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Universidad del País Vasco

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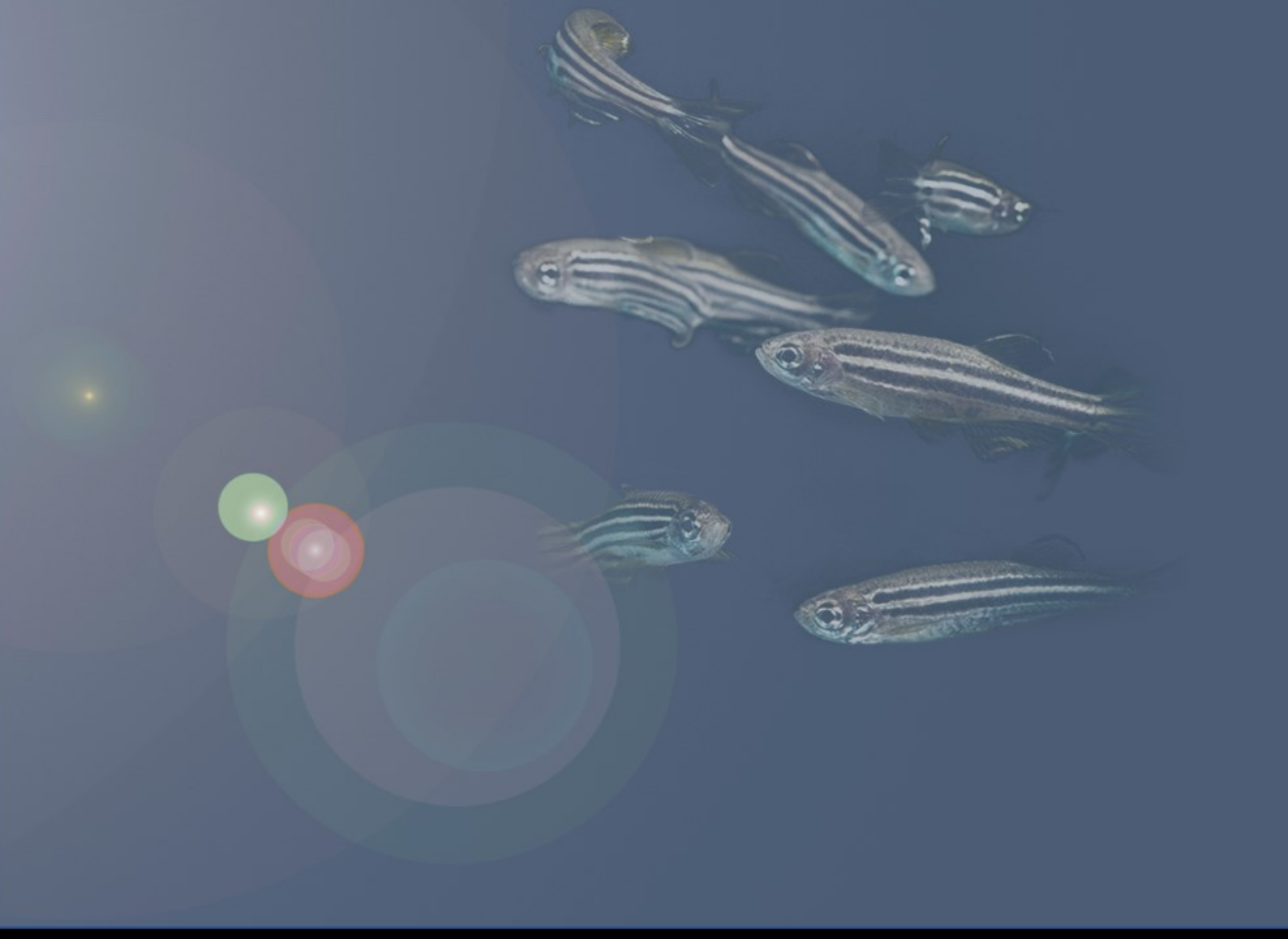
Use of zebrafish to evaluate the probiotic efficacy of lactic acid bacteria



Ignacio Iturria Gallego

PhD Thesis
2017

PhD THESIS

Use of zebrafish to evaluate the probiotic efficacy of lactic acid bacteria

Ph. D. Thesis submitted by

IGNACIO ITURRIA GALLEGO

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- U. Oyarbide, **I. Iturria**, S. Rainieri, M. A. Pardo, I. Aki Iturria, S. Rainieri, and M. A. Pardo, "**Use of Gnotobiotic Zebrafish to Study *Vibrio anguillarum* Pathogenicity**," Zebrafish, vol. 0, no. 0, pp. 18–24, (2014)

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ABREVATIONS

AA	auto-aggregation
Act	β-actine
Agdi	agmatine deiminase
BA	biogenic amines
BHI	brain heart infusion bacterial growth medium
BSA	bovine serum albumin
BSH	bile salt hydrolase
CCL20	chemokine (C-C motif) ligand 20
CD	chron´s disease
CFU	colony forming units
Cm	chloramphenicol
Ct	threshold cycle
DC	dendritic cells
DNA	deoxyribonucleic acid
dpf	days post fertilization
DSS	dextran sulfate sodium
EFA	*Enterococcus faecalis*
EFSA	European food safety association
EPS	exopolysaccharide
EW	embryo water
G	glucose
GALT	gut-associated bowel disease
GFP	green fluorescent protein
GIT	gastrointestinal tract
Hdc	histidine decarboxylase
HMWB	high-molecular-weight bacteriocin
hpce	hours post continuous fertilization

hpf	hours post fertilization
hpi	hours post infection
hpse	hours post short fertilization
IBD	intestinal bowel disease
IEC	intestinal epithelial cells
IgA	immunoglobulin A
IL	interleukin
LA5	*Lactobacillus acidophilus 5*
LAB	lactic acid bacteria
LBr	*Lactobacillus brevis*
LCA	*Lactobacillus casei*
LcM	*Leuconostoc mesenteroides*
Lf	*Lactobacillus fermentum*
LMWB	low-molecular-weight bacteriocin
Lp	*Lactobacillus plantarum*
Lyz	lysozyme
MAMP	microbial-associated molecular patterns
MIC	minimum inhibitory concentration
MPO	*myeloperoxidase*
MRS	De Man, Rogosa and Sharpe bacterial growth medium
MUB	mucus adhesion gene
MUC	mucin gene
MyD88	myeloid differentiation primary response gene 88
NK-κβ	nuclear factor kappa-light-chain-enhancer of activated B cells
NRI	neutrophil recruitment index
NTP	nucleoside triphosphate
OD	optical density
Odc	ornithine decarboxylase
PAMP	pathogen-associated molecular patterns

PBS	phosphate-buffered saline
PCA	plate count agar bacterial growth medium
PCR	polymerase chain reaction
Pt	putrescine
PTU	phenylthiourea
qPCR	quantitative polymerase chain reaction
QPS	qualified presumption of safety
RIN	RNA integrity index
RNA	ribonucleic acid
ROI	region of interest
RT	reverse transcriptase
rt-qPCR	real-time quantitative polymerase chain reaction
S	sucrose
SCFA	short chain fatty acid
TIF	tagged Image File
TLR	toll-like receptors
TNBS	2,4,6-Trinitrobenzenesulfonic acid
TNFα	Tumor necrosis factor alpha
Ty	tyramine
Tyrdc	tyrosine decarboxylase
UC	ulcerative colitis
VAN	*Vibrio anguillarum*
WHO	world health organization

SUMMARY

Humans have a complex digestive system, able to process and assimilate components present in the food to provide the host with nutrients and energy. But at the same time, the gastrointestinal tract (GIT) is continually exposed to microorganisms, mainly bacteria but also archaea, and microbial eukaryotes. The majority of gut microbiota are commensal microorganisms, although some of them can be harmful and their proliferation can result in an infectious process.

Gut microbiota is composed of approximately 100 trillion cells, and expresses at least 100-fold more unique genes than their host. Some species have developed a symbiotic relationship with the host, providing digestive enzymes and other benefits in exchange for a stable and nutrient-rich place to live. Gut-associated lymphoid tissue (GALT) can identify specific molecular sequences from the cell surface of bacteria to discriminate between commensal and pathogenic bacteria, and produce a tolerance or combat response. Nevertheless, dysfunctions of this mechanism can lead to the onset of various pathologies. The incidence of digestive inflammatory diseases, such as food allergies and inflammatory bowel disease (IBD), has increased over the last years due largely to the widespread of the "Western diet" and its influence on the microbiota composition. The dysbiosis caused by this diet, coupled with other environmental factors and genetic predisposition, may produce a loss of tolerance towards commensal bacteria, and ultimately the onset of IBD. This chronic inflammatory disease is treated with antibiotics and anti-inflammatory therapy administered in acute episodes of the disease; however, new approaches are necessary.

In recent years, probiotics have gained interest for the many benefits they can bring. Probiotics are living microorganisms which, when administered in adequate amounts, confer a health benefit to the host. The beneficial effects include the processing of non-digestible fibers of vegetal origin, the production of essential nutrients like vitamins, the prevention of infectious diseases, and a modulatory effect over the immune system, which can be beneficial in pathologies such as IBD.

Lactic acid bacteria (LAB) are a group of Gram-positive commensal bacteria which have been used for food fermentation processes for a long time. Those organisms are present in many fermented foods, such as yogurt, kefir, cheese, bread or fermented meat products. In addition, the probiotic effect of many LAB species is well known. The main probiotic species belong to the genus *Lactobacillus*, *Leuconostoc*, and *Bifidobacteria*.

Nevertheless, the potential health benefits of probiotic administration are often strain specific, and health claims can only be made for strains or species in which the effect has been scientifically demonstrated. Different assays are carried out to evaluate their safety, phenotypic traits and probiotic efficacy. Simple and cost-effective *in vitro* assays

are performed to discard the less effective candidates, and subsequent *in vivo* assays allows to predict the probiotic potential in humans.

The aim of this study is to explore the potentiality of the zebrafish animal model, to assess the probiotic efficacy of LAB strains.

The use of the zebrafish as animal model presents several advantages in comparison to other models due to its small size, external fertilization, embryo transparency and rapid embryonic development. This animal model is extensively used in many biomedical researches, helping to bridge the gap between simple invertebrate models, and more complex mammals. Zebrafish shares a high genetic homology with mammals and presents a similar physiology of the main organs.

To achieve the aim of this work, an initial colonization study was performed to evaluate the suitability of the zebrafish model for evaluating the colonization ability of LAB. The exposure procedure was optimized to ensure the optimal conditions for the digestive colonization. Several recombinant *Lactobacillus* strains, which express a fluorescent protein, were used. Their use, together with the transparency of zebrafish allowed to monitor in real time the evolution of the colonization of the GIT, and the differential effect of the bacterial phenotype over the colonization potential. The results showed that bacteria presenting a ropy, or mucous, phenotype had a reduced colonization ability, comparing to non-ropy strains.

Following, different pro-inflammatory substances were assessed to produce a chemical model of IBD, in order to evaluate the immunomodulatory effect of the LAB strains. TNBS (2,4,6-Trinitrobenzenesulfonic acid) was selected to this end. The immune response to the substance was characterized by different methods. Intestinal mucus overproduction was evaluated by histological staining. The immune cellular recruitment at GIT level was evaluated using a zebrafish transgenic line, which produces fluorescent neutrophils. Subsequently, the transcriptomic response was evaluated analyzing the differential expression of several genes related to the innate immune system (*Il1β, Il10, Il22, CCl20, Myd88, NF-κβ, Tnfα, Tlr1, Tlr2, and Tlr22*). The immunomodulatory influence of probiotic administration in zebrafish larvae was assessed and the validity of the model was confirmed by observing the strong anti-inflammatory effect produced by the *L. acidophilus LA5* strains, a bacterial strain with a well-established probiotic aptitude. When probiotic-pretreated zebrafish larvae were exposed the pro-inflammatory substance, the inflammatory response was lower than in TNBS-only exposed larvae, evidencing an anti-inflammatory effect of the mentioned probiotic strain.

In the same way, the protective effect of probiotics against digestive pathogens was evaluated in the zebrafish larva model. *Vibrio anguillarum* was selected as a digestive pathogen. This bacterial strain was also fluorescently tagged, allowing to observe the evolution of the infection, and the effect of co-exposure with probiotic strains. Results

showed that treatment with *Lactobacillus sakei MN1,* a LAB isolated from meat products, exerted a protective effect, increasing the survival rate after the infection.

Finally, a combination of *in vitro* tests and the above-mentioned *in vivo* assays were carried out. The *in vitro* tests included the evaluation of safety features such as antibiotic resistance and production of biogenic amines, survival ability to digestive conditions and epithelial adhesion. A selection of 20 LAB strains, isolated from various food products, were evaluated using this combination of *in vivo* and *in vitro* tests, and one strain (*L. plantarum 4*) was identified as a potential candidate to be used as probiotic for human administration.

In conclusion, the research performed in the present work demonstrates that zebrafish is an appropriate model for the evaluation of probiotic efficacy of LAB strains. The combination of tests carried out, *in vivo* assays developed in the present work, and *in vitro* evaluation of safety features and adhesion potential, allowed to perform a preliminary screening of the probiotic ability of LAB strains and to identify strains with probiotic effect.

I. Introduction 29

1. Digestive system 31
   1.1. Gut Microbiota 32
   1.2. Digestive Inflammatory Pathologies 34
2. Probiotics 36
   2.1. General description 36
   2.2. Health benefits of probiotic administration 37
   2.3 Screening techniques 41
      2.3.1. In vitro probiotic screening. 41
      2.3.2. Animal Models 43
3. Zebrafish Animal model 45
   3.1. Phylogeny and general features. 45
   3.2. Life Cycle 47
   3.3. Use of the zebrafish as an animal model 48
      3.3.1 The zebrafish as a human disease model 49
      3.3.2. Zebrafish in intestinal immunity and infectious disease research 50
4. Hypothesis and objectives 53
5. Bibliography 54

II. Results 69

Chapter I 71
1. Introduction 73
2. Materials and Methods 75
   2.1. Experimental organisms 75
      2.1.1. Zebrafish breeding 75
      2.1.2. Axenic embryo production 76
      2.1.3. Bacteria strains 76
   2.2. Probiotic administration 78
      2.2.1. Bacterial dose 78
      2.2.2. Probiotic exposure 78
   2.3. In vivo imaging 79
      2.3.1. Wide-field fluorescence 79
      2.3.2. Confocal microscopy 79
      2.3.3. Image analysis 79

2.4. Bacteria load determination 80
2.4.1. rt-qPCR 80
2.4.2. Plate count 81
2.5. Statistical Analysis 81
3. Results 82
3.1. Bacterial dose determination 82
3.2. Colonization of larva GIT 82
3.3. Bacterial load 89
3.3.1. rt-qPCR 89
3.3.2. Plate count 90
4. Discussion 92
5. Conclusions 97
6. References 98
Chapter II 105
1. Introduction 107
2. Materials and Methods 110
2.1. Experimental organism 110
2.2. Bacterial strains and exposure 110
2.3. Toxicological evaluation 110
2.4. Mucus staining 111
2.5. Neutrophil recruitment 111
2.6. rt-qPCR 112
3. Results 114
3.1. Toxicological evaluation 114
3.2. Mucus production 115
3.2 Analysis of neutrophilic inflammation response by live imaging 117
3.3. rt-qPCR 120
4. Discussion 124
5. Conclusions 127
6. References 128
Chapter III 133
1. Introduction 135

2. Materials and Methods 137
2.1. Experimental organism 137
2.1.1. Zebrafish breeding 137
2.1.2. Axenic embryos production 137
2.2. Bacterial strain and exposure 137
2.2.1. Bacterial strains 137
2.2.2. Bacterial exposure 137
2.3. Infection monitorization 138
2.4. Survival rate 139
2.5. Acidic mucin production 139
2.6. qPCR 139
2.7. Probiotic administration 140
2.8. Statistical analysis 140
3. Results 141
3.1. Infection monitorization 141
3.2. Survival rate 143
3.3. Acidic mucin production 144
3.4. rt-qPCR 144
3.5. Probiotic administration 145
3.5.1. Infection monitorization 146
3.5.2. Survival rate 147
3.5.3. Acidic mucin production 148
3.5.4. rt-qPCR 148
4. Discussion 150
5. Conclusions 152
6. References 153
Chapter IV 157
1. Introduction 159
2. Materials and Methods 161
2.1. Lactic acid bacteria strain and media 161
2.2. Safety properties 162
2.2.1. Antimicrobial susceptibility test 162

2.2.2. Biogenic amines 162
2.3. Phenotypic tests 162
2.3.1. Resistance to gastrointestinal Stress. 162
2.3.2. Biofilm production 163
2.3.3. Autoaggregation assay 163
2.4. In vivo Zebrafish assays 163
2.4.1. Zebrafish maintenance and breeding 163
2.4.2. Zebrafish exposure to bacteria 164
2.4.3. Vibrio anguillarum challenge test 164
2.4.4. Chemical induction of enterocolitis 164
2.4.5. Gene expression analysis by rt-qPCR. 164
2.4.6. Statistical Analysis 165
3. Results 166
3.1. Safety properties 166
3.2. Phenotypic tests 168
3.2.1. Resistance to gastrointestinal stress 168
3.2.2. Biofilm production 169
3.2.3. Autoaggregation assay 170
3.2.4. Strain selection 171
3.3. In vivo assays 173
3.3.1. Protective effect of probiotic candidates against VAN infection 173
3.3.2. Immunomodulatory effect of probiotic candidates in a chemical enterocolitis model 173
4. Discussion 175
5. Conclusions 178
6. References 179

III. Conclusions 187
1. Conclusions 189
2. General conclusion 190
3. Future research 191

I. INTRODUCTION

1. Digestive system

Human beings, like any other heterotroph, need to obtain their energy and carbon from the surrounding media. To do so, humans need to consume other organisms, i.e. animals or vegetables. However, it is essential for us to protect ourselves from harmful elements (either chemical or biological). To this end, evolution has shaped a highly complex digestive system that is able to process and assimilate nutrients from food while providing mechanisms for detecting and fighting harmful elements.

The digestive system is divided into the gastrointestinal tract (GIT), which is composed of the mouth, esophagus, stomach and intestines, and the accessory organs of digestion (salivary glands, pancreas, liver, and gall bladder), which provide secretions necessary for digestion[1] (Figure 1). Since the GIT is continually exposed to microorganisms, the immune system should always function in the GIT. The gut-associated lymphoid tissue (GALT) represents almost 70% of the entire immune system[2]. There is a dynamic interplay between the GALT and the gastrointestinal function. For example, the intestinal permeability may be modified in response to specific immune signals, and in the same way, the immune response can be orientated towards the luminal content, thus producing a tolerance or "fighting" response to antigens. Nevertheless, an excessive response can provoke damage to the intestinal mucosa, e.g., in the case of allergies or autoimmune inflammatory pathologies.

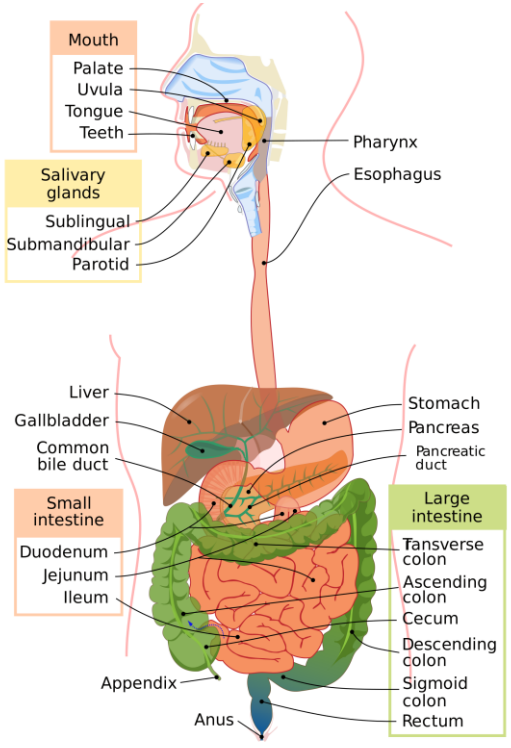

Figure 1. The human digestive system. (Wikimedia commons)

1.1. Gut Microbiota

The digestive system has evolved in the bacterial world, as a matter of fact coevolution occurs between bacteria and superior organisms[3]. Multicellular organisms can be considered as meta-organisms composed of a macroscopic host and its symbiotic microbiota. With an estimated composition of 100 trillion cells, human symbionts outnumber host cells by at least a factor of 10 and express at least 100-fold more unique genes than their host's genome[4]. Some microorganisms developed a symbiotic strategy by cooperating with their host, which provides them with a stable and nutrient-rich ecosystem. In return, they offer enzymes to process non-digestible carbohydrates, provide essential nutrients, and contribute to the defense against pathogenic microbes[5]. Besides, the immune system must distinguish between beneficial and pathogenic microorganisms. Discriminating between these organisms is accomplished by recognizing specific molecular sequences and subsequently delivering a pro- or anti-inflammatory response according to the nature of the identified antigen.

