *L. delbrueckii* subsp. *lactis*, seguida de otra incubación con *A. salmonicida* consiguió proteger el intestino de salmón de los daños causados por esta última bacteria.

8.- Las células del tejido linfoide asociado a la mucosa intestinal de dorada, caracterizadas por una escasez de células fagocíticas (granulocitos y macrófagos) y la abundancia de linfocitos, se pueden aislar satisfactoriamente usando protocolos de bajo

coste y duración. El uso de columnas de nylon-algodón y gradientes de Percoll permiten purificar los linfocitos intestinales con una alta viabilidad.

9.- Los valores *in vitro* de las actividades inmunitarias innatas (fagocitosis, peroxidasa y estallido respiratorio) de las células del GALT de dorada son bajas comparadas con las de los leucocitos del riñón cefálico. La respuesta natural citotóxica fue, por el contrario, elevada y mayor que la observada en células del riñón cefálico.

10.- La combinación de FDA y PI es un método efectivo para la cuantificación de apoptosis por citometría de flujo en cultivos de leucocitos de dorada. La cinética de apoptosis de linfocitos y fagocitos de riñón cefálico muestra un patrón distinto siendo en general los fagocitos menos susceptibles de entrar en apoptosis.

11.- Los extractos citoplasmáticos de *L. delbrueckii* subsp. *lactis* y 51M6 inhiben la proliferación de las líneas celulares SAF-1 y EPC, generalmente de manera dosis dependiente. Las células de la línea EPC son más resistentes al efecto de los extractos quizás como consecuencia de su origen tumoral o bien a diferencias interespecíficas (carpa versus dorada).

12.- Los efectos antiproliferativos de *L. delbrueckii* subsp. *lactis* son más fuertes que los de 51M6 sobre las dos líneas celulares estudiadas. Las bacterias autóctonas derivadas de la superficie mucosa de los peces no son tan efectivas como las bacterias del ácido láctico.

13.- La disminución de la proliferación celular de las líneas celulares epiteliales de peces inducida por los extractos citoplasmáticos de los probióticos es seguida de la inducción de apoptosis y necrosis lo que indica un posible mecanismo de control de las respuestas inflamatorias de los probióticos en peces teleósteos.

XIII. PUBLICATIONS DERIVED FROM THE THESIS

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