The inner surface of the GIT has an estimated surface of 200 m^2. This surface is densely populated by bacteria, archaea, and microbial eukaryotes, which form the gut microbiota[6]. This microorganism community represent one of the more crowded ecosystems on the planet, due to relatively stable conditions and continuous supply of nutrients in the GIT. Gut microbiota is a complex system formed by several species of bacteria; microbial eukaryotes, such as protozoa, yeast, and fungi; as well as viruses. Nevertheless, the major proportion of the species belongs to the bacteria domain. The majority of the species is strict anaerobes and belongs to two phyla: *Bacteroidetes* and *Firmicutes*. Nonetheless, species from the phyla *Proteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Fusobacteria*, and *Cyanobacteria* are also present [7]. The total estimated number of species present in gut microbiota differs among different studies, and it ranges between 500 and 1500 species. Nevertheless, recent studies suggest that the collective human gut microbiota may be composed of more than 35000 species[6] (Figure 2).

Environmental conditions vary markedly among different sections of the GIT, and so do the density and composition of the microbiota. The stomach harbors a relatively low bacterial density (10^1–10^3 bacteria per mL) due to its extreme pH conditions. The main bacterial groups are *Lactobacillus, Veillonella* and *Helicobacter.* The number of bacteria increases gradually through the duodenum, jejunum and ileum, with a predominance of *Bacteroides, Bifidobacterium, Streptococcus*, Enterobacteriaceae, and *Ruminococcus.* The distal colon harbors the maximum bacterial density, with up to 10^{12} bacteria per mL. The microbiota composition also varies transversally through the intestinal section. The section proximal to the epithelium and the adjacent thick mucus layer is almost sterile[8]. The bacterial density increases through the luminal space, and the outer mucus has a high bacterial density[6,8].

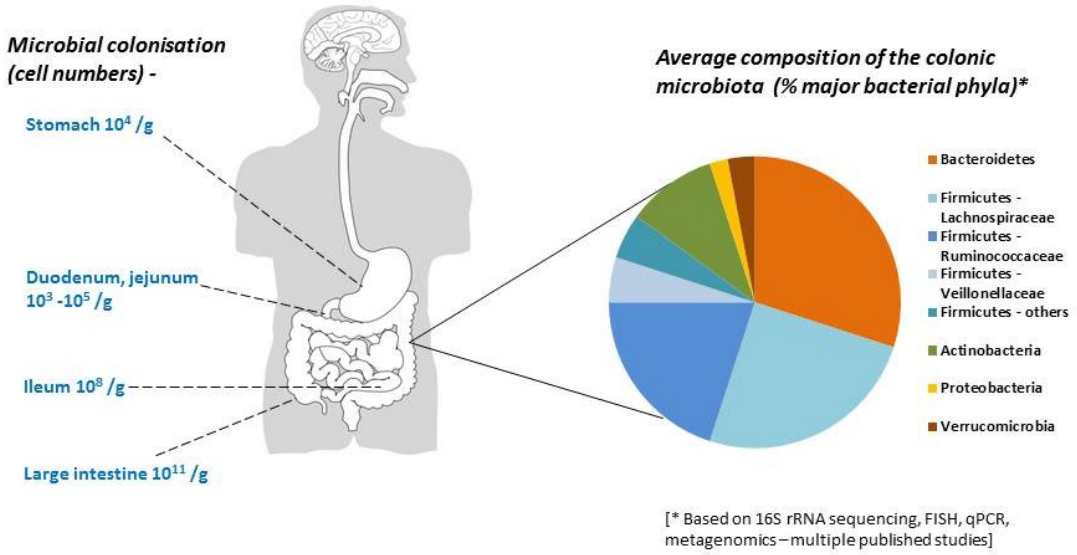

Figure 2: Digestive microbiota composition in healthy adults. (Wikimedia commons)

The microbiota is almost considered as an additional organ, for its valuable contributions to the host's health and physiology[9]. The microbiota acts as the first barrier of physical defense by occupying the ecological niches present on the GIT and impairing the adhesion of pathogenic bacteria[10]. Gut microbiota also provides the host with numerous nutritional functions, such as the digestion of complex polysaccharides found in plants or the production of essential amino acids and vitamins[11]. In addition, recent studies have suggested an essential role of the microbiota in the development of the immune system. Several studies conducted on germ-free animal models have demonstrated that the absence of microbiota leads to immune deficiencies, such as the incomplete development of the humoral immune system, reduction in lymphocyte count and of GALT size[12,13]. In addition, the presence of microbiota promotes the development of the GI vasculature. Research by Stappenbeck et al. demonstrated that adult germ-free rodents presented an immature intestinal vascular development, which could be developed with the addition of normal microbiota[14].

Gut microbiota can also play an important role in the pathogenesis of various diseases. Recent genetic studies have revealed that the contribution of host genetic factors in various pathologies is usually less than 50%, thus indicating the role of environmental factors[15]. This fact is confirmed by the important increase in the incidence of several diseases over the last decades, mostly in industrialized nations. The current Western diet, which is rich in red meat, dairy products, salt, and processed and artificially sweetened foods but with reduced presence of fruits, vegetables, fish, legumes, and whole grains, has a profound impact on the digestive microbiota composition[16]. Furthermore, this diet and the alteration it produces in the microbiota has been

associated with chronic inflammatory states[17] that are related to the onset of pathologies such as diabetes, atherosclerosis, colon cancer and inflammatory bowel disease[18].

1.2. Digestive Inflammatory Pathologies

Inflammatory bowel disease (IBD) is a group of pathologies characterized by inflammation, ulceration, and stenosis of the GIT. The main symptoms are abdominal pain, diarrhea, and bleeding. Transmural inflammation is observed on the affected intestinal epithelium, indicating lymphoid hyperplasia, submucosal edema, ulcerative lesions and fibrosis[19]. The disease can be classified as Ulcerative Colitis (UC) or Chron´s Disease (CD), depending on the exact symptoms. Ulcerative Colitis produces a continual mucosal inflammation through the extension on the colon, whereas in CD, inflammatory injuries can be located in any zone of the GIT. The pathology of CD is mediated by a T helper Th_1 cell response, which produces proinflammatory cytokines such as tumor necrosis factor (TNFα) and Interferon γ[19]. The pathology of UC is characterized by a Th_2 response[20].

IBD is caused by a combination of genetic and environmental factors. Recent research suggests that microbiota plays a major role in the onset of IBD (Figure 3). Defects in the symbiotic relationship between the digestive microbial community and the mucosal immune system are thought to be the main cause of chronic intestinal inflammation[21]. Dysbiosis and decreased complexity of the gut microbiota are usually observed in IBD patients[22], who also exhibit an increased production of IgG and hyper-reactivity of T-cells against commensal bacteria[23,24]. Studies of cultures of intestinal mucosa samples of IBD patients in the presence of non-pathogenic *E. coli* have reported an increase in the production of proinflammatory cytokines (TNFα, IFN-γ, Il_6, Il_{23}, Il_{12}, and Il_7) and chemokines (Il_8, $CXCL_1$ and $CXCL_2$), which leads to the activation of the inflammatory cascade[25,26]. This inflammatory process elicits matrix metalloproteins' production, causing matrix disorganization, loss of epithelial union and ulceration of the tissue[27]. Administration of broad-spectrum antibiotic therapy reduces the mucosal inflammation. These observations suggest that in such conditions the physiological tolerance towards commensal bacteria is lost[21].

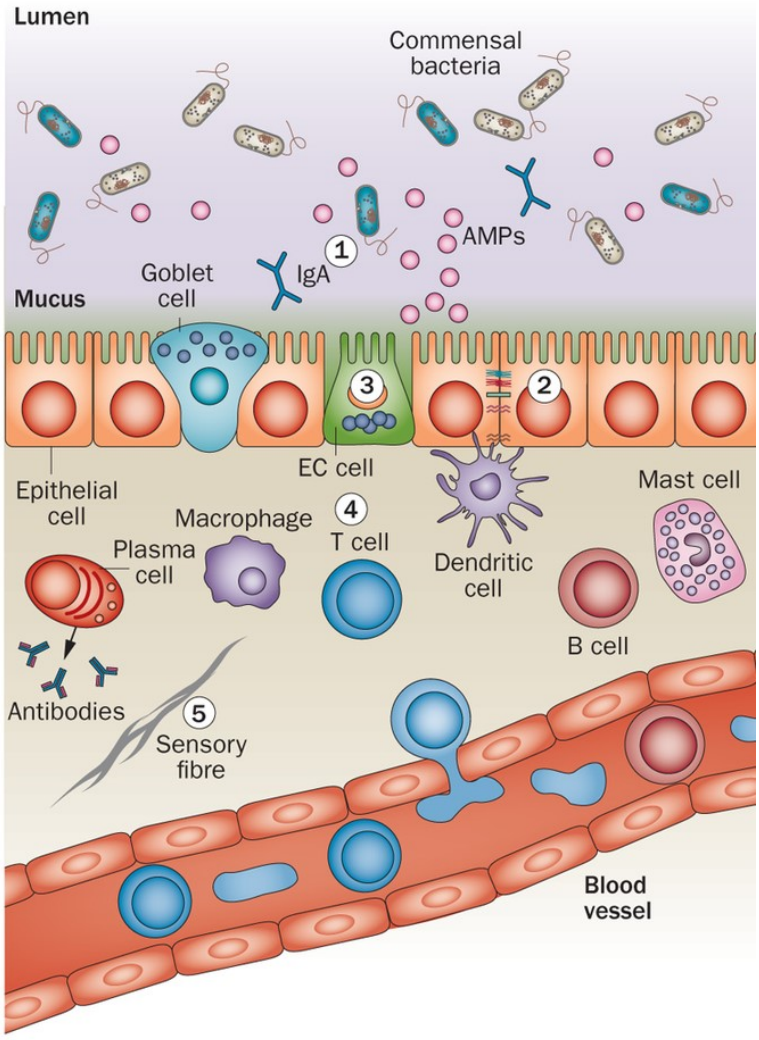

Figure 3: The role of microbiota on the etiopathology of IBD. 1. An alteration in the microbiota composition can lead to the loss of epithelial junction (2). The translocated bacteria trigger an inflammatory response (3, 4). Additionally, endocrine activity and hyper-stimulated sensory fibers in response to stress can also influence the disruption of epithelial continuity (5) (Öhman 2015).

Several studies have demonstrated the important role of commensal bacteria in maintaining the inflammatory response at physiological levels[28]. A study conducted on mice demonstrated that some *Clostridium* species induce the expansion of T_{reg} cells[29,30], whereas others may inhibit the activation of the NF-κβ cellular mediator, thus decreasing the production of proinflammatory cytokines[31]. In addition, several LAB strains are able to downregulate the expression of proinflammatory cytokines and chemokines in a host with an activated inflammatory state[25,26,32]. Moreover, some LAB are able to upregulate the production of anti-inflammatory cytokine IL-10[33–35] and thus contribute to the mitigation of inflammatory reactions

2. Probiotics

2.1. General description

According to World Health Organisation, probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host"[36]. Many probiotic strains are commercially available as functional food or nutraceutical preparation.

The most used probiotic strains belong to the heterogenous group of lactic-acid bacteria (LAB). The main representative genus are Lactobacillus *(L. acidophilus, L. casei, L. plantarum, L. reuteri, L. rhamnosus, and L. salivarious),* Leuconostoc *(L. mesenteroides),* Bifidobacteria *(B. breve, B. longum, and B. lactis*), Pediococcus (*P. acidilactici*), Streptococcus (*S. thermophilus*) and Enterococcus (*E. faecium)*[37] (Figure 4). Lactic acid bacteria are aerotolerant Gram-positive bacteria, non-sporing cocci and rods that produce lactic acid as a result of carbohydrates fermentation. They are classified as homofermentative when the main fermentation product of sugar is lactic acid or heterofermentative when they also produce ethanol, acetic acid and CO_2.

Lactic acid bacteria have been used for food fermentation for a long time, to increase nutritional value and enhance storage time of dairy products such as yogurt, kefir, cheese, and bread as well as meat products such as fermented sausages[38–42]. Many LAB species are commensals in the human digestive microbiota and have been associated with health benefits.

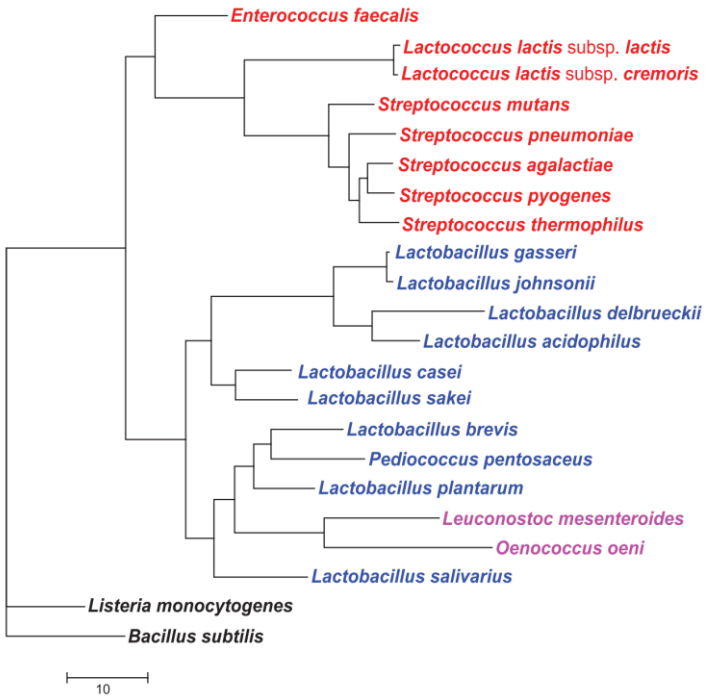

Figure 4: Phylogenetic tree of *Lactobacillales* constructed from concatenated alignments of four subunits of the DNA-dependent RNA polymerase (Makarova 2007).

Other microorganisms, such as B*acillus subtilis* or the yeast *Saccharomyces boulardii*, are also used in powder or capsule form for nutraceutical preparations[43]. *Saccharomyces boulardii* administration has proven to have a beneficial effect on antibiotic-associated diarrhea, travelers' diarrhea, and IBD[43]. In addition, the natural resistance of yeast to antibiotic therapy makes this organism suitable for patients undergoing antibiotic treatment.

2.2. Health benefits of probiotic administration

Probiotic microorganisms may confer several health benefits to the host (Figure 5). Those effects are the result of three modes of action[44]: i) Immune modulation. Probiotics can exert their modulating effect on the host's defenses, i.e. both the innate and adaptive immune system. This property might be helpful in the prevention and therapy of infectious diseases and the treatment of inflammatory diseases[45]. ii) Microbiota modulation. Probiotics can have a direct influence on other components of the microbiota, either commensals or pathogens. Thus, they can be beneficial in dysbiosis or infectious diseases[46]. iii) Compounds transformation. Probiotics may modify molecular products produced by other microorganisms, i.e. toxins, produced by the host, i.e. bile salts, or from food origin, *i.e*. indigestible fibers[47].

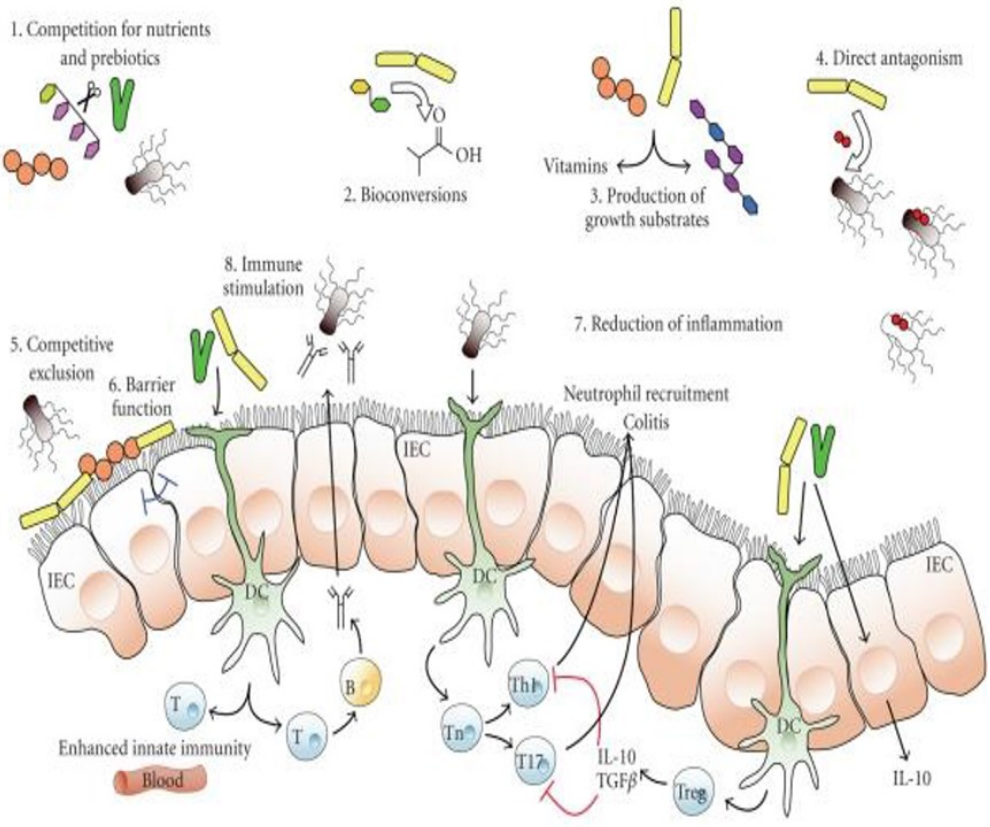

Figure 5: Schematic diagram of probiotic mechanisms of action (Khalighi 2016).

i) Immune modulation.

The immunomodulatory effect of probiotic microorganisms over the host is mediated by specific components of the cell, such as metabolites, DNA fragments or cell wall components. Host epithelial and mucosal immune-related cells present surface receptors, which specifically recognize microbe-associated molecular pattern (MAMP) receptors[48]. The activation of receptors triggers a signaling cascade that leads to immune response. In physiological conditions, probiotics and other commensal bacteria do not adhere to the epithelium surface but rather colonize the outer mucus layer of the epithelium[49]. Nevertheless, host immune cells can be in direct contact with bacterial cells in different ways. M cells absorb antigens from the luminal space and transfer them to dendritic cells, where they are processed[50]. Dendritic cells are also able to directly phagocyte bacterial cells or antigens[51]. Brat el al. observed in an *ex vivo* study that when DC derived from human monocytes matured in the presence of *L. rhamnosus,* they were able to modulate naïve CD4+ T cells, thus resulting in a decrease of proinflammatory cytokine production[52]. Components of bacterial cell wall may also produce an immunomodulatory effect through TLR_2 receptors on the host. Grangette et *al.* produced a mutant *L. plantarum* strain, expressing a modified teichoic-acid structure on the cell wall[53]. When this mutant strain was administered to murine models, an important reduction of proinflammatory cytokine production as well as an increase of anti-inflammatory cytokine Il_{10} in monocytes and peripheric blood mononuclear cells was observed.

Another important mechanism of immunomodulation by probiotics is their influence on the signal transduction system. Some strains produce a reduction of the Iκβ inhibitor by reducing the ubiquitination of this mediator. This effect is achieved by modifying the proteasome function or influencing RelA localization through the γ-dependent signal cascade[54,55]. In the same way, probiotics have a positive effect on enhancing the epithelial barrier integrity. Likewise, probiotics can increase the production of occluding proteins located between epithelial cells[56] as well as defensins and cryptidins on Paneth cells[57].

Some studies reported a direct anti-inflammatory effect of DNA molecules from probiotic bacteria, mediated by TLR_9 receptors[58]. Also, a study evidenced an induction of the proliferation of regulatory T cells (T_{reg}) when probiotics were applied subcutaneously, with a promising effect in arthritis treatment[59]. Finally, a placebo-controlled cross-over trial exhibited the effect of *L. casei* Shirota on natural killer cells (NK) in humans. This effect was mediated by the induction of IL_{12} cytokines[58].

ii) Microbiota modulation

Probiotic administration can modify the composition and metabolic state of microbiota components through different mechanisms:

Competition for resources. One of the most limited resources in the digestive ecosystem is iron. This element is essential for the majority of bacteria, and species such as *L. acidophilus* and *L. delbrueckii* are able to absorb ferric hydroxide from the luminal space, thus making it unavailable to other microorganisms[60]. Similarly, the probiotic *E. coli* Nissle 1917 strain is able to chelate present iron by secreting siderophores to assimilate it[61].

Inhibition of pathogen adhesion to the intestinal epithelium. Probiotic bacteria are able to adhere to mucin-related structures and even increase MUC_3-mucin production, as revealed by Mark et *al.*[62]. An inhibitory effect against adhesion of pathogenic *Salmonella, Clostridium* and *E. coli* strains has been observed in the presence of *B. brevis* Bb12 and *L. rhamnosus* LGG on pig intestinal models[63]. The adhesion of probiotic cells to human mucus is mediated by surface adhesines present in *Lactobacillus*, such as *Mub* protein of *L. reuteri* 1063[64]. Other mechanisms, such as biofilm formation, degradation of carbohydrate receptors, and biosurfactant production may also be involved in this process[44].

Production of antimicrobial substances. Probiotic strains secrete diverse substances with an inhibitory effect on the growth of pathogenic organisms. The main produced components are lactic acid, hydrogen peroxide, low-molecular-weight bacteriocins (LMWB) and high-molecular-weight bacteriocins (HMWB) (Figure 6). Low-molecular-weight bacteriocins are antimicrobial proteins that can be divided in three classes; class I antibiotics, class II heat-stable non-cyclic, and class III cyclic antimicrobial peptides[65]. High-molecular-weight bacteriocins, on the other hand can be divided into two groups: contractile phage tail-like (R-type) and flexible non-contractile type[66]. Broad spectrum class II *Abp118* bacteriocins, produced by *L. salivarius* UCC118, for example, have exhibited a protective effect against *Listeria monocytogenes* in rodent models[67]. The influence of *Abp118* on the inhibitory process was confirmed by the absence of effect observed when Abp118-defective mutants were administered under the same conditions. The *L. reuteri* ATCC55730 LAB strain produces the antibiotic reuterin (3-Hydroxypropanal), which is a broad-spectrum antibiotic active against bacteria, yeast, fungi, protozoa, and viruses[68].

iii) Compound transformation

Antitoxin effect. The antitoxin effect of probiotics is evidenced by their beneficial effect on toxin-mediated diarrhea. *Bacillus breve* Yakult and *B. pseudocatenulatum* DSM20439 inhibit the expression of the Shiga toxin in *E. coli O157:H7* pathogenic strain, both in *in vitro* and *in vivo* rodent models[69]. Likewise, the probiotic yeast *S. boulardii* exhibited a protective effect against Toxin A from *Clostridium difficile* in cell-culture assays[70]. In addition, the aforementioned yeast elicits an anti-Toxin A specific IgA secretion by the host[71]. Finally, some probiotic strains present a protective effect against fungal toxins. *Lactobacillus rhamnosus* strain LC-705 is able to bind to aflatoxins and other mycotoxins

by modulating its intestinal absorption and increasing fecal excretion, thus reducing toxicity in the host[72].

Classification/features	Bacteriocins	Molecular weight (Da)	Producing strain	References
CLASS I				
The bacteriocins are post-translationally modified, linear or globular peptides containing lanthionine, β-methyl lanthionine and dehydrated amino acids	Nisin A	3352	*Lactococcus lactis* subsp. *lactic*	Field et al., 2012
	Nisin U	3029	*Streptococcus uberis*	Wirawan et al., 2006
	Nisin Z	3493	*Lactococcus lactis* subsp. *lactic*	Mulders et al., 1991
	Mersacidin	1824	*Bacillus* sp. *Y85,54728*	Chatterjee et al., 1992
	Labyrinthopeptin A2	1922	*Actinomadura* sp.	Meindl et al., 2010
	subtilosin A	3399	*Bacillus subtilis* 168	Babasaki et al., 1985
CLASS II				
Heat stable, unmodified, non-lanthionine-containing bacteriocins, heterogeneous class of small peptides				
Class IIa (pediocin PA-1like bacteriocins)	pediocin PA-1	4629	*Pediococcus acidilactici* PAC-1.0	Henderson et al., 1992
	carnobacteriocin X	3602	*Carnobacterium maltaromaticum* C2	Tulini et al., 2014
Class IIb (composed of two peptides)	lactacin F	4755	*Lactobacillus* spp.	Fremaux et al., 1993
	ABP-118	4096	*Lactobacillus salivarius* subsp. *salivarius* UCC118	Flynn et al., 2002
Class IIc (circular peptide)	carnocyclin A	5862	*Carnobacterium maltaromaticum* UAL307	Martin-Visscher et al., 2008
	enterocin AS-48	7149	*Enterococcus faecalis*	Samyn et al., 1994
Class IId (linear, non-pediocin like, single-peptide)	epidermicin NI01	6074	*Staphylococcus epidermidis*	Sandiford and Upton, 2012
	lactococcin A	5778	*Lactococcus lactis* subsp. *Cremoris*	Holo et al., 1991
CLASS III				
Large, heat unstable proteins	Caseicin 80	~42000	*Lactobacillus casei* B80	Muller and Radler, 1993
	Enterolisin A	34501	*Enterococcus faecalis* LMG 2333	Nilsen et al., 2003
	Helveticin J	37511	*Lactobacillus helveticus* 481	Joerger and Klaenhammer, 1990

Figure 6: Classification of bacteriocins produced by LAB.

Bile salt deconjugation. A number of bile salt hydrolase enzymes (BSH) have been identified and characterized in several probiotic strains[73]. The BSH activity confers probiotic bacteria with a competitive benefit since the amino acids released by deconjugation can be used as a carbon and energy source[74] and to decrease the bile salt's toxicity[75]. Thus, the GIT persistence of the probiotic bacteria is increased. This trait can also exert a beneficial cholesterol-lowering effect on the host since deconjugated bile salts are less efficiently reabsorbed than their conjugated counterparts, thus resulting in the excretion of a larger quantity of bile acids in feces[76].

Prebiotic fibers. Prebiotics are "selectively fermented ingredients that allow specific changes in both the composition and activity of the digestive microbiota that confer health benefits to the host's health"[77]. Prebiotics are generally non-digestible fiber compounds of vegetable origin that pass undigested through the upper part of the GIT and are fermented by bacteria on the large bowel[62]. The fermentation of these compounds by probiotic bacteria produces butyric acid and other short-chain fatty acids

(SCFAs), which serve as an energy source for colonocytes and contributes to increasing the barrier integrity[78].

2.3 Screening techniques

The potential health benefits of probiotic administration are often strain specific, and health claims can only be made for strains or species in which the effect has been scientifically demonstrated[79]. In addition, most of the species used as probiotics are apparently safe, although some microorganisms could be potentially harmful. Several cases of opportunistic pathogen infection have been reported for the enterococci *E. faecium*, causing endocarditis and bacteremia as well as intra-abdominal, urinary-tract and central-nervous-system infections in the hospital environment[80]. Similarly, some species may harbor transmissible antibiotic-resistance determinants, thus increasing the risk of infection persistence by other pathogens[81].

Therefore, potential probiotic candidates should undergo a series of *in vitro* and *in vivo* assays aimed at evaluating their safety and phenotypic traits as well as assessing their influence on host functions, such as immune response or metabolic functions[82]. Different animal models should be used to this end. Finally, the most promising strains should be evaluated in clinical trials to confirm their beneficial effects on human health.

2.3.1. *In vitro* probiotic screening.

In vitro screening techniques are the first choice in probiotic selection, due to their simplicity and low cost.

Safety. The European legislation specifies microorganisms that are safe for use in food[83]. Nevertheless, additional safety aspects have to be evaluated prior to the commercialization of new probiotic strains[84]. Those aspects include the assessment of antibiotic resistance, production of biogenic amines (BAs) and the expression of enzymes or toxins with a pathogenic potential (i.e. glycosidases, proteases, gelatinases or cytolysins). The European Food Safety Authority (EFSA) details a procedure to evaluate the minimum inhibitory concentration (MIC) for the most used antibiotics[85]. Furthermore, microdilution assays performed on 96-well plates[38] and commercial kits are also available[86]. Biogenic amines are produced by decarboxylation of amino acids and may present a hazard for the host's health [87]. *In vitro* assays can be performed to evaluate the BA production capacity of probiotic strains. Growth test on solid media, containing amino-acid substrates and pH-shifting indicators, are carried out to screen for decarboxylase activity[88]. The quantification of BA production can be performed by chromatographic techniques[89]. Finally, the screening of bacterial genome for the presence of antibiotic resistance or BA metabolism genes is an efficient method of predicting these safety aspects[90,91].

Survival. To exert their beneficial effect on the host, probiotics must remain viable and survive under specific conditions found in the GIT. Different stress tolerance assays can be carried out to assess their ability to survive in those conditions. After being ingested, probiotic bacteria must face stressful conditions in the stomach. In this compartment, pH can vary between 1 and 5, and several digestive enzymes are secreted. In the same manner, the passage to the duodenum exposes the bacteria to high pH levels, pancreatic enzymes, and bile salts. Simple assays may be performed that expose bacteria to extreme pH conditions in different tests[92]. A more elaborate approach includes the use of artificial gastric and intestinal juices to better represent *in vivo* conditions. Those synthetic juices are composed of adjusted pH and osmolarity as well as pepsin for the gastric juice and pancreatin and bile salts for the intestinal juice. A procedure comprising sequential exposure to gastric juice followed by exposure to intestinal juice produces a more precise representation of physiologic conditions[93]. Furthermore, several GIT simulators have been developed over the last years. Multicompartmental dynamic models that simulate the oral cavity, stomach and small intestine are available to quantify the survival rate of probiotic candidates[94]. Lately, a complete GIT simulator has been developed[95]. The SHIME system consists of five reactors that sequentially simulate the stomach, small intestine and the three regions of the large intestine. The computer-controlled environmental parameters not only allow for simulating the physicochemical conditions but also harbor a complex and stable microbial community that mimics the different microbiota compositions present in the different regions of the human colon. A mucus layer is also present in the colon compartment for the colonization of microbial community. This model is used for the evaluation of LAB strains and to study the biotransformation of pharmaceutical compounds and the food-metabolization process.

Colonization. One of the most important traits of a probiotic strain is its colonization ability to the GIT. Adhesion to mucus and the epithelial cell is, nevertheless, a debated matter in probiotic evaluation. The ability of a probiotic strain to make this adhesion increases the probiotic's colonization potential; however, this ability may be considered to be a risk for immune-depressed patient, since it may increase the probiotic's translocation capacity and facilitate the infection process[96]. Initially, cell-surface hydrophobicity was considered to be an indicator of the adhesion capacity to epithelial cells[97]. A number of tests were developed to evaluate the partition rate in hydrophobic/water mixtures (i.e. hexadecane, xylene or toluene)[98]. However, controversial results have made this approach obsolete[99]. Several studies indicated that the autoaggregation capacity of LAB is correlated to their adhesion capacity to epithelial cells[100]. In that sense, self-aggregation tests are performed to measure the absorbance of resting bacterial suspensions in specific conditions[101]. A good correlation is observed between autoaggregation assays and adhesion to epithelial cells[102]. A more complete approach is based on the use of intestinal epithelial cell lines to evaluate the colonization ability. Several epithelial cell lines are available (i.e. HT-29, HT29MTX and Caco2) for this purpose[103]. Nonetheless, these assays are technically difficult and have low

reproducibility due to the use of different variants of cell lines, and the results are controversial for the use of cancer cells, with very dissimilar surface properties[104]. The use of whole-tissue models, including epithelial tissue with a mucus layer that harbors commensal microbiota, is a promising line of work in the *in vitro* evaluation of probiotic colonization ability[49].

Antimicrobial assays. Antimicrobial activity of probiotic strains is related to the production of organic acids and specific antimicrobial compounds as well as the competitive exclusion of pathogens from binding sites[82]. The ability to produce antimicrobial compounds is usually evaluated by means of a simple inhibition test on solid media. The agar-spot test[105], paper-disk diffusion[106], and well-diffusion assay[107] are commonly used for the evaluation of antimicrobial activity against the most common digestive pathogens, such as *E. coli, Salmonella, Campylobacter* and *Listeria*. Assays for the evaluation of the inhibitory effect of probiotic-culture filtrates are also performed to assess the effect of secreted bacteriocins and other antimicrobial compounds[108]. The competitive exclusion of pathogens by probiotics can be evaluated using the *in vitro* adhesion assays detailed above. The coaggregation assays between different bacterial strains are of interest in this subject. Radiolabeling and fluorescence detection are often used in such assays[63].

2.3.2. Animal Models

Preferably, the selection of probiotic candidates should be performed in human clinical trials, with healthy population, and specific groups with targeted pathologies[109]. However, important ethical and economic considerations certainly restrain this approach; as a replacement, several *in vivo* models are available to further refine probiotic candidate selection, to carry out final human clinical trials.

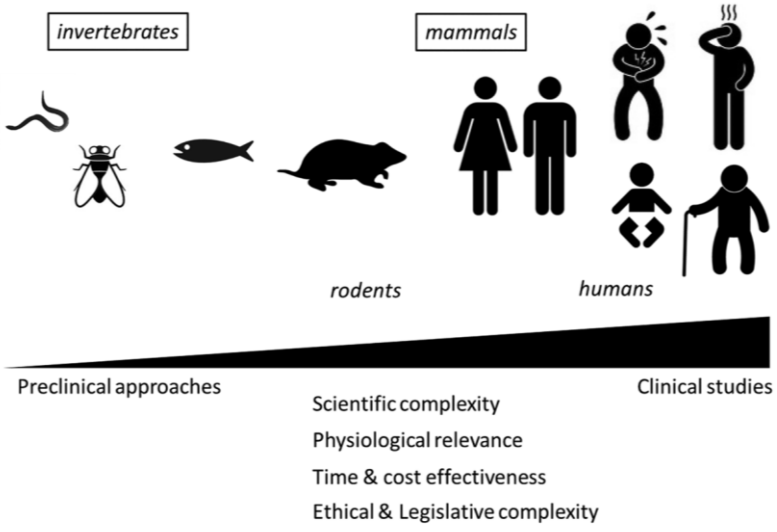

Figure 7: Schematic view representing the progressive complexity of *in vivo* models (Papadimitriou et *al.* 2015).

Animal-model testing is aimed at proving the safety of the evaluated therapy and helping to demonstrate the beneficial effect of a specific health-promotion claim[96]. An ideal model should comply with the following considerations. The model should be scientifically validated to confirm that the studied mechanisms are shared between the animal model and humans (Figure 7). The information provided could not be obtained by *in vitro* assays, and the procedures should minimize animal suffering by reducing the number of organisms used and improving the experimental conditions, to adhere to 3R guidelines[110].

Simple animals, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, are cost-effective and ethically acceptable solutions, even if their complexity is distant from mammalian physiology. *Drosohpila melanogaster* has been usefully used to study the influence of probiotics on main metabolic signaling pathways related to innate immunity[111]. Studies on *C. elegans* demonstrated the antioxidative and lifespan-extending effect of *Lactobacilli*[112,113] and their antitumoral activity[114].

In an intermediate level of complexity, zebrafish is increasingly used as a human-disease model[115]. The fitness of this vertebrate model for high-throughput screening has encouraged its use as a drug-discovery model[116]. In addition, recent works have suggested that the zebrafish may be useful for evaluating probiotic activity. Several studies have demonstrated a beneficial effect of probiotic administration on zebrafish's innate immune response[117,118], hepatic stress tolerance[119], and reproduction[120,121]. Models of IBD on zebrafish have also been developed, which can be useful for the immunomodulatory evaluation of probiotic strains[122].

Rodents are the most used models in biomedical research, and the same can be stated for probiotic evaluation[123]. Several colitis models are available on rodents[46]. Chemical models, using pro-inflammatory substances, are the first choice for evaluating the anti-inflammatory effect of probiotics[40]. The characteristics of those substances and their use on zebrafish are discussed later. Besides, knock-out (KO) rodent models are also available. Knock-out Nod_1/Nod_2 mutants are useful to model CD, even if the lack of those receptors limits the study of bacteria producing a immunomodulatory effect over this signaling route[124]. Similarly, other KO rodent models such as Il_{10} and Tlr-deficient models are useful for the evaluation of specific mechanisms of action.

The mouse-infection model with *Citrobacter rodentium* is extensively used due to the inflammatory response it elicits[125]. This immune response, which depends on the mouse strain, shares a high similarity to human enteropathogenic *E. coli* infection. Studies performed on this model demonstrated a protective effect of some probiotic *Lactobacilli* and *Bifibacterium* strains[126,127]. Other human infection models on rodents require the administration of broad-range antibiotics, thus hampering the evaluation of bacterial probiotics[128]. Thus, *Clostridium difficile* and *Salmonella Typhimurium*[129] infection models are suitable models to study the probiotic aptitude of yeast, such as *S. boulardii*[130].

3. Zebrafish Animal model

3.1. Phylogeny and general features.

Zebrafish (*Danio rerio)* is a small subtropical freshwater fish that belongs to the *Cyprinidae* family, of the order of *Cypriniformes*. The former scientific name was *Brachydanio rerio*, but it was changed to *Danio rerio* in 1981. Zebrafish are indigenous from South Asia and can be found in India, Pakistan, Nepal, Myanmar and Bangladesh[131]. Their natural habitat is located in water ponds, rice fields, and rivers of slow-moving waters, with slightly alkaline (pH 8) and clear water[132]. This species is generally found in shoals of between 5 and 20 individuals in slow-moving or standing waters and are generally located in sections with aquatic vegetation. Given that tropical areas are subject to severe climate fluctuations due to the monsoon season, zebrafish presents high adaptability to fluctuations of water physicochemical conditions. Zebrafish can tolerate a temperature range of 15–35 °C, albeit its optimal temperature is around 28 °C[133]. Likewise, it can survive in water with pH values between 5.5 and 9 and tolerate salinity values between 10 to 1000 µS[134].

Zebrafish can indeed tolerate significant variations in water quality in nature; however, the physicochemical properties of the water in laboratory conditions should be maintained within an ideal range to the ensure its optimal physiological conditions and guarantee the reproducibility of experimental results (Table 2).

Variable	Tolerated	Ideal
Temperature (°C)	6.7–41.7	26–28
Hardness (Ca^{2+}, mg L^{-1})	75–200	100
Acidity (pH)	5.5–9	7–8
Salinity (NaCl, ppt)	0.1–14	0.25–0.75
Ammonia (NH_4^+, mg L^{-1})	0–0.05	0.02
Nitrite (NO_2^-, mg L^{-1})	0–0.5	0.2
Nitrate (NO_3^-, mg L^{-1})	15–200	50
Phosphate (PO_4^{3-}, mg L^{-1})	150–300	250
Dissolved oxygen at 28 °C (mg L^{-1})	5.5–8	7.5
Conductivity (µS)	200–4000	750–1500
Alkalinity (carbonate and borate specimens, mg L^{-1})	60–120	80
Copper (Cu, pg mL^{-1})	0–100	0–100
Chloride (Cl^-, mg L^{-1})	<0.1	<0.1

Table 2: Tolerated and optimal physicochemical properties of rearing tank water for zebrafish (Ribas 2014).

An adult zebrafish measures approximatively 4 cm in length, and its weight ranges between 0.5 and 0.9 g[135] . Its body presents a laterally compressed and fusiform shape with five horizontal blue stripes on the side. The species exhibits a sexual dimorphism[136]. The two sexes can be distinguished based on differences in body size, shape, and pigmentation. Males are usually smaller and have a more elongated body with gold stripes, whereas females are larger, have a larger abdomen with silver stripes and a small genital papilla in front of the anal fin[137]. These differences are difficult to visualize in young fish but are more visible when the animals reach adulthood. The fish reaches sexual maturity within 3 months, and its lifespan in captivity is about three years[138]. Zebrafish are omnivorous, with a varied diet of benthic and planktonic crustaceans as well as worms and insect larvae[134]. Although the optimal nutritional requirement of zebrafish is not fully defined, current feeding practices on zebrafish-husbandry facilities involve a diet based on rotifers and dried-food pellets used for other freshwater aquarium species. Fulfillment of nutritional requirements is essential for the fish's survival during the larval stage. It is necessary to provide food several times a day between 6 and 15 days to ensure proper development and avoid major survival reduction[139]. During this stage, the diet is based on *Artemia Nauplii* and dried food. Later, during the juvenile stage, the food supply interval can be extended to 1–3 takes a day. Adult zebrafish seek food in the water column, but they are also able to feed on the surface (Figure 8). Unlike those in natural conditions, laboratory-reared zebrafish do no feed on the bottom of the tanks, so excessive food accumulation at the bottom can lead to water contamination[140].

Figure 8: Zebrafish breeding facility at AZTI.

3.2. Life Cycle

Under laboratory conditions, zebrafish embryonic development is very rapid. Hatching occurs at 2.5–3 days post-fertilization (dpf). Embryos exhibit a low level of activity until around 5 dpf, when their swim bladder is inflated and they start to actively seek for food. Until then, embryos subsist on yolk-sac reserves[141]. As soon as 24 hours post-fertilization (hpf), the neural tube is formed, and a vascular system as well as a beating heart is visible. Pigmentation is present and muscular contraction begins. At 120 hpf, the fish's major organs are already functional[142] (Figure 9).

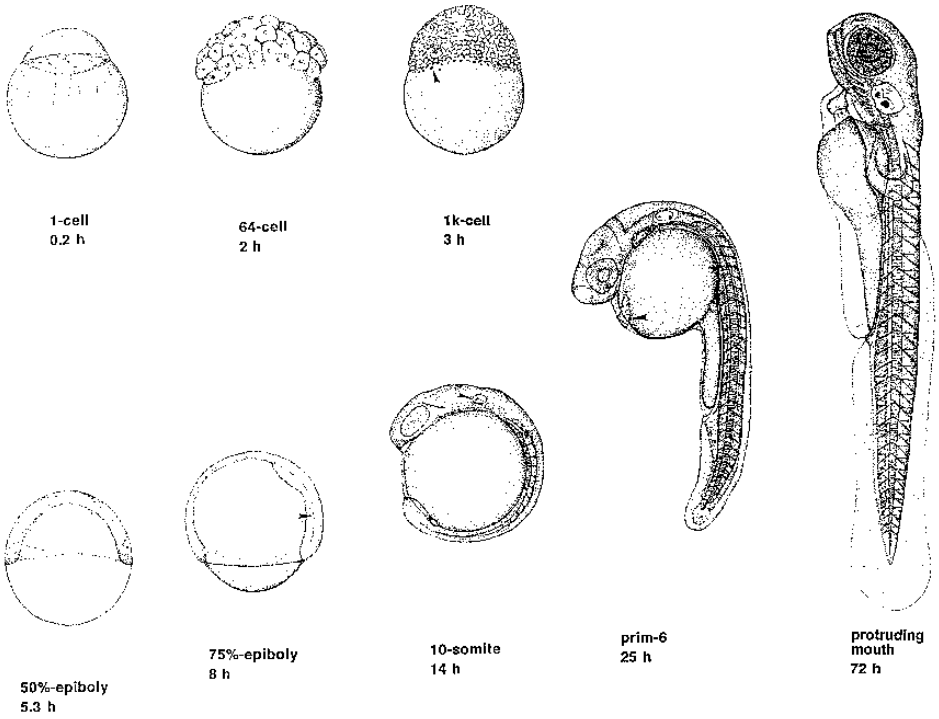

Figure 9. Stages of embryonic development (Kimmel *et al.* 1995).

Offsprings are considered embryos until they can feed externally, which occurs around 72–96 hpf. An individual offspring is described as a larva when it is no longer an embryo but has yet to become a juvenile. The transition to juvenile occurs around 15 dpf. In this state, individuals have acquired most adult characteristics but not sexual maturity. The transition to the adult stage occurs at 2–3 months post-fertilization when viable gametes are produced and secondary sexual characteristics appears[142] (Figure 10). Under favorable conditions, females are able to spawn every two days, even if the egg number produced may be variable due to stress factors[143]. The best spawning performance is attained with a frequency of approximately 10 days[144]. Mating is controlled by

photoperiod, and spawning generally occurs at dawn within the first few hours of light, even though it is not strictly limited to this period. Each female can spawn between 100 to 300 eggs, depending on the physicochemical and nutritional conditions.

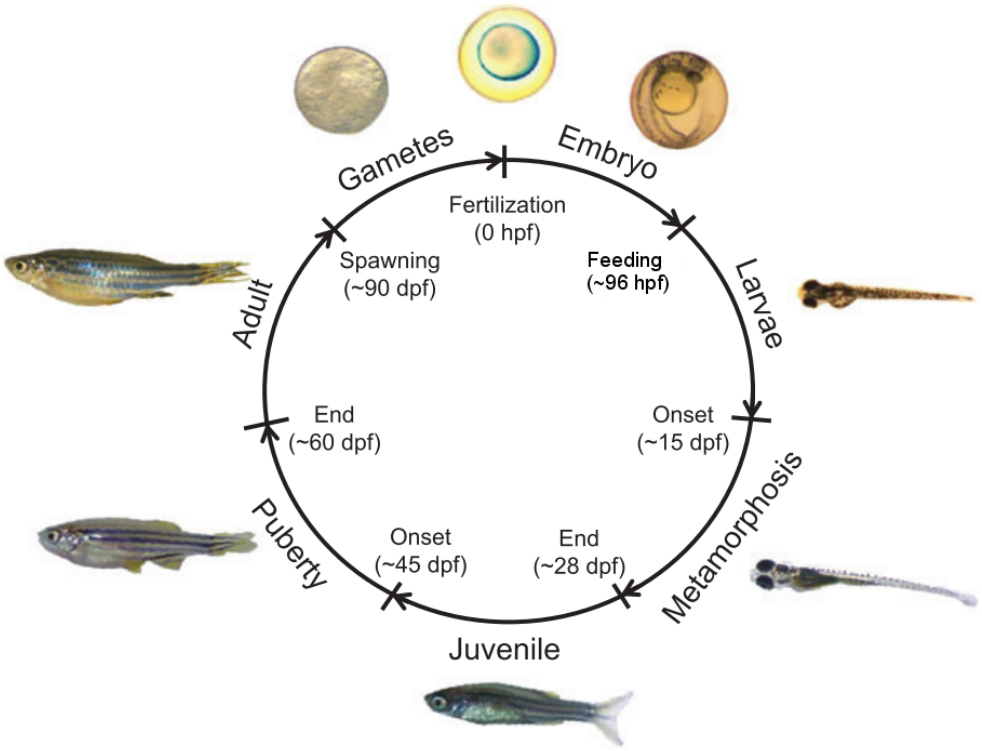

Figure 10: Life cycle of zebrafish (Modified from Ribas 2014).

3.3. Use of the zebrafish as an animal model

The use of the zebrafish as a vertebrate model commenced in 1972 when George Streisinger opted for this small fish for development and genetics studies, due to its small size, high fecundity, external development and optical clarity. Dr. Streisinger developed the first techniques for genetic manipulation; nowadays, numerous genome-based techniques and mutant lines are available[145]. Zebrafish share high genetic homology with humans, and even though this model cannot replace mammals and higher vertebrate models, it offers a cost-effective and more ethical solution for many biomedical studies. Therefore, this model organism is used in many research fields, such as organogenesis, genetics, toxicology, disease modeling and drug discovery.

The zebrafish species has been fully characterized morphologically and physiologically, and the genome is completely sequenced and annotated. Likewise, several new research methods and technologies have been developed, which allow for new experimental approaches[146]. During the 1980s, numerous genetic techniques, such as cloning[147], mutagenesis, transgenesis, and the mapping approach[148], were developed

and thus allowed the use of forward genetic approaches, which were only used on invertebrates until then. Later, several early developmental zebrafish mutants were identified through genetic screening, thus establishing zebrafish as a powerful model in developmental biology[149]. In addition, according to the EU directive 2010/63/EU, zebrafish embryos are considered to be an alternative animal model, so their use is not restricted by regulation for animal welfare[150].

3.3.1 The zebrafish as a human disease model

The use of animal models in biomedical research is essential to comprehend the molecular and cellular pathologic mechanisms involved in disease and make available systems for the identification and testing of new therapeutic approaches. Mammalian models such as rodents have been extensively used because of their high homology with humans at physiological and genetic levels. Several methodological approaches and powerful tools have been developed based on mice, thus allowing the creation of accurate models of many human diseases.

Although those models make possible gathering invaluable knowledge to understand several pathologies, other economical and practical aspects should be considered. Besides, the high degree of the functional conservation of basic cellular processes observed across invertebrates and vertebrates allows modeling those molecular mechanisms at cellular and whole-organism levels on invertebrate organisms such as flies and worms[151,152]. Invertebrates offer a series of practical benefits, such as lower operating costs, increased fecundity, and ease of manipulation. Despite these advantages, the phylogenetic distance between invertebrates and mammalians imposes some critical limitations on the use of invertebrate models, considering that invertebrates lack many structures and physiological systems involved in many human pathologies, so their role in human disease modeling is limited.

In this context, the zebrafish animal model emerges as a smart solution that bridges the gap between the complexity of vertebrate models and the convenience of invertebrates. In the recent years, zebrafish has appeared to be an attractive human-disease model. Many studies have been conducted on metabolic, neurodegenerative, cancer-related and genetic diseases[153]. The optical transparency during the first embryonic stages allows for high-resolution imaging of internal organs *in vivo* and for prolonged times[154].

Forward genetic studies identified several zebrafish mutants with heart defects similar to human defects. Stainier et al. described a mutant with a heart defect analogous to human dilated cardiomyopathies, with an enlarged ventricle, atrium, or both and decreased myocardial contractility[156]. The *silent heart* and *pickwick* mutants present a defective heart contractility, and the study of the mutations leads to the identification of cardiac troponin T (tnnt2) and the large sarcomeric Titin protein[157,158]. Mutations related to laminin α-4 and integrin-linked kinase are the cause of heart failure in zebrafish, as they affect cardiomyocytes and endothelial cells, and mutations in these

genes have been associated with hereditary dilated cardiomyopathies in humans[159]. Similarly, mutants with syndromes of QT alterations have been identified in mutagenesis screens[160].

Furthermore, the anatomical simplicity of the zebrafish kidney and the capacity of nephron regeneration represent an asset in the modeling of renal diseases. Polycystic kidney disease and nephronophthisis have been modeled in zebrafish[161]. Several ciliopathies including Bardet-Biedl syndrome (BBS); nephronophthisis (NPHP); as well as Jeune, Joubert, oro-facial-digital (OFD1), and Meckel (MKS) syndromes have also been investigated[161].

Numerous models for human neurological, neurodegenerative and behavioral diseases have been developed, such as Alzheimer's, Huntington's and Parkinson's diseases[162–164]. Several cancer studies have been conducted over the last years in zebrafish models[165,166]. The first model of leukemia was described in 2003 in a transgenic zebrafish line-expressing mouse *c-Myc* gene under the *rag2* promoter that produced T- and B-cell lymphoblastic and myeloid malignancies[167]. Other blood tumor models were later developed, and they expressed mutated proto-oncogenes such as *TEL-AML* and *NOTCH1*[168,169]. Those leukemia models are currently extensively used to screen and identify selective inhibitors of human lymphoblastic malignancies[170]. Similarly, a melanoma model was developed by Patton *et al.* in a transgenic line that expresses the human oncogene *BRAF* in a neural cell crest[171]. These transgenic zebrafish develop melanomas when the *p53* gene is silenced[172].

Recent technological innovations have enabled the zebrafish model to become an invaluable assay system for the drug-discovery process. The above-stated benefits of this organism make it a good candidate for a low-cost, high-throughput whole-animal platforms. The high fecundity and low maintenance costs allow providing a large number of replicates. Besides, the optical clarity makes possible the use of a fluorescent reporter to observe *in vivo* the physiopathologic response to evaluated molecules. Thus, several zebrafish assays have been developed to help predict drug safety in humans[173,174,175] and test the efficacy of drug candidates on diverse disease models[176],[116,154,177–181]

3.3.2. Zebrafish in intestinal immunity and infectious disease research

The zebrafish digestive system is fundamentally analogous to humans in structure and function[182]. The main human gastrointestinal organs (i.e. the intestine, pancreas, gall bladder and liver) can be found in zebrafish, and the embryonic developmental programs are similar[183] (Figure 11). The intestine is organized as a long tube folded twice in the abdominal cavity and can be divided into three morphologically differentiated sections: the intestinal bulb, the mid intestine, and the posterior intestine. Studies using gene expression analysis on intestinal tissue samples indicated that the differentiated function of intestinal segments resembles the small and large intestines of mammals[184]. The intestinal epithelium forms irregular elevations consisting of absorptive

enterocytes, endocrine cells, and mucus-producing goblet cells. Beneath the epithelium is the *lamina propia*, which harbors several cellular types involved in gut immunity[185]. Nevertheless, the zebrafish intestine does not have submucosa or Paneth cells, although high levels of β-defensine are detected[186]. Zebrafish hepatocytes are also equivalent to human liver cells, although there are some differences in hepatic structure. The pancreas presents a more accused morphological difference, but zebrafish has the same pancreatic cell types as those of mammals. Zebrafish pancreas has exocrine cells that secrete lipase and peptidase digestive enzymes as well as insulin-producing Langerhans islands[187].

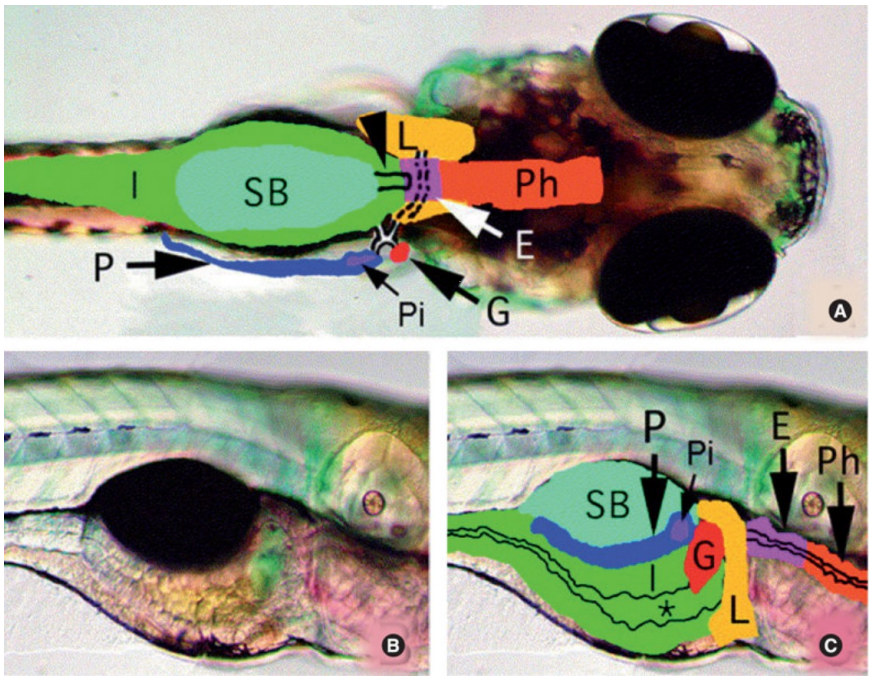

Figure 11: (A) Dorsal view of larval digestive organs. (B) and (C) Lateral view. I: Intestine, P: Pancreas, SB: Swim bladder, G: Gall bladder, E: Esophagus, L: Liver, Ph: Pharynx, *: Intestinal lumen (Farber *et al.* 2003).

Zebrafish has innate and adaptive immune systems equivalent to mammals. This species also has an active complement system with three activation routes: the classical, alternative, and lectin pathways[188]. Microbial-associated molecular pattern (MAMP) receptors, which detect conserved molecular patterns in bacteria such as lipopolysaccharides and double-stranded RNA fragments, and the associated signal-transduction pathways are present in zebrafish. Several Tlr receptor homologs to mammals have been described in zebrafish by *in silico* analyses[189]. The adaptor proteins *myd88*, *mal*, *ticam1* and *sarm* have also been described. Co-transfection studies of zebrafish *tlr3*, *irak4* and *traf6* genes with an *NF-kβ* reporter construct demonstrated a strong induction in *NF-kβ* activity upon bacterial infection[190]. Phylogenetic studies observed strong conservation among Tlr proteins and their pathway components[191,192],

although detailed functional analysis of those genes is still incomplete. Several cytokine homologs have been identified in zebrafish. Homologs of mammalian TNF[193,194], LIF[195], $Il_{1\beta}$[194], Il_{10}[196], Il_{11}[197], Il_{15}[198], Il_{22}[199], and Il_{26}[199] have been described in zebrafish. Similarly, a number of chemokines have been identified recently[200–203], although their complete characterization is still in progress.

Immune cell types are similar to those found in mammals, with the presence of leucocytes, macrophages, and granulocytes. The adaptive T and B immune cell types are functionally equivalent and undergo recombination of *Ig* and *TCR* genes during their development. Zebrafish lacks adaptive immunity during the first 4–6 weeks of life; therefore, it is possible to study the innate immune system avoiding the interference of adaptive immunity[204]. Macrophages and neutrophils have been partially characterized[205–207], but dendritic cells have not been identified yet. In their study, Herbomel *et al.* visualized the activity of macrophages in *E. coli*-infected zebrafish embryos[208]. Macrophages were observed while they were approaching and phagocyting bacterial cells a short time after the onset of the infection. Other studies used *in vivo* fluorescence-based assays for the detection of reactive oxygen species produced in the respiratory burst during the phagocytic activity[209]. Mobile macrophages can be observed as early as 3 hpf[208]. When circulation is initiated, some of the cells migrate to the blood stream and are distributed through the embryo. At 20 hpf, the expression pattern of macrophages' molecular patterns can be detected in zebrafish embryos. Likewise, zebrafish neutrophils share many similarities with their mammalian counterparts; a segmented nucleus and myeloperoxidase positive cytoplasmic granules have been described[204,207]. Renshaw *et al.* developed a transgenic zebrafish line with GFP-labeled neutrophils, which have been useful in studying the role of neutrophils in infection and inflammatory response[210]. Given so, zebrafish appears as an attractive animal model for the evaluation of the influence of probiotic bacteria over the host health and immune response. Gioacchini *et al.* observed that the administration of the probiotic strain *Lactobacillus rhamnosus IMC 501* enhanced the zebrafish welfare by modulation the innate immune response and improving hepatic stress tolerance[211]. Rendueles *et al.* screened a selection of 37 bacterial strains, and identified three strains with a protective effect over the *Edwardsiella ictaluri* pathogen infection[212].

4. Hypothesis and objectives

In recent years, there has been an increasing interest in the use of probiotic bacteria to improve the digestive health and prevent intestinal imbalance. Many LAB strains are available, not only in fermented food but also as pharmaceutical preparations as a substitute for conventional therapeutics. Nevertheless, health claims in functional foods such those containing probiotic bacteria, need to be scientifically demonstrated at strain level before they can be stated on commercial products. The vast amount of candidate species that should be tested to demonstrate their efficacy as probiotics, and the consequent high cost of animal and clinical studies, has favored the performance of preliminary screenings *in vitro* to reduce the number of strains to be finally tested in mammals and humans. However, the actual *in vitro* assays bring limited information, so an intermediate vertebrate model would be useful. In this context, the following hypothesis is stated:

zebrafish is a suitable animal model for the evaluation of the probiotic efficacy of LAB strains.

The following objectives were established to prove this hypothesis:

1. To evaluate the suitability of the zebrafish model for evaluating the colonization ability of LAB (Chapter I).

2. To evaluate and optimize available methods for a zebrafish colitis model for the assessment of the immunomodulatory effect of LAB (Chapter II).

3. To evaluate different intestinal infection models and available methods of determining the antimicrobial effect of new probiotic strains (Chapter III).

4. To set up a novel array of *in vivo* assays with zebrafish and combine it with the existing *in vitro* methodologies to determine the probiotic properties of new LAB strains (Chapter IV).

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II.RESULTS

CHAPTER I

Evaluation of zebrafish larvae digestive colonization by lactic acid bacteria

1. Introduction

Lactic acid bacteria are one of the most important bacterial groups in the food industry due to their substantial role in many fermentation processes. Lactic acid bacteria contribute to the fermentation of many traditional products, such as dairy products[1], meat products,[2,3] or fermented beverages of vegetal origin[4,5]. Likewise, some species of LAB are involved in the ripening and maturation of wine, cider, and cheese[6,7]. In recent years, the growing knowledge about the beneficial influence of these microorganisms on digestive health has increased interest in LAB as probiotics. The potential health benefits comprise a protective effect against digestive pathogens, a reduction of antibiotic-associated diarrhea[8], an enhancement of the immune response, the maintenance of the remission of ulcerative colitis and intestinal bowel disease[9], an improved tolerance to lactose, and a reduction of cholesterol levels[10]. However, those health claims should be demonstrated in a strain-specific way. Therefore, individual strains have to be evaluated to confirm their potential benefits. Most probiotic organisms belong to the genera *Lactobacillus* and *Bifidobacterium*, although other microorganisms have been proven to benefit the health of the host. Bacteria from the *Bacillus* genus have been demonstrated to have immunomodulatory activity, producing high anti-spore immunoglobulin G titers[11] and an antimicrobial effect against *Staphylococcus aureus*, *Enterococcus faecium*, and *Clostridium difficile*[12]. In the same way, the yeasts *Saccharomyces boulardii* and *S. cerevisiae* exhibited probiotic activity[13], particularly in the treatment of Crohn´s disease[14].

The ability to colonize a host's gastrointestinal tract (GIT) is a prerequisite to producing any health benefit. Given this, a mandatory attribute of any potential probiotic is the ability to adhere to the digestive epithelium of the host. Several approaches are used to determine the adhesion properties of potentially probiotic strains. One of the evaluations consists of measuring the bacterial surface hydrophobicity through the microbial adhesion to hydrocarbons assay (MATH) or the adhesion to hydrophobic surfaces[15]. Nevertheless, adhesion assays to intestinal epithelial cells (IEC) produces a more precise representation of the GIT conditions. Numerous studies have been performed using Caco-2 or HT29 cells to evaluate the adhesion of potential probiotic strains[16]. Moreover, there are commercially available kits of intestinal mucus for high-throughput screening of probiotics[17]. *In vitro* models produce useful and cost-effective information for the prescreening of new probiotic strains, but *in vivo* models provide a complete knowledge of the interplay between the host immune system and the bacteria.

Zebrafish (*Danio rerio*) is a widely-used model for developmental biology, oncology, toxicology, or genetic researches. The zebrafish possesses several advantages as an animal model, such as high fecundity and rapid development. Zebrafish embryos develop externally and are transparent, allowing for the visualization of internal organs

in real time. Its genome has been fully sequenced, and there are also numerous mutant and transgenic strains available[18]. Its small size and rapid growth make it useful for high-throughput screening of new drugs[19–21], as well as efficacy evaluation of food bioactive compounds[22]. Recently, this vertebrate model has been the subject of interest as a human disease model[18,23–25], since the development and function of zebrafish organs are similar to mammalians. Recently, a digestive disease model has been developed for intestinal bowel disease (IBD) that partially reproduces the IBD clinic in human disease[31]. This model can be valuable in the prescreening of new anti-inflammatory treatments[32]. The use of gnotobiotic zebrafish embryos has also led to a better understanding of the role of the microbiota on gut development[33], demonstrating the existence of a common microbiota core[34] and its influence on intestinal inflammation[35]. The work of Rawls et al. has suggested the existence of a conserved response of the immune system towards the gut microbiota among all vertebrates[36]. Moreover, He et al. have investigated and characterized the intestinal microbiota dysbiosis in zebrafish produced during the onset of IBD[37].

In the last decade, the availability of fluorescent proteins has greatly improved microscopy techniques in many biological disciplines[38]. Fluorescent proteins, such as GFP and its derivatives, are far less toxic than the earliest fluorescent probes, allowing for the observation of living cells in real-time for extended periods of time[39]. The inclusion of genes coding for these proteins in precise regions of DNA enables their expression to be controlled by a specific regulatory sequence, thus being expressed in different subcellular localizations[40]. The expression of fluorescent proteins can be inherited in a stable way and does not interfere with biological processes. Currently, many different fluorescent proteins are available, covering the entire visible spectrum and allowing for the use of multicolor labeling[41]. In this work, “mCherry” fluorescent protein is employed[42]. This marker is a red protein monomer that has many advantages over GFP, such as rapid maturation, higher photostability, and increased resistance to photobleaching.

The present work aims to assess the suitability of the gnotobiotic zebrafish model to evaluating the colonization ability of potentially probiotic bacteria. To this end, two probiotic exposure procedures were developed and compared. The use of fluorescent bacteria allowed for the monitoring the colonization of zebrafish GIT in real time. Finally, conventional microbiology, as well as qPCR techniques, were studied to evaluate the probiotic concentration present in the digestive tract of zebrafish larvae.

2. Materials and Methods

2.1. Experimental organisms

2.1.1. Zebrafish breeding

Zebrafish embryos were obtained from wild-type adult zebrafish (*Danio rerio*) bred in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain). Adult zebrafish were maintained at 27 ±1 °C with a 12 h light/dark cycle in 60 L tanks. Each tank was fitted with an external filtration system (biological, chemical, and physical filtration). Zebrafish were maintained according to standard protocols[43]. Fish were fed with *Artemia nauplii* and a pellet-formulated diet (Gemma Micro 300, Skretting). Fertilized eggs were collected on spawning traps just after fertilization and maintained in embryo water (EW) (sterilized deionized water solution containing $CaCl_2$ 294 $mg.L^{-1}$, $MgSO_4$ 123 $mg.L^{-1}$, $NaHCO_3$ 64.7 $mg.L^{-1}$, KCl 5.7 $mg.L^{-1}$) at 27 ±1 °C (Figure 1). All experimental procedures were approved by the regional animal welfare body.

Figure 1. View of spawning traps placed on breeeing tanks at the AZTI zebrafish facility.

2.1.2. Axenic embryo production

Axenic zebrafish. The procedure was carried out in a laminar flow cabinet. Embryos of 3 hpf were washed repeatedly in EW supplemented with methylene blue 0.01% (w/v). They were then washed several times with antibiotic solution[44] (AB solution). The AB solution contained a mixture of antibiotics at the following final concentrations[45]: kanamycin 15 $\mu g.ml^{-1}$, ampicillin 300 $\mu g.ml^{-1}$, and amphotericin B 1.25 $\mu g.ml^{-1}$. Subsequently, embryos were immersed in 0.02% (v/v) polyvinylpyrrolidone (PVP) for 2 min. The embryos were then incubated in 0.003% (v/v) bleach solution for 1h and washed 10 times in EW. They were then incubated overnight in AB solution supplemented with 75 µM 1-phenyl-2-thiourea (PTU) to inhibit melanogenesis[46]. Thereafter, all media contained PTU to maintain the inhibition. The following day, embryos were washed repeatedly in EW and transferred to Petri plates in groups of 50. Finally, they were treated with two UV light pulses of 1.6 kV (Pulsed UV System XeMatica 1:2L, SteriBeam Systems, GmbH).

Sterility test. Sterility was routinely assessed at two time-points: after the UV light pulse and at 96 h post-fecundation (hpf). 100 µL of liquid media and a pool of fifteen homogenized zebrafish embryos were cultured under aerobic and anaerobic conditions on Plate Count Agar (PCA) and brain-heart infusion (BHI) broth at 30 ºC for 72 h. Samples were also cultured on 10 mL of Sabouraud Dextrose Broth (Sab-Dex) to detect fungi and yeast. In addition, a PCR amplification using primers targeting 16S ribosomal RNA gene was carried out. Primers 63f (CAGGCCTAACAGATGCAAGTC) and 1387r (GGGCGGWGTGTACAAGGC) were used[33]. Embryos were solubilized in 0.5 mL of extraction buffer [1% (w/v) SDS, 150 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 8.0, 50 µL of 5 M guanidinium thiocyanate, and 25 µL of proteinase K solution (Applied Biosystems)]. The solution was incubated at 56 ºC overnight and then centrifuged at 8000 g for 5 min. DNA was isolated with a Wizard-DNA CleanUp Extraction Kit (Promega). Quantitative PCR was carried out with a Light Cycler 480 sequence detection system (Roche Diagnostics) in 10 µL containing 300 nM of primers, 5 µL 2x SYBR Green PCR master mix (Roche Diagnostics), and 20 ng of DNA template. Reaction conditions were as follows: 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, followed by a final extension of 72 °C for 5 min.

2.1.3. Bacteria strains

The following bacterial strains were used in the present study (see details in Table 1): *Lactobacillus plantarum Lp90,* a ropy strain isolated from wine[47]; *Lactobacillus plantarum B2 (CECT8328)* and *Lactobacillus fermentum PCB11.5 (CECT8448*), two riboflavin overproducer strains isolated from sourdough[48]; and *Lactobacillus sakei MN1* strain, isolated from meat product[49]. The cited bacterial strains carried the pRCR12 plasmid. This plasmid contains the *mrfp* gene, encoding for mCherry fluorescent protein[50] (Figure 2). Bacteria were routinely grown in MRS containing chloramphenicol

(Cm) at 10 µg.ml^{-1} at 37 ºC. *Lactobacillus sakei* MN1 was grown in MRS with 2 % glucose (Ls/G) or MRS supplemented with 2 % sucrose, and lacking glucose (Ls/S) and incubated at 30 ºC. This strain produces dextran, presenting a mucoid or ropy phenotype when growing in the presence of sucrose as carbon source[51].

Table 1: Strains and growing conditions used in this study.

Code	Strain	Growing media	Phenotype
Lp 90	*L. plantarum 90*	MRS + Cm	Ropy
Lp B2	*L. plantarum B2*	MRS + Cm	Non-ropy
Lf 11.5	*L. fermentum PCB11.5*	MRS + Cm	Non-ropy
Ls/G	*L. sakei MN1*	MRS + Cm + Glucose	Non-ropy
Ls/S	*L. sakei MN1*	MRS + Cm + Sucrose	Ropy

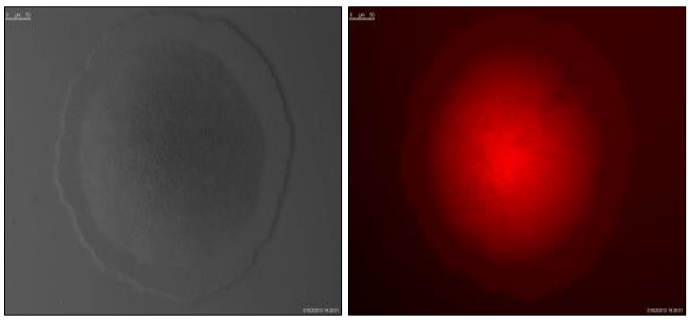

Figure 2. *L. plantarum* 90 colony observed on bright field (left) and fluorescence microscopy (right).

2.2. Probiotic administration

2.2.1. Bacterial dose

A preliminary assay was performed to determine the maximal concentration of bacteria not leading to complete dead of the zebrafish cohort. Overnight cultures of each strain were centrifuged at 3500 rpm for 5 min and re-suspended in PBS. Ten-fold dilutions were then prepared in PBS. The following concentrations were assessed: 5.10^9 cells.mL^{-1}, 1.10^9 cells.mL^{-1}, 5.10^8 cells.mL^{-1}, 1.10^8 cells.mL^{-1}, 5.10^7 cells.mL^{-1}, 1.10^7 cells.mL^{-1}, 5.10^6 cells.mL^{-1}, and 1.10^6 cells.mL^{-1}. One embryo of 4 dpf (days post-fertilization) per well was placed in a 12-wells microplate (Corning, USA) along with 1 mL of the above-mentioned dilutions. Twelve embryos per condition were evaluated. Embryos immersed in sterile PBS were used as a negative control. Cumulative mortality was determined at 12, 24, and 48 h. The experiment was repeated twice.

2.2.2. Probiotic exposure

Two exposure procedures were assessed (Figure 3):

Short exposure: Ten embryos of 4 dpf were immersed in 15 mL of bacterial dilution in a Petri dish and incubated for 18 h at 27 ± 1ºC with gentle shaking (60 rpm). Ten embryos were placed in sterile PBS solution as a control. After 18 h embryos were washed three times in sterile PBS and analyzed. At the end of the experiment the embryos can be considered larvae. (0 **hpse**: hours post-short-exposure)

Continuous exposure: Ten embryos of 4 dpf were immersed in 15 mL of bacterial dilution with 50 µL of sterilized 1 % (w/v) larva feed dilution (Zfin Biolabs, Spain) in a Petri dish and incubated at 27 ±1 ºC with gentle shaking. Ten embryos were placed in sterile PBS with 50 µL of sterilized 1 % (w/v) embryo feed dilution as a control. The media was replaced with a fresh bacterial dilution daily. The exposure was conducted until 9 dpf. At the end of the experiment the embryos can be considered larvae. (0 **hpce**: hours post-continuous-exposure)

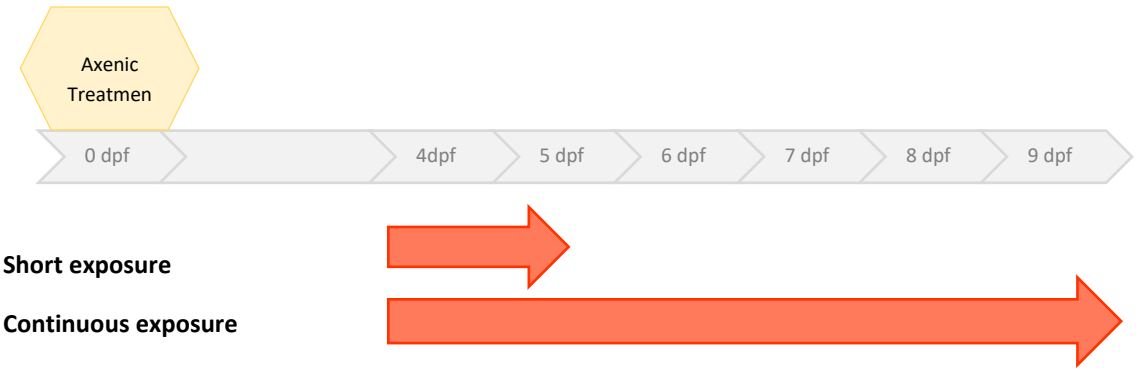

Figure 3. Schematic view of experimental procedures. Zebrafish embryos were exposed to probiotic bacteria for 18 h on **short exposure**, and 120 h on **continuous exposure**.

2.3. In vivo imaging

2.3.1. Wide-field fluorescence

After exposure living larvae were observed in lateral view using a Leica M205 FA stereomicroscope (Leica Microsystems GmbH, Germany). Larvae were anesthetized using tricaine (MS- 222) (Sigma-Aldrich, USA) at 120 $mg.ml^{-1}$ and placed laterally in 3% (w/v) methylcellulose. Larvae images were captured using a Leica DFC 360FX camera. mCherry fluorescence was detected by exposure to ultraviolet light in the excitation range of 545/30 nm. All images were captured using a Planapo 1x objective, with the same acquisition parameters (Exposure: 200 ms; gain: 1; Intensity: 800). Images were registered in lossless TIF format using the LAS-AF software suite (Leica Microsystems GmbH, Wetzlar, Germany).

2.3.2. Confocal microscopy

The detailed structure of larvae digestive tract was visualized using confocal microscopy. Larvae were anesthetized using tricaine and mounted in 0.8% (w/v) low melting point agarose. The intestine was observed with an Olympus FluoViewFV500 confocal laser scanning microscope. The images were acquired using a Plan Apo 20×/0.70 objective with an excitation wavelength of 580 nm. The images were processed using Olympus FluoView 5.0.67 software (Shinjuku, Japan).

2.3.3. Image analysis

The obtained images were subjected to manual evaluation to determine the fluorescence prevalence, as well as automatic image analysis to quantify the fluorescence intensity. The image analysis was realized using ImageJ 1.48 software[52].

Prevalence of mCherry fluorescence on digestive tract. Larva exposed to the above-mentioned bacterial strains were observed to determine the bacteria colonization of GIT. Three groups of 12 larvae for each treatment were observed under the fluorescent microscope to determine the presence of significant fluorescence at 6, 24, and 48 hpe. Larvae presenting fluorescence intensity levels above the threshold value of 200 (a.u.) in the intestinal region were considered positive.

Automatic quantification of mCherry fluorescence. Initially, a region of interest (ROI) was manually specified in every image to frame the intestinal region and discard the rest of the larva. The area and shape of the ROI remain constant within all images. A macro was set up to automatically determine fluorescent area, mean intensity, and shape descriptor value. The macro program performs an initial rolling ball background subtraction operation to reduce the background noise[53]. Subsequently, the image is thresholded using “Yen” algorithm[54]. The resulting binary image is used to produce a selection area, which is then applied to the original image. Finally, the total area,

fluorescence mean intensity, and shape descriptor of selections are determined. Three groups of six larvae were quantified for each treatment and timepoint.

2.4. Bacteria load determination

Two methods were used to determine the LAB concentration present in the digestive system of the zebrafish larva: rt-qPCR and microbiological plate count.

2.4.1. rt-qPCR

Calibration curve from bacterial cultures. A standard curve was generated from pure cultures for the quantification of bacterial load in zebrafish larvae. Ten-fold dilutions from overnight cultures of each LAB strain were prepared in sterile PBS. A 100 µL sample of each dilution was plated in triplicated in MRS + Cm agar plates. After 48 h of incubation, colonies were counted and the mean value of each dilution calculated. Simultaneously, DNA was extracted from the serial dilutions using the QIAamp DNA Mini Kit (Qiagen), following the manufacturer's instructions. DNA concentration and quality were determined with a Nanodrop ND-1000 spectrophotometer (Wilmington, DE). Real-time PCR analysis was performed as stated below. A standard curve was created by plotting the bacterial CFU per mL against the corresponding C_T (threshold cycle) value for each dilution.

Bacterial DNA extraction from zebrafish larvae. Larvae were individually homogenized with a Pellet Pestle Cordless Motor (Sigma) and solubilized in 0.5 mL of extraction buffer [1 % (w/v) SDS, 150 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl, pH 8.0, supplemented with 50 µL of 5 M guanidinium thiocyanate, and 25 µL of proteinase K solution (Applied Biosystems)]. The mixture was incubated at 56 ºC overnight and then centrifuged at 8000 g for 5 min. The supernatant was treated with the Wizard-DNA CleanUp Extraction Kit (Promega). The purified DNA was resuspended in DNase-free sterile distilled water. DNA concentration was determined with a Nanodrop ND-1000 spectrophotometer (Wilmington, USA).

Real-time PCR. Quantitative PCR was performed in a LightCycler 480 system (Roche Applied Science). The reaction volume was 10 µL containing 5 µL 2x SYBR Green PCR master mix (Roche Applied Science), 0.5 µL of each primer, and 1 µL of sample containing 20 ng of DNA. Specific primers for the genus *Lactobacillus,* were used[55] (Forward TCCTACGGGAGGCAGCAGT; reverse GGACTACCAGGGTATCTAATCCTGTT). The reaction conditions were 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 10 s and 60 ºC for 30 s. A dissociation step was performed at the end of the analysis to determine the specificity of amplification: 95 ºC for 1 min, 65 ºC for 1 min, and 95 ºC for 15 s.

2.4.2. Plate count

Zebrafish larvae were euthanized with tricaine at 200 mg.mL^{-1} and washed in three different baths of sterile PBS supplemented with 0.1 % (w/v) Tween 20 to remove any bacteria attached to the skin. In each experimental condition, ten larvae were individually homogenized with a Pellet Pestle Cordless Motor in 100 µl of PBS. Only larvae with a visually confirmed fluorescence were selected. Finally, serial dilutions of the recovered suspension were spotted onto MRS agar plates. Fluorescent colonies were counted after 48 h incubation at 37 ºC. Three independent experiments were performed.

2.5. Statistical Analysis

Results were expressed as the mean ± SEM. The significance of differences was determined using a one-way ANOVA, followed by Tukey's test for multigroup comparisons, using statistical software, GraphPad Prism 7 (GraphPad Software Inc.). A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Bacterial dose determination

The cumulative mortality of larvae exposed to bacterial dilutions is represented in figure 4. Exposure of larvae to 10^9 CFU.mL^{-1} led to a mortality increase of 80% after 6 h, comparing to non-treated larvae. The mortality reached 100 % after 24 h. When larvae were exposed to 10^8 CFU.ml^{-1}, the cumulative mortality after 48 h ranged between 27.7 % for *Lf 11.5* and 44 % for *Lp 90*. Bacterial dilutions up to 5.10^7 CFU.ml^{-1} of LAB did not cause any significant mortality or malformations. Therefore, a concentration of 1.10^7 CFU.ml^{-1} was selected for the subsequent assays.

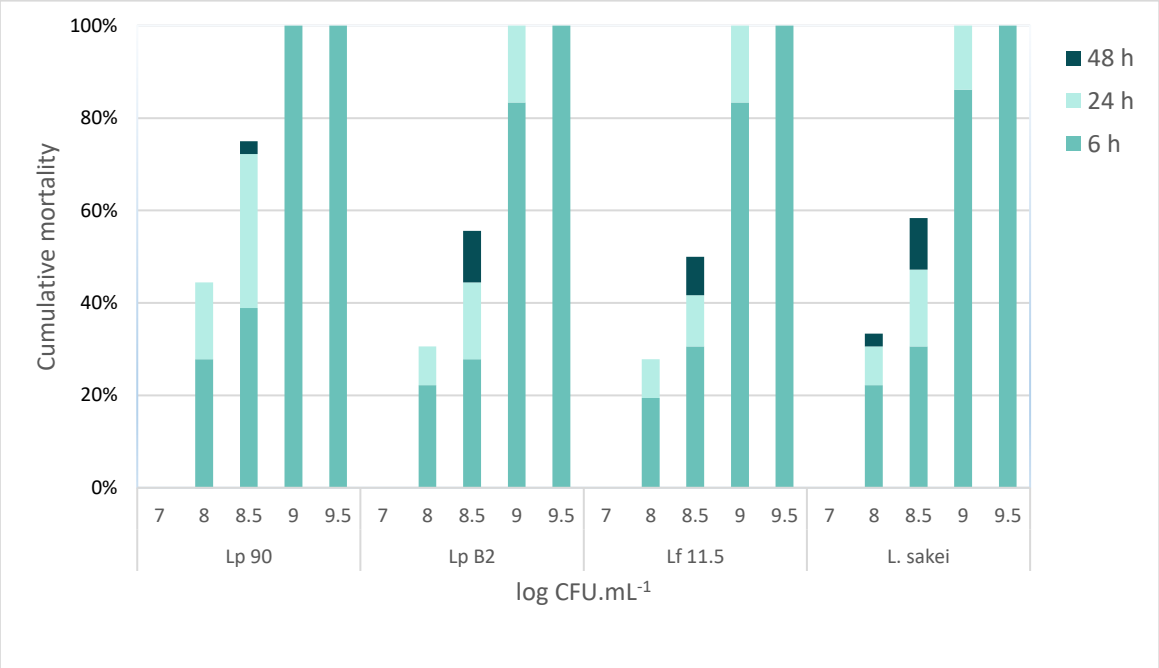

Figure 4. Cumulative mortality of 5 dpf zebrafish exposed to the evaluated bacterial dilutions. Bacterial concentration is expressed as $\log_{10}$ cfu.mL^{-1}

3.2. Colonization of larva GIT

The fluorescent labeling of bacteria allowed to monitor the *in vivo* colonization of the intestinal tract of the zebrafish larva by LAB. The adhesion of bacterial cells to the intestinal epithelium of the larva was confirmed by confocal microscopy (Figure 5). The images show that 12 hpe, some bacterial cells are close to the intestinal epithelial cells, while others are located on the luminal space. After 48 h, only adhered bacterial cells are present.

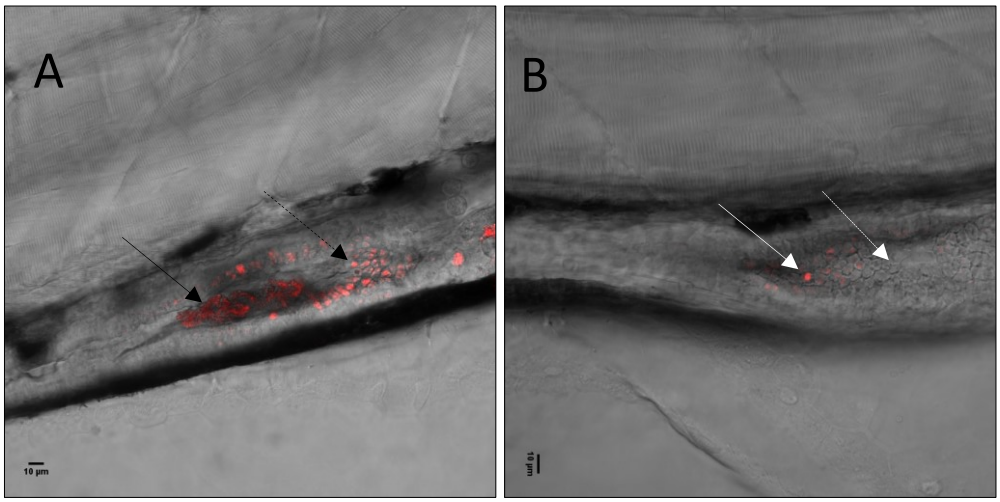

Figure 5. Zebrafish detailed intestinal structure images obtained by confocal microscopy. **A.** 12 h after exposure to *Lp 90* strain, abundant fluorescent cells can be observed in the digestive track. Bacterial cells adhered to the epithelium (black dashed arrow) can be distinguished from bacterial aggregates located in the luminal space (black arrow). **B.** After 48 h, the fluorescence observed is significantly reduced. Fluorescent LAB cells (white arrow) adhered to zebrafish intestinal epithelial cells (dashed white arrow) are visible.

Prevalence of mCherry fluorescence on zebrafish larva digestive tract. At 6 hpse, red fluorescence revealed the presence of LAB in the GIT of larvae (Figure 6). However, in the case of *Lf 11.5*, the prevalence of fluorescence appeared much lower than other strains at 6 hpse and non-existent at 24 hpse. Less than 5 % of fluorescent larvae were detected 6 h after exposure to *Lf 11.5*. By contrast, during the same period, abundant red fluorescence was visible inside the digestive tract of larvae exposed to both *L. plantarum* and *L. sakei*. At 6 hpse, 71 % of larvae exposed to *Lp 90* exhibited significant fluorescence, whereas 55 % of larvae exposed to *Lp B2* did the same. With regard to larvae exposed to *L. sakei MN1*, 55 % of larvae exposed in the presence of glucose showed fluorescence, whereas 47% did so when exposed in presence of sucrose. At 24 hpse, the fluorescence prevalence monitored in larvae exposed to *Lp B2* and *Ls/*G suffered a minor decrease (37 % and 40 % respectively). The fluorescence decrease observed in larvae exposed to *Lp90* and *Ls/S* (22 % and 14 % respectively) was more accentuated. After 48 h, a very low number of larvae (≈ 1-9 %) displayed any fluorescence. No fluorescence was visible on larvae exposed to heat-killed bacterial dilutions.

The prevalence detected in the continuous exposure followed a similar trend (Figure 7). Overall, the percentage of larvae presenting fluorescence was higher than those obtained with the short exposure. The prevalence observed at 6 hpce was nearly 100% for all strains. At 24 hpce, the observed fluorescence diminished to 61% and 55% for *Lp 90* and *Ls/S*, respectively, while the values for *Lp B2* and *Ls/*G maintained over 75%. At 48 hpe, the same trend was observed; the prevalence values of the strains *Lp B2* and

*Ls/*G decreases to 48% and 50%, respectively, whereas the ropy strains *Lp90* and *Ls/S* experienced a more acute reduction (29% and 27%). Moreover, the fluorescence could be detected on larvae up to 72 hpce. Figure 8 presents the prevalence results obtained with each exposure procedure at 24 hpe. The continuous exposure produced significantly higher bacterial prevalence than the short exposure for all bacterial strains evaluated.

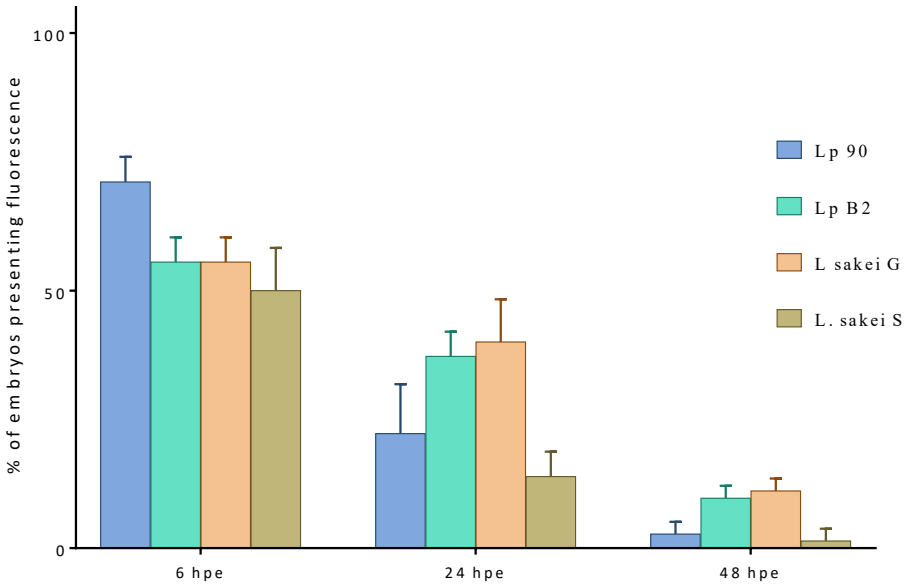

Figure 6. Prevalence of zebrafish s displaying fluorescence after a **short exposure to LAB strains**. The mean ±SEM are represented.

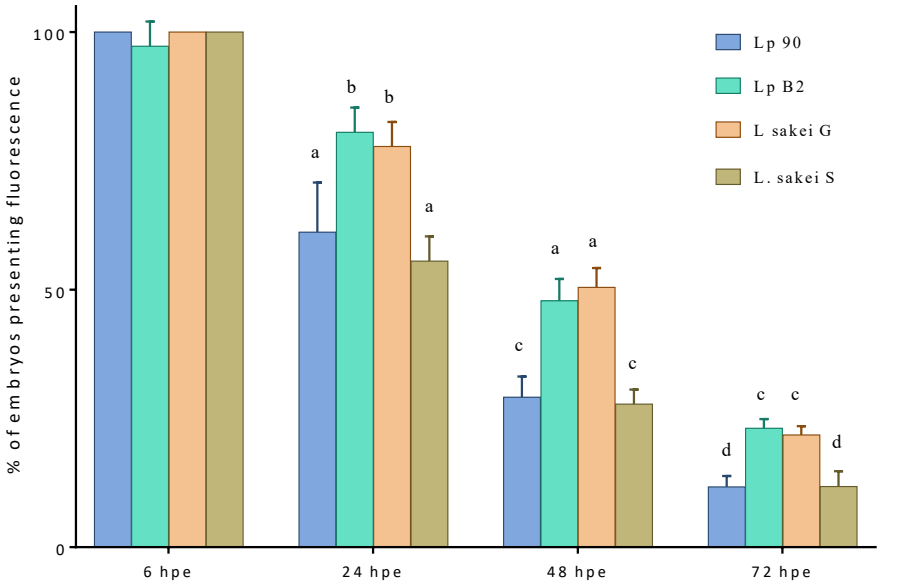

Figure 7. Prevalence of zebrafish s displaying fluorescence after **continuous exposure to LAB strains**. The mean ±SEM are represented. Columns that do not share the same letter are statistically different ($p < 0.001$).

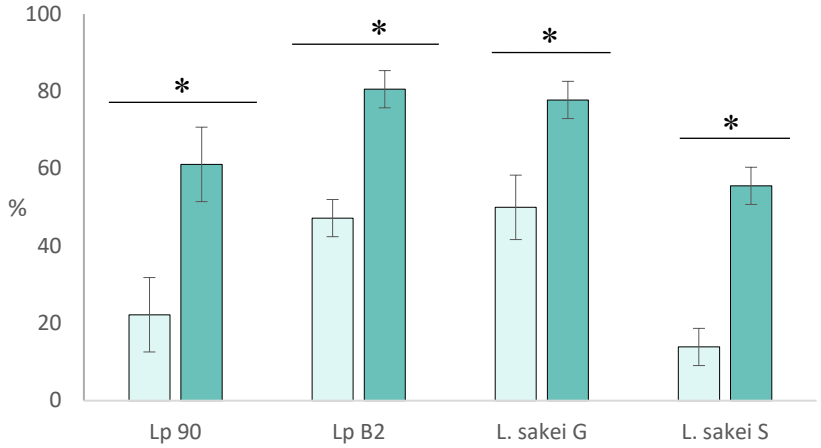

Figure 8. Fluorescence prevalence obtained for the compared procedures at 24 hpe. The prevalence value for every strain and procedure is represented (mean ± SEM). Light green columns represent the short exposure results. Dark green columns represent the values obtained in the continuous exposure. An asterisk indicates a significant difference between the values obtained for each strain. ($p < 0.001$).

Automatic quantification of fluorescence in larva GIT. The evaluation of the images from larvae exposed to LAB strains under the short exposure procedure reveals a considerable variability in the intensity of fluorescence located in the intestinal region (Figure 9). The mean fluorescent area reduction from 6 hpse to 24 hpse for Lp *B2* was from 697.97 ±85.15 to 442.24 ±38.5, and from 878.69 ±72.02 to 485.82 ±46.25 for *Ls/G*. The fluorescent area decreased from 800.19 ±77.47 to 323.38 ±44.65 for Lp90. Finally, the area reduction for *Ls/S* was from 721.64 ±66.98 to 388.02 ±38.05. Overall, fluorescence values declined between 24 and 48 hpse for all the evaluated strains. However, the extent of the reduction was not influenced by the colonizing LAB strain.

Regarding the continuous procedure, larvae exposed to the LAB strains presented a similar intensity at 6 hpce, regardless of the strain. The detected values were also similar to the results obtained at 24 hpce, so only the second timepoint was evaluated. At 24 hpce, the detected fluorescence of exposed larvae in the continuous procedure did not display any significant difference between LAB strains. (Figure 10). Nevertheless, an overall statistically significant reduction ($p \leq 0.001$) in fluorescence area between 24 hpce and 48 hpce was detected for each strain. The mean fluorescent area experienced a decrease from 2825.2 ±223.6 to 1578.7 ±98.2 between 24 hpce and 48 hpce in larvae exposed to *Lp B2* strain. The detected reduction for the *Ls/G* was between 2670.9 ±252.3 and 1260.8 ±84.8. The reduction for *Lp 90* was from 3292.9 ±281.9 to 747.6 ±67.9, and between 3598.7 ±249.7 and 762.1 ±100.1 for *Ls/S*. Overall, ropy strains exhibited a sharper decrease of the fluorescence area than non-ropy strains ($p \leq 0.05$). Remarkably, the mean area and circularity value displayed by the ropy strains presented a significant increase that was not observed in non-ropy strains. (Table 2). Furthermore, bright dots could be visualized directly under the microscope. They were mainly located in the

anterior intestine and could be found on larvae exposed to strain *Lp 90* and *Ls/S*. Figure 11 illustrates this behavior, comparing the fluorescence intestinal localization between a ropy phenotype strain (Lp 90) and a non-ropy strain (Lp B2).

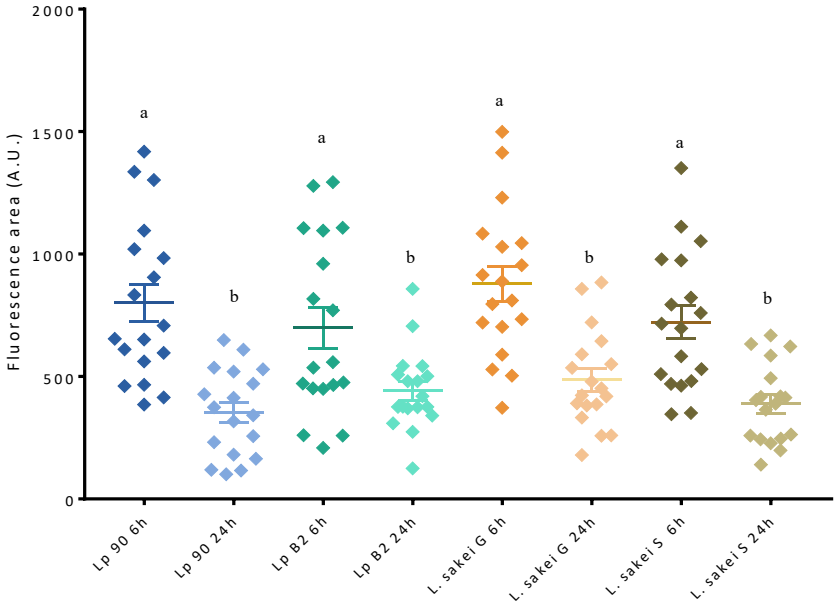

Figure 9. Fluorescence area of s exposed to LAB strains following the **short exposure procedure**. Error bars indicate the standard error of the mean. Multiple comparisons within ANOVA tests were carried out through the post hoc Tukey´s test. Columns that do not share the same letter are statistically different (p ≤0.05).

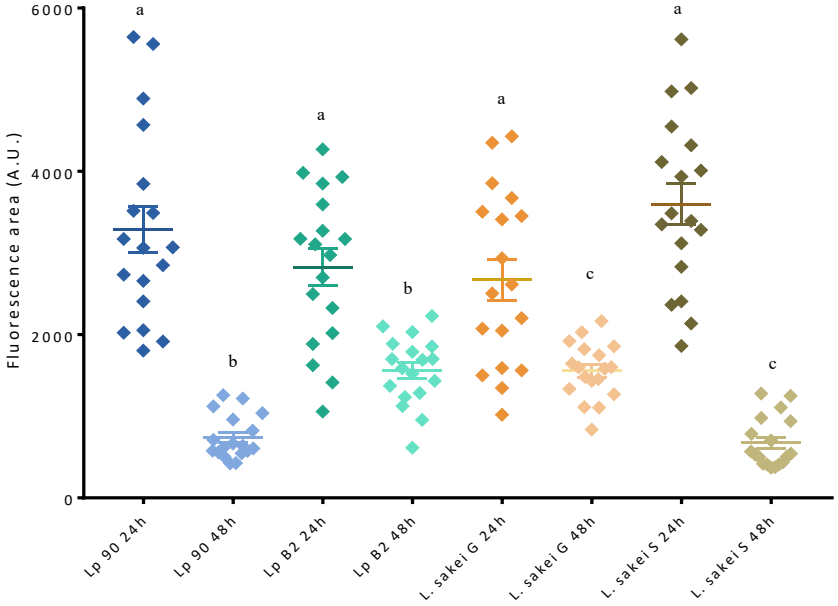

Figure 10. Fluorescence area of larvae exposed to LAB strains following the **continuous exposure procedure.** Error bars indicate the standard error of the mean. Multiple comparisons within ANOVA tests were carried out through the post hoc Tukey´s test. Columns that do not share the same letter are statistically different (p ≤0.05).

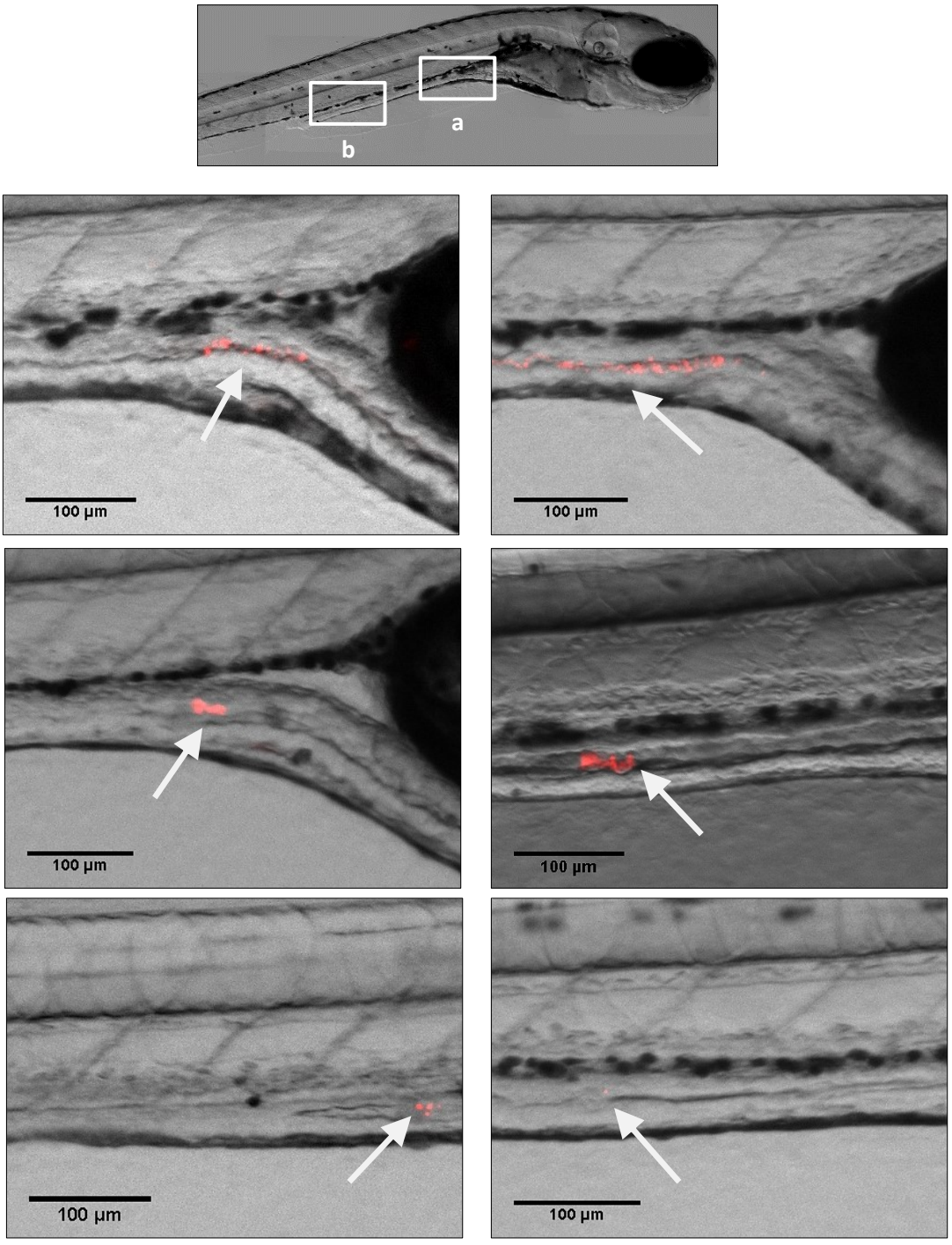

Figure 11. Detection of intestinal distribution of fluorescent LAB. Zebrafish larvae infected with either *Lp 90* (left, ropy phenotype) or *Lp B2* (right, non-ropy phenotype) observed under a fluorescence stereomicroscope at 6, 24, and 48 hpe. White arrows mark the localization of LAB strains in the medium (a) or posterior (b) intestinal tract.

Table. 2. Fluorescence quantification values comparison between bacterial strains presenting a ropy (*Lp 90* and *L. sakei* /S) and non-ropy (*Lp B2* and *L. sakei* /G) phenotype.

	24 h		48 h	
Strain	Area	Circ.	Area	Circ.
Lp B2	2825.2 ± 223.6	0.19±0.03	1578.7±98.2	0.13±0.01
Ls/G	2670.9±252.3	0.22±0.04	1260.8±84.8	0.14±0.05
Lp 90	3292.9±281.9	0.24±0.02	747.6±67.9	0.43±0.05
Ls/S	3598.7±249.7	0.28±0.05	762.1±100.1	0.45±0.09

3.3. Bacterial load

3.3.1. rt-qPCR

The cell count and C_T values obtained for each serial dilution are represented in Figure 12. A linear relation between bacterial cell count and C_T values was obtained for the evaluated LAB strains in the range 10^4 –10^9 cell/mL (R^2=0.99). Bacterial DNA extracted from homogenates was quantified by qPCR. All the evaluated samples resulted in C_T values over 35 and were consequently dismissed. Certainly, the bacterial concentrations of exposed larvae obtained by plate count were lower than 10^4 cell/mL, falling outside of the sensibility range.

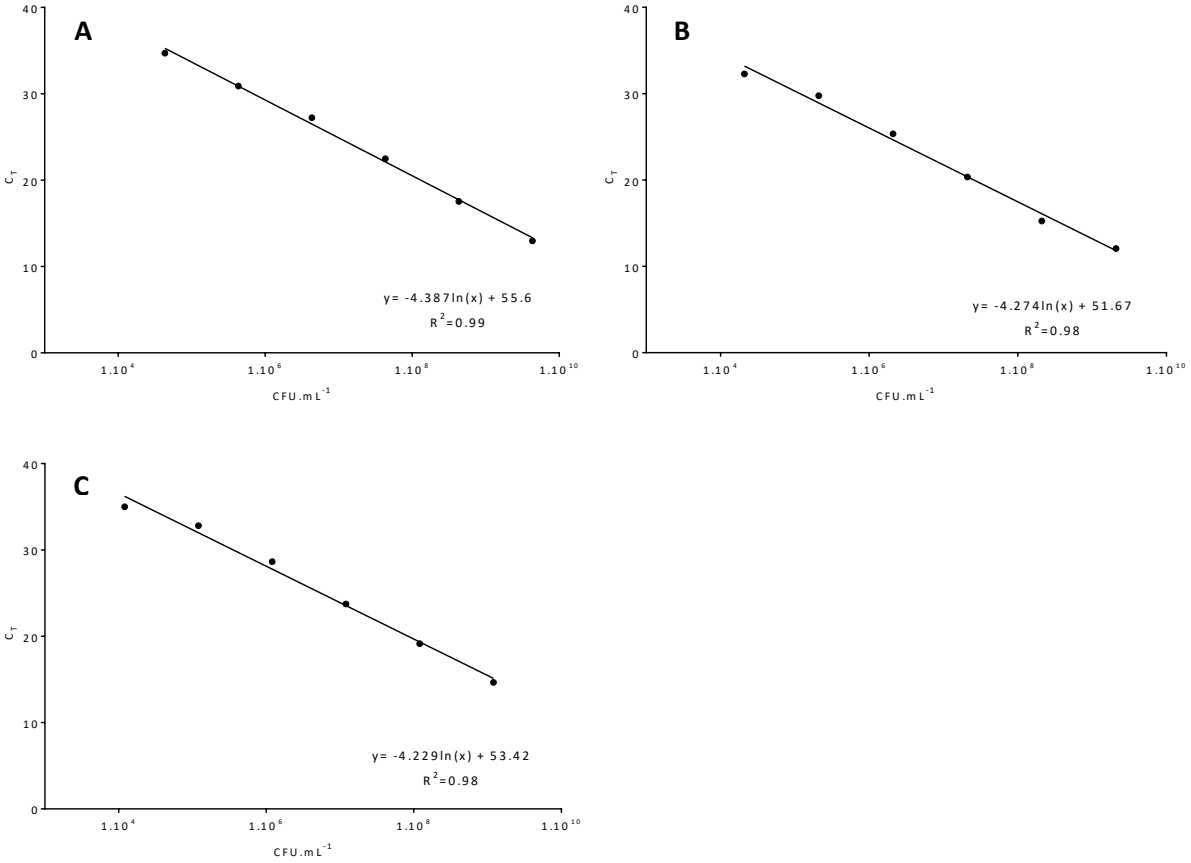

Figure 12. Calibration curve obtained after DNA amplification with *Lactobacillus* genera-specific 16S rRNA gene targeting primer. The curve displays the result of the linear regression between C_T values obtained by rt-PCR and cell density obtained by agar plate count. The calibration curves correspond to **A**. *Lp 90*. **B**. *Lp B2*. **C**. *L. sakei*

3.3.2. Plate count

Enumerations of bacteria from whole homogenates are shown in Figures 11 and 12. Larvae exposed following the short procedure exhibited bacterial loads between 1.10^2 and 2.10^2 cells per larva at 6 hpe (Figure 13). At 6 h, the bacterial load detected in larvae exposed to *Lp 90* ($2{,}7.10^2$ CFU.mL^{-1}) doubled the count found in other strains. However, while the cell count for *Lp B2* and *Ls/G* remained nearly constant after 24 h, the bacterial load of larvae exposed to *Lp 90* and *Ls/S* experienced an accentuated decrease. After 72 h, most larvae did not harbor any detectable bacteria.

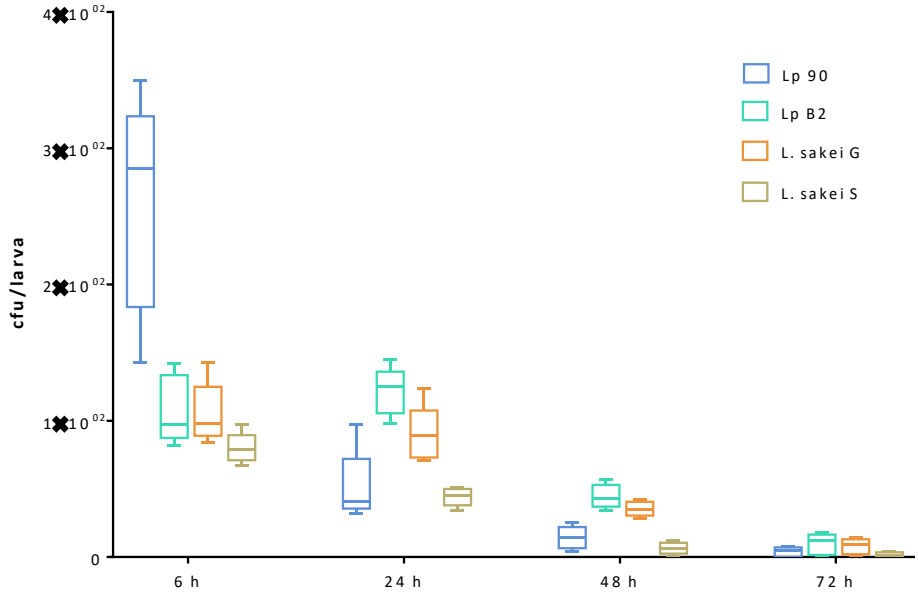

Figure 13. Bacterial enumeration of homogenate larvae exposed to the evaluated LAB following the **short procedure**. n= 10 biological duplicates. Multiple comparisons by ANOVA test with Turkey´s post hoc test. * indicates p ≤0.001.

The bacterial counts observed in larvae exposed to the continuous exposure were overall higher that the values obtained with short exposure. Nevertheless, a similar decreasing trend over time was observed (Figure 14). At 6 hpce, the bacterial count ranged between $1.26x10^3$ CFU.mL^{-1} for *Ls/S* and $3.37x10^3$ CFU.mL^{-1} for *Lp 90*. After 24 h, the bacterial load for *Lp 90* decreased to $9.53x10^2$ CFU.mL^{-1}, while the cell count for *Lp B2* conserved similar values to 6 h ($1.65x10^3$ CFU.mL^{-1}). In the same way, *Ls/S* bacterial counts decreased between 6 h and 24 h (from $1.26x10^3$ CFU.mL^{-1} at 6 h, to $6.15x10^2$ CFU.mL^{-1} after 24 h). After 72 hpe, the bacterial load detected was low (between 22 CFU.mL^{-1} for *Ls/S* and $1.81x10^2$ CFU.mL^{-1} for *Lp B2*). Bacteria count was detected in larvae at up to 72 hpe.

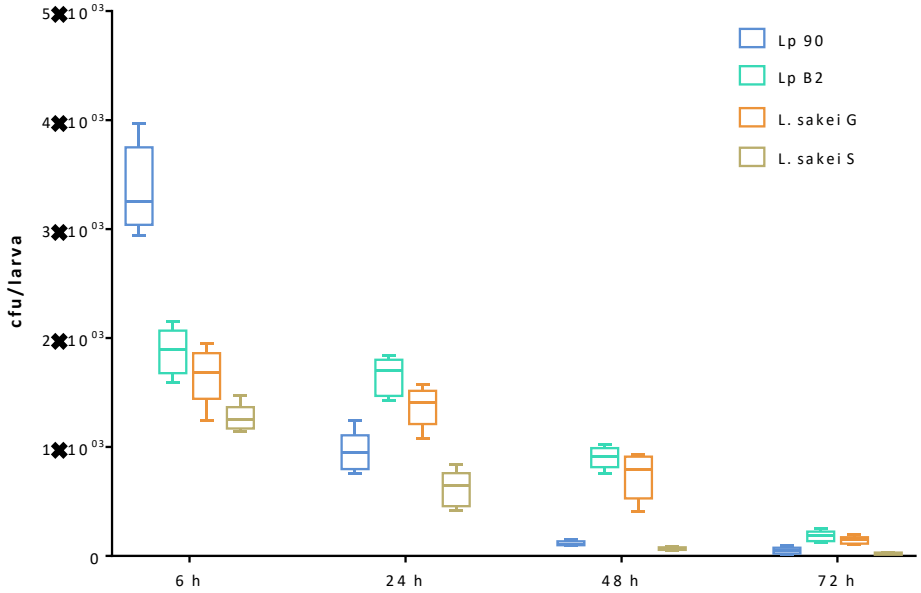

Figure 14. Bacterial enumeration of homogenate larvae exposed to the evaluated LAB following the **continuous procedure**. n= 10 biological duplicates. Multiple comparisons by ANOVA test with Turkey´s post hoc test. Columns that do not share the same letter are statistically different ($p \leq 0.05$).

4. Discussion

Lactic acid bacteria have the potential to produce various health benefits to the host but require certain traits to survive and compete in the intestinal niche. Several studies of the colonization dynamic of probiotic bacteria have highlighted the transient nature of the GIT colonization[56]. Thus, the adhesion capacity to the intestinal epithelium and subsequent colonization are among the most desirable properties of a probiotic strain[57]. The aim of this research has been to develop a procedure for the evaluation of the colonization potential of new probiotic strains, using the zebrafish as an animal model.

Several administration routes are utilized for probiotic screening assays on animal models. The most used method on rodents and adult zebrafish is the feed supplementation regime, where dry feed pellets are soaked in a dilution containing the desired bacterial strain[58]. Probiotic administration on zebrafish larvae is usually done through direct immersion in a media containing the bacterial dilution[59]. In addition, oral micro-gavage is also performed in different animal models[60]. The latter allows the researcher to deliver a precise dose directly onto the GIT; however, it is a time-consuming method and not suitable for zebrafish larvae. In this work, the immersion procedure was chosen for the exposure of larvae to probiotic bacteria. This technique is less invasive and time-consuming than micro-gavage, allowing the bacteria and dissolved chemical compounds to access the intestine through a natural route[60]. Moreover, bacterial concentration is a crucial factor in ensuring the adhesion of an appropriate number of cells to the epithelium[61], meaning that the highest dilution may be preferable[62]. Nevertheless, the risk of extra-intestinal effect represents a constraint in the selected administration route[63]. An optimal bacterial exposure concentration assay was carried out to overcome this constraint. The assay showed that larvae immersed in bacterial dilutions higher than 10^8 CFU.mL^{-1} suffered a decrease of 50 % in their survival rate after 24 h. The mortality reached 100 % when larvae were immersed in solutions with a bacterial load higher than 10^9 CFU.mL^{-1} after 6 h. Remarkably, the exposure to bacterial strains presenting a ropy phenotype produced a higher mortality with respect to the non-ropy strain. It is speculated here that the EPS concentration increases the viscosity of the media, hindering respiratory gas interchange at the gills. Therefore, a bacterial dilution of 10^7 CFU.mL^{-1} was chosen for the subsequent procedures. The exposure of larvae to the selected concentration did not increase mortality rates or produce any malformation.

Once the optimal concentration was defined, 4 dpf embryos were exposed to different LAB strains following two different procedures. Embryos were exposed to bacteria for 24 h in the short exposure and 120 h in the continuous exposure. Even if the assessment of fluorescent LAB prevalence in digestive system reveals a variable adhesion degree, the continuous procedure displayed higher fluorescent values than those observed with

the short procedure. Fluorescence prevalence 24 hours after the short exposure protocol ranged between 14 and 40 %, while the continuous exposure exhibited values ranging between 55 and 75 %. After 48 h, the prevalence on the short procedure was less than 9 %, whereas between 25 % and 50 % fluorescence prevalence was detected with the continuous procedure. In the same way, the automatic fluorescence quantification revealed that the continuous procedure resulted in an increased fluorescent area for all evaluated LAB when compared to the short exposure. Interestingly, fluorescent bacteria could only be detected at 72 hpe on larvae exposed to the short procedure. The bacterial load count confirmed that the continuous exposition generates an increased probiotic colonization of zebrafish GIT. The plate count assay revealed that the continuous exposure produced tenfold greater CFU per larva count and that colonies could be recovered up until 96 hpe. Meanwhile, the bacterial load in the short exposure did not exceed 10^2 CFU.mL^{-1}, and only a few colonies could be detected after 72 h. Additionally, the variability of the results was reduced with the continuous exposure. As expected, when compared to the short and continuous exposure procedures, the increased contact time between the bacteria and intestinal epithelium in the continuous procedure lead to improved colonization results[64]. Although the short exposure procedure is less laborious and uses 5 dpf embryos, thus complying with EU directives of alternative animal model, the continuous exposure was selected for the subsequent researches performed in this work for the improved colonization results. To our knowledge, this is the first published procedure of an extended probiotic exposure in zebrafish larvae beyond 48 h.

Fluorescence quantification is a useful tool to assess the bacterial colonization in zebrafish larvae, but it also presents some constraints. The bacterial strain employed in this study emits a strong and consistent fluorescent signal. Nevertheless, the evaluation of fluorescence signal of pure cultures during the exponential phase revealed significant intensity differences between strains. In a previous work performed by the authors of this research, the optical density and mCherry fluorescence levels of pure cultures in exponential growth were monitored[50]. The results showed an important variability in fluorescence intensity between strains, *Lf 11.5* being the most significant example. In the present research, less than 5 % of fluorescent larvae were detected after exposure to *Lf 11.5*. This behavior could be explained by the low level of fluorescence previously observed for this strain in the *in vitro* assays[50]. Moreover, the environmental condition found in the intestine may influence the metabolic state of bacteria, modifying the fluorescent protein synthesis or degradation rate and thus the fluorescence intensity. The natural variability in the development rate of larvae also poses a limitation to the evaluation procedure[65]. Even if embryos of similar developmental stages are carefully selected prior to the test, the growth stage varies markedly among the test organisms at 5 dpf. At this timepoint, some larvae may not yet have a fully developed digestive system[66]. This limitation is reduced in the continuous exposure assessing larvae of 9-10

dpf to ensure the complete development of the intestinal tract. In addition, Field et al. have demonstrated the existence of intrinsic variation in intestinal transit speed among individuals[67]. These authors have fed zebrafish larvae with a fluorescent marker and observed that, once the fluorescent marker administration was suspended, the GIT of some larvae was cleared from fluorescence after 6 h, while other individuals required more than 24 h to transit all material through their gut. These authors suggested the existence of individual variability in gastrointestinal motility. All the mentioned factors account for the variability displayed in the results. While widefield microscopy is a fast and cost-effective technique for imaging and analyzing small living specimens, it also presents some limitations. In this technique, the sample is fully illuminated with the excitation light, meaning that fluorescent proteins present in whole organisms are excited. During the image acquisition, the camera coupled to the microscope registers both in-focus and out-of-focus light, resulting in a blurrier image and hindering the subsequent image analysis[68]. Computational image processing techniques, such as deconvolution, can increase the resolution eliminating the light produced in different focal plans, thus enhancing the contrast and resolution of obtained images[69]. Besides this, new microscopy imaging techniques have been developed to overcome the limitations of wide-field microscopy. Confocal microscopy increases lateral and axial resolution and eliminates the out-of-focus light. Lately, Selective Plane Illumination Microscopy (SPIM) has garnered great interest in the context of zebrafish imaging[70]. This technique offers a resolution and optical sectioning capacity comparable to confocal techniques, but images can be acquired at a much higher rate due to the use of a plane of light instead of a point[71]. This characteristic allows for the registering of 4D data (3D data over time) for extended periods. Light sheet fluorescence microscopy has recently been used to study the heart[72], eye[73], and whole-brain[74] development of zebrafish over time with a sub-cellular resolution. SPIM techniques are suited for HTS and are thus useful for screening studies such as the present one[75].

Furthermore, the comparison of the probiotic colonization extension revealed a differential outcome correlated to the phenotypic characteristics of the evaluated LAB strains. Overall, a decrease in bacterial presence at GIT was detected over time after the probiotic administration is suspended in all strains[76]. However, remarkably, the ropy-phenotype species (*Lp 90* and *Ls/S)* displayed a swifter bacterial load reduction than larvae exposed to the non-EPS producing strains (*Lp B2* and *Ls/G)*. In the same way, a spatial displacement of bacterial location from the medium to posterior intestinal tract was observed over time, suggesting a transient colonization by the probiotics. After 6 hpe, almost no larva showed red fluorescence in the posterior intestine. The percentage increased after 24 h for all the bacterial strains, but remarkably, larva exposed to the ropy strains retained a significantly higher fluorescence on the anterior intestine. The shape descriptor values obtained in the automatic quantification also showed an increase in the circularity index, as well as in the maximal fluorescence intensity values

of ropy bacteria compared to non-ropy ones. This suggests the formation of "bacterial clumps" composed of aggregates of bacterial cells. This behavior may explain the initial high bacterial count found at 6 hpe in larvae exposed to ropy bacteria. The CFU and fluorescence values obtained at 6 hpe for the ropy strains are certainly considerably higher than other strains. However, after 24h, the registered values for the ropy strains fall. It is here hypothesized that this phenomenon is due to an unspecific mechanical retention at the luminal space, caused by the surrounding EPS layer, and that it is not related to epithelial adhesion. Overall, the results obtained suggest an adverse effect of EPS on bacterial intestinal adhesion ability. Nevertheless, the influence of EPS on the intestinal colonization is still unclear. Nikoli et al. have found that the adherence of non-ropy derivatives to human intestinal cells was significantly increased, as compared to the parental ropy strain[77]. These authors suggest the surrounding EPS layer precludes the interaction between bacterial adhesins and epithelial cells. Other reports have found a negative dose-dependent influence of EPS on *in vitro* adhesion to human intestinal mucus[78]. In the same way, Chen et al. have suggested that the inability of *Lactobacillus kefiranofaciens M1* strain to colonize the intestine of germ-free mice was related to EPS production[79]. However, a positive correlation between EPS production and epithelial adhesion to Caco-2 cells was found in strains isolated from cider and wine[80,81]. Thus, the EPS influence on the adhesion capacity seems to be strain- and structure-dependent. Moreover, exopolysaccharides produced by LAB have been reported to present many other interesting traits. The EPS layer exerts a protective effect for bacterial cells against environmental stresses like extreme pH conditions, digestives enzymes, or antimicrobial molecules[82]. Those compounds may also have direct benefits for the host's health. Mazmanian et al. have suggested that EPS production may modulate the immune response, contributing to the clonal expansion of CD4(+) T-cell population[83]. Stier et al. have shown that 1,3/1,6-D-Glucans have an immunostimulant effect when administered orally, through the activation of pathogen recognition receptors[84]. Exopolysaccharides can also exert a prebiotic effect on the microbiota, serving as a carbon source for probiotic bacteria and thus modulating the composition of the microbiota. Salazar et al. have shown that EPS produced by *Bifidobacterium longum IPLA E44* stimulated the growth of *Lactobacilli, Enterococci, Bifidobacteria*, and *Bacteroidetes* using an *in vitro* fermentation system[85]. Dal Bello et al. have reported a probiotic activity of levan-type EPS produced by *Lactobacillus sanfranciscensis*[86]. Recently, EPS have also been shown to exhibit a promising antioxidant effect; the preliminary *in vitro* experimental data indicated strong antioxidant activity of EPS from *Bifidobacterium bifidum WBIN03* and *Lactobacillus plantarum R315*[87].

Recently, molecular techniques have been developed to determine the bacterial load of a sample without the need for culturing. In this work, the use of quantitative real-time PCR (rt-qPCR) to determine the bacterial load of a sample have been evaluated. Although a strong correlation was observed between this method and the culturing

plate count, the molecular method was not able to detect the low bacterial count present in exposed larvae. Indeed, the research where the primers were designed[88] quantified different *Lactobacillus* strains present in carious dentine. The authors reported that the concentration of bacteria found in the dental lesions ranged from $1x10^4$ to $1.4x10^8$ cells.mg^{-1}. Herbel et al.[89] have also developed a similar rt-qPCR assay, based on heat shock protein 60 gene for identification and quantification of *Lactobacillus* directly from yogurt. The detection limit (10^5 cell.mL^{-1}) was adequate for the quantification of probiotic load found on dairy products (10^6 to 10^{12} cells.mL^{-1}). However, the bacterial count registered in the present work is more than one order of magnitude lower and thus falls outside of the sensibility range of the molecular method. Given this, the rt-qPCR method is not suited for the evaluation of the bacterial load present in zebrafish larvae. Consequently, the plate count method will be used for subsequent works.

5. Conclusions

A novel procedure to evaluate the colonization potential of candidate probiotic strains using zebrafish larvae as a vertebrate animal model was developed in the present work. This is the first continuous exposure procedure published, in which the probiotic administration is prolonged until 9 dpf, increasing the contact time and improving probiotic colonization. Following this procedure, the evaluated LAB strains were able to adhere and colonize the digestive track up to 96 h after the exposure. The use of recombinant strains containing the pRCR12 plasmid (encoding the red fluorescent protein "mCherry")[50,90] allowed the researcher to track the evolution of bacteria inside the GIT in real time, as well as to evaluate the influence of phenotypic characteristics, such as the production of exopolysaccharides, in the colonization process over time. Furthermore, this approach can be combined with GFP-expressing reporters, such as zebrafish immune cells[91] or pathogens[92], for co-localization studies. The probiotic zebrafish assay presented in this work is a fast and cost-effective system that is useful for the screening of potential probiotic strains.

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CHAPTER II

Evaluation of probiotic immunomodulatory effect on a chemical enterocolitis zebrafish model

1. Introduction

The digestive system contains the largest number of immune cells in the whole organism. This is because it is continually exposed to a wide number of microorganisms (up to 10^{11} bacterial per gram), antigens, and other immunomodulatory agents.[1] The immune system must be able to discriminate between commensal and pathogenic bacteria in order to prevent any excessive inflammatory response towards the former while combating the latter. In basal conditions, commensal bacteria are recognized and tolerated through specific recognition by TLR receptors.[2] In this way, the interplay between the gut microbiota and the host mucosal immune system maintains the intestinal homeostasis through a balance between pro- and anti-inflammatory mediators. Nevertheless, several factors can produce a disbalance. This leads to a number of pathologies, including autoimmune diseases, allergies, obesity, diabetes, colon cancer, and intestinal bowel disease (IBD).[3] The prevalence of IBD, which includes ulcerative colitis (UC) and Chron's disease (CD), has increased globally in recent years. Despite the significant morbidity and mortality caused by this pathology, its etiology is still poorly understood. The most widely held view, points towards a genetic predisposition leading to microbiota dysbiosis and causing chronic inflammation of intestinal epithelial tissues. Several studies have claimed that the microbiota composition is altered in IBD patients, with an increase in the number of *Bacteroidetes,* invasive *E. coli*, and *Enterococcus*, as well as a decrease in the presence of *Lactobacillus* and *Bifidobacterium.*[4] Conventional therapeutic approaches aim to suppress the immune hyper-reaction through the use of aminosalicylates, corticosteroids, immunosuppressants (such as azathioprine, methotrexate, or cyclosporine), and anti-TNFα agents.[5] Antibiotic therapy is also useful for decreasing disease activity and maintaining remission of UC, as well as in the treatment of suppurative complications of CD.[6]

Recently, probiotics have received increased attention in the context of IBD treatment and prevention.[7] Probiotic bacteria have a modulatory effect on the mucosal immune system, enhancing membrane permeability through the production of SCFA and the modulation of cytokine production.[8] *Lactobacillus* and *Bifidobacteria* are able to limit pathogen outgrowth by producing antimicrobial substances and competing for epithelial adhesion sites.[9] Several studies have assessed the efficacy of probiotics in IBD therapy. Dunne et al. have conducted an *in vitro* screening of 1500 strains isolated from surgical resected segments and have selected one strain (*Lactobacillus salivarius subsp. Salivarius* strain UCC118) for further studies in mouse and clinical trials. The probiotic mixture VSL#3 (composed of *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus*)[10]

exhibited a protective effect over the mucus layer in several *in vitro* and *in vivo* models. Likewise, Caballero-Franco et al. have demonstrated a stimulating effect of VSL#3 over the colonic mucin (MUC) secretion and *MUC2* gene expression in colonic epithelial cells.[11] Moreover, studies conducted in the colitis murine model have demonstrated a beneficial effect of VSL#3 on the epithelial barrier integrity. This can be useful in the treatment of IBD since the increased epithelial permeability produced by defective tight junction proteins is one of the leading factors of this pathology.[12] Several researchers have conducted clinical trials with probiotic therapy for IBS treatment. Mimura et al. have demonstrated the efficacy of VSL#3 therapy in maintaining remission in pouchitis[13]. Twenty pouchitis patients whose remission was induced by antibiotic therapy received a daily dose of probiotic mixture. In 85% of them, the remission was maintained for one year, compared to 6% in the placebo group.

In the present work, the authors evaluated the utility of a set of assays using a zebrafish colitis model to assess the immunomodulatory effect of several lactic acid bacteria (LAB). Initially two proinflammatory agents were compared. The first of these is trinitrobenzene sulfonic acid (TNBS), which is a haptenizing agent that renders luminal proteins immunogenic to the host immune system. This substance has been extensively used to explore the mechanisms causing IBD pathology,[14–16] as well as the beneficial effect of probiotics on rodent[17,18] and zebrafish colitis models.[19–23] The second agent is dextran sulfate sodium (DSS). This sulfated polysaccharide is toxic to the intestinal epithelium, producing a disruption of its integrity. Each of those models has its own specific characteristics. TNBS triggers an inflammatory process involving T cells, while the DSS model is more likely to induce epithelial barrier disturbances.[24] The inflammatory response to the inflammatory agents is highly dependent on the model´s specific strain, housing conditions, and local microbiota, meaning that the concentrations must be optimized for each laboratory.[25] To this end, a toxicologic assay was conducted to evaluate the optimal dose that leads to consistent intestinal inflammation without severe extra-intestinal damage or increased mortality.

The mucus layer coating the intestinal epithelium represents the first defense against chemical and microbial aggressions.[26] The mucus is composed mainly of secreted O-glycosylated mucin, containing bioactive compounds like defensins and immunoglobulin A.[27] In physiological conditions, microbiota only colonize the exterior zone of the mucus layer, while the inner part next to the intestinal epithelium remains sterile. Alteration in goblet cell physiology and mucus production is a consistent marker of the onset of IBD.[28] Defects in the mucus layer may expose the intestinal epithelium to bacteria, leading to an inflammatory response. Histological staining of whole larvae was performed to evaluate the mucus production in response to an inflammatory insult and the potential effect of probiotic administration.

Neutrophils are among the first mobilized immune cells during the onset of inflammation. In addition to the phagocytic activity, neutrophil cells also contribute to the recruitment of other immune cells to the site of injury through Il_8 and TNFα cytokine secretion[29]. Although the role of neutrophils in IBD remains unclear; some studies indicate that they have a beneficial role,[30] while others point to a harmful contribution.[31] Nevertheless, the intestinal disruption and subsequent bacterial invasion of the *lamina propia* triggers a massive recruitment and proliferation of neutrophils [32,33]. A zebrafish transgenic line Tg(mpo:GFP) was used in the present work to evaluate the neutrophil recruitment at the intestinal level in inflammatory conditions, as well as the potential effect of probiotic administration.[34,35]

Finally, a gene expression analysis was carried out to further characterize the immune response prompted by the enterocolitis agents and the probiotic administration. The differential expressions of 10 innate immune system related genes (Il1β, Il10, Il22, CCl20, Myd88, NF-κβ, Tnfα, Tlr1, Tlr2, and Tlr22) were determined in 9-dpf larvae exposed to different combinations of colitis agents and probiotic bacteria.

2. Materials and Methods

2.1. Experimental organism

Zebrafish embryos were obtained from wild-type adult zebrafish (*D. rerio*), bred in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain). Adult zebrafish were maintained at 27 ± 1 °C with a 12 h light/dark cycle in 60 L tanks. Each tank was fitted with an external filtration system (biological, chemical, and physical filtration). Zebrafish were maintained according to standard protocols. Fish were fed with *Artemia Naupli* and a pellet- formulated diet (Gemma Micro 300, Skretting). Fertilized eggs were collected and maintained in embryo water (EW) (sterilized deionized water solution containing $CaCl_2$ 294 mg.L^{-1}, $MgSO_4$ 123 mg.L^{-1}, $NaHCO_3$ 64.7 mg.L^{-1}, KCl 5.7 mg.L^{-1}) at 27 ± 1°C. The transgenic zebrafish line Tg(mpo:GFP) was maintained in the same conditions[34] (Figure 2). All experimental procedures were approved by the regional animal welfare body. When embryos were subjected to image analysis, Phenylthiourea (PTU) was added continuously to the media (starting from 8 hpf) to inhibit melanogenesis.

2.2. Bacterial strains and exposure

The following bacterial strains were used in the present study: *Lactobacillus plantarum Lp90,* a strain isolated from wine and presenting a ropy phenotype; *Lactobacillus plantarum B2 (CECT8328)*, a riboflavin overproducer strains isolated from sourdough; and *Lactobacillus sakei MN1*, isolated from meat product.[36-38] *Lactobacillus acidophilus 5* was used as a reference probiotic strain.[39] All the evaluated strains carried the pRCR12 plasmid, which contains the *mrfp* gene, encoding for mCherry fluorescent protein.[40] Bacteria were routinely grown in MRS media containing chloramphenicol (Cm) at 10 µg.ml^{-1} at 37 ºC. *Lactobacillus sakei* MN1 was grown in MRS with 2 % glucose (Ls/G) or MRS supplemented with 2 % sucrose, lacking glucose (Ls/S), and incubated a 30 ºC. This strain produces dextran, presenting a ropy phenotype when growing in the presence of sucrose as carbon source.[41] Zebrafish embryos were exposed to the LAB strains following the continuous exposure procedure described in Chapter 1. Ten embryos of 4 dpf were immersed in a Petri dish in 15 mL of bacterial dilution with 50 µL of sterilized 1 % (w/v) larva feed dilution (Zfin Biolabs, Spain). They were incubated at 27 ± 1 ºC with gentle shaking. Ten embryos were placed in sterile PBS with 50 µL of sterilized 1 % (w/v) larva feed dilution as a control. The media was replaced daily with a fresh bacterial dilution. The exposure was conducted until 9 dpf. At that stage, the embryos are considered larvae.

2.3. Toxicological evaluation

An effective dose determination test was carried out to assess the most suitable concentration leading to digestive inflammation without producing significant mortality

or extra-intestinal damage. Dilutions of TNBS were prepared to achieve final concentrations of 25, 50, 75, and 100 µg.mL^{-1} from a 5 % (w/v) stock solution (picrylsulfonic acid solution, Sigma-Aldrich, USA). DSS dilutions of the following final concentrations were prepared: 0.25 %, 0.5 % 0.75%, and 1 % (w/v). Twenty embryos of 4 dpf were placed individually in 1 mL of solution for each treatment. The media was renewed daily with fresh solutions. Mortality and malformations were assessed at 24, 48, and 72 h. Twenty embryos immersed in 1 mL of EW were used as a negative control. The assay was repeated twice.

2.4. Mucus staining

Mucus secretion in response to inflammation was assessed in zebrafish larvae. To this end, Alcian blue staining was performed on whole mount larva[42]. Fifteen 9 dpf larva per condition were stained and observed through a Nikon SMZ1000 stereomicroscope (Nikon, Japan). Images were captured with a Nikon DN100 digital camera and stored in lossless TIF 16-bit color format. Manual cropping of the GIT area was performed with an ROI selection. A macro was set up for automatic image analysis with ImageJ 1.48 software[43]. According to Landini et al., the macro performs an initial background subtraction with color correction.44 Subsequently, a color deconvolution tool based on the Ruifrok and Johnston method was used to determine the staining dye separation.45 Specific vectors were determined from control stained larvae. The resulting image was thresholded automatically. Finally, the total stained area was calculated.

2.5. Neutrophil recruitment

Zebrafish larvae of 9 dpf from the Tg(mpo:GFP) transgenic line[34], kindly provided by S. Renshaw from the University of Sheffield, were imaged to monitor neutrophil recruitment at the intestinal level in response to inflammation. This transgenic line expresses high fluorescence levels in circulating neutrophils, thus allowing for epifluorescent imaging. Larvae were anesthetized with tricaine and mounted laterally in 3 % (w/v) methylcellulose for live imaging. Images were captured using a Leica MZFL III stereomicroscope (Leica Microsystems GmbH, Germany). All images were captured using the same acquisition parameters. The intestinal region was photographed using a Plan Apo 1.00x objective, with the same exposure time and intensity for all larvae. Images were registered using the LAS-AF software suite, and the neutrophil number was quantified using the ImageJ 1.48 software. A region of interest (ROI) was defined, containing the anterior and posterior intestine, from the end of the intestinal bulb through the cloaca. Cropped zones were automatically thresholded and converted to binary images, where fluorescent cells were pictured in black against a white background. A watershed filter was then applied and the particle count was automatically determined. The results are expressed through the neutrophil recruitment index (NRI), according to the following formula:

$$\text{NRI} = \frac{\text{Mean neutrophil count in exposed larvae}}{\text{Mean neutrophil count in negative controls}}$$

2.6. rt-qPCR

A gene expression analysis was carried out to further determine the immune response of larvae to inflammatory agents. The transcript levels of ten genes related to innate immunity were monitored after exposure to the inflammation inductor agents. This was done to select the most appropriate markers. The selected genes were then monitored in enterocolitic larvae exposed to the evaluated probiotic strains. The evaluated genes are listed in Table 1 where the correspondent PCR primers are also shown. Zebrafish larvae of 9 dpf were euthanized and triturated in 500 µL of $_{dd}H_2O$. Total RNA was isolated with TRIzol®, following manufacturer instructions (Invitrogen Life Technology, Belgium). The RNA concentration and integrity were assessed with an Agilent 2100 Bioanalyzer (Agilent Technology, USA). RNA samples with an RIN (RNA Integrity Number) lower than 8.5 were discarded. The reverse transcription was carried out with TaqMan® Reverse Transcription, following the instructions of the manufacturer. cDNAs were synthesized from the RNA samples in a reverse transcription reaction (RT) containing 20 ng of RNA per assay. RT was performed in a mix containing 1x TaqMan RT buffer, 5.5 mM MgCl2, 500 µM dNTPs, 2.5 µM oligo-dT, RNase inhibitor (0.4 U/µL), and 1.25 U/µL MultiScribe reverse transcriptase (Applied Biosystems, Belgium). The mixture was incubated at 25 °C for 10 min and at 48 °C for 30 min. The enzyme was inactivated at 95 °C for 10 min. Quantitative PCR was performed with SYBR Green PCR master mix (Roche Diagnostic Rotkreuz, Switzerland) on a Roche Light Cycler 480. The reaction conditions were as follows: 50 ºC for 2 min and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1min. β-actin was used as a housekeeping gene. Each sample was tested in triplicate. The mean Ct of each sample was normalized against the housekeeping gene and the corresponding control. The relative quantification of each gene was calculated by the 2-ΔΔCt method, using the REST 2009 software (Qiagen, Germany).

Table 1: Primers used in this study for gene expression analysis

ABBREVIATION	GENE	DESCRIPTION	NCBI ID	SEQUENCE
ACT	Beta actin	Housekeeping gene	NM_131031.1	TGCTGTTTTCCCCTCCATTG TTCTGTCCCATGCCAACCA
IL_{1B}	Interleukin 1β	Proinflammatory cytokine	NM_212844.2	CATTTGCAGGCCGTCACA GGACATGCTGAAGCGCACTT
IL_{10}	Interleukin 10	Anti-inflammatory cytokine	NM_001020785	ATATTTCAGGAACTCAAGCGGG ACTTCAAAGGGATTTTGGCAAG
IL_{22}	Interleukin 22	Proinflammatory cytokine	NM_001020792.1	TGAGGGAGGGTCTGCACAG CACAAGCGGATGGCTGG
CCL_{20}	C-C Motif Chemokine Ligand 20	Inflammation-induced cytokine	NM_001113595.1	GAGTCCGCGATATGCTGTTTG GGCAGGCAGCCATTCATAAA
MYD88	Myeloid differentiation primary response gene 88	Inflammation mediator	NM_212814.2	CACAGGAGAGAGAAGGAGTCACG ACTCTGACAGTAGCAGATGAAAGCAT
NF-KB	Nuclear factor kappa beta	Immune response regulator	NM_001003414	AGAGAGCGCTTGCGTCCTT TTGCCTTTGGTTTTTCGGTAA
TNF_A	Tumor necrosis factor alpha	Proinflammatory cytokine	NM_131840.2	CTGGAGGAAACACCTGCATGT CACCGCATGCCCACAAA
TLR_1	Toll-like receptor 1	Pathogen-associated molecular pattern receptor	NM_001130593.1	GGAAGGTGGCACTAAGAGCCT TGATCGGTCGTGGAGGAGTT
TLR_2	Toll-like receptor 2	Pathogen-associated molecular pattern receptor	NM_212812.1	AGTCATTGTTCCTACGAGTCTCATC CCTCTTACCTCAGTTACAATTTATA
TLR_{22}	Toll-like receptor 22	Pathogen-associated molecular pattern receptor	NM_001128675.2	CCAGCTCTCGCCGTACCA TTGGGCCAGCGGATGT

3. Results

3.1. Toxicological evaluation

The survival rate of larvae exposed to different concentrations of TNBS and DSS are shown in Figure 1. Larvae exposed to a TNBS dose of 25 µg.ml^{-1} displayed a survival rate of 66 % after 72 hpe. The survival rate was 50 % after an exposure to 50 µg.ml^{-1}. Exposure to higher doses (75 and 100 µg.ml^{-1}) resulted in the death of all in the cohort. The DSS exposure at the lowest concentration assayed (0.25 %) did not significantly reduce the survival rate. The survival rate was 88 % after larvae were exposed to 0.5 % DSS. Larvae exposed to higher doses of TNBS or DSS largely resulted in the death of the entire cohort. Therefore, no further assays were carried out that included these concentrations.

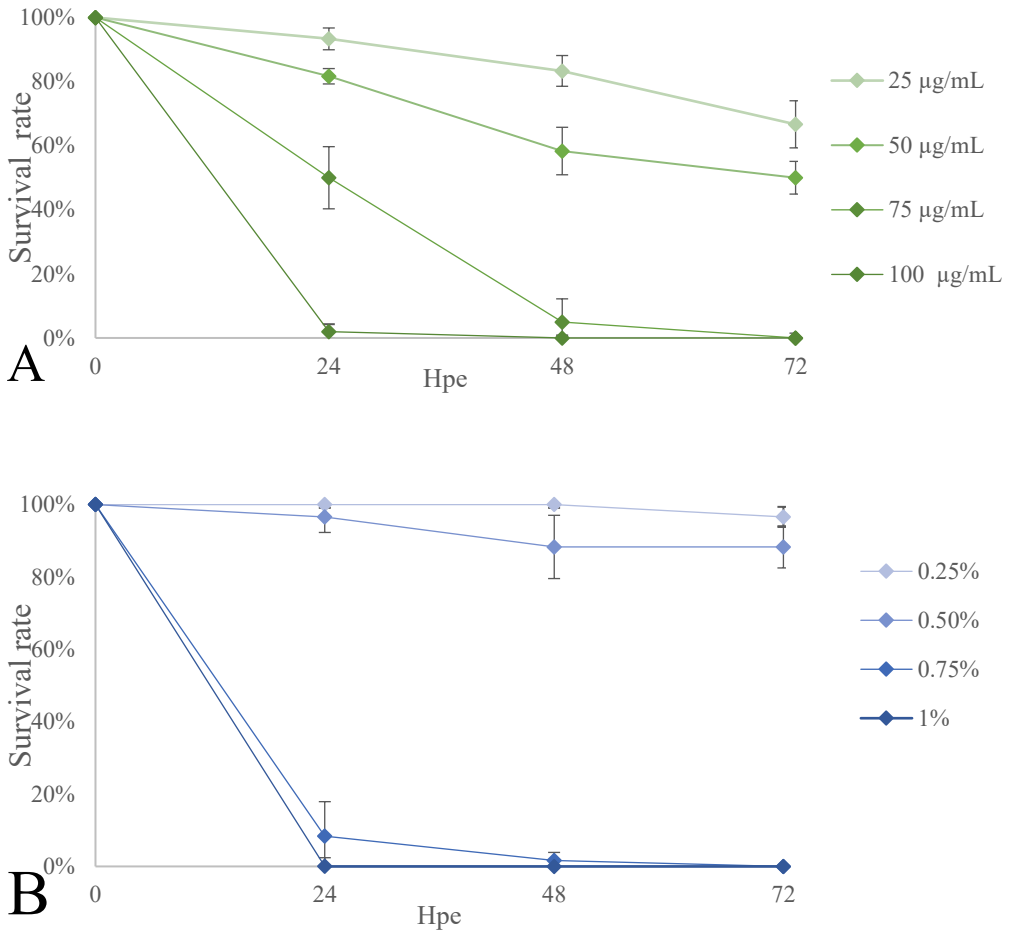

Figure 1. Percentage of surviving larvae after exposure to enterocolitis agents for 72 h. A. Survival rate after TNBS exposure. B. Survival rate after DSS exposure. Mean ±SEM is represented for each time-point. (n=60)

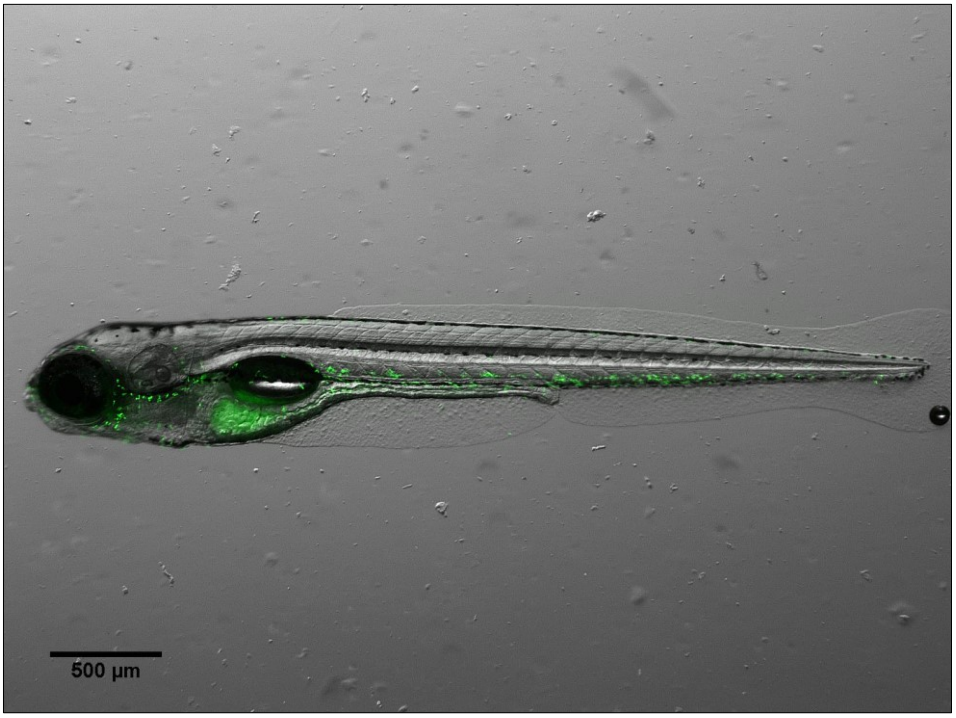

Figure 2. Micrography of Tg(mpo:GFP) zebrafish transgenic line in basal conditions. Neutrophils are located in the caudal hematopoietic tissue, situated between the caudal artery and the caudal vein.

3.2. Mucus production

Alcian Blue staining was performed on whole-mount larvae to visualize and quantify the acidic mucus production (Figure 3). The exposure to a 25 µg.ml^{-1} concentration of TNBS did not produce any significant increase in intestinal mucus. Mucus production in larvae exposed to 50 µg.ml^{-1} of TNBS was significantly increased compared to non-treated larvae. In contrast, the exposure to DSS did not significantly increase the mucus production at any concentration. However, exposure to 0.5 % DSS produced a high variability in mucus secretion (Figure 4). A dose of 50 µg.mL^{-1} TNBS was used to evaluate the potential effect of probiotic administration in the colitis model. Subsequently, larvae were incubated in the presence of TNBS and each of the evaluated LAB strains. Colonization by the evaluated probiotic strains did not have any significant effect on mucus secretion. The calculated NRI index did not significantly differ from larvae that were exposed to TNBS only.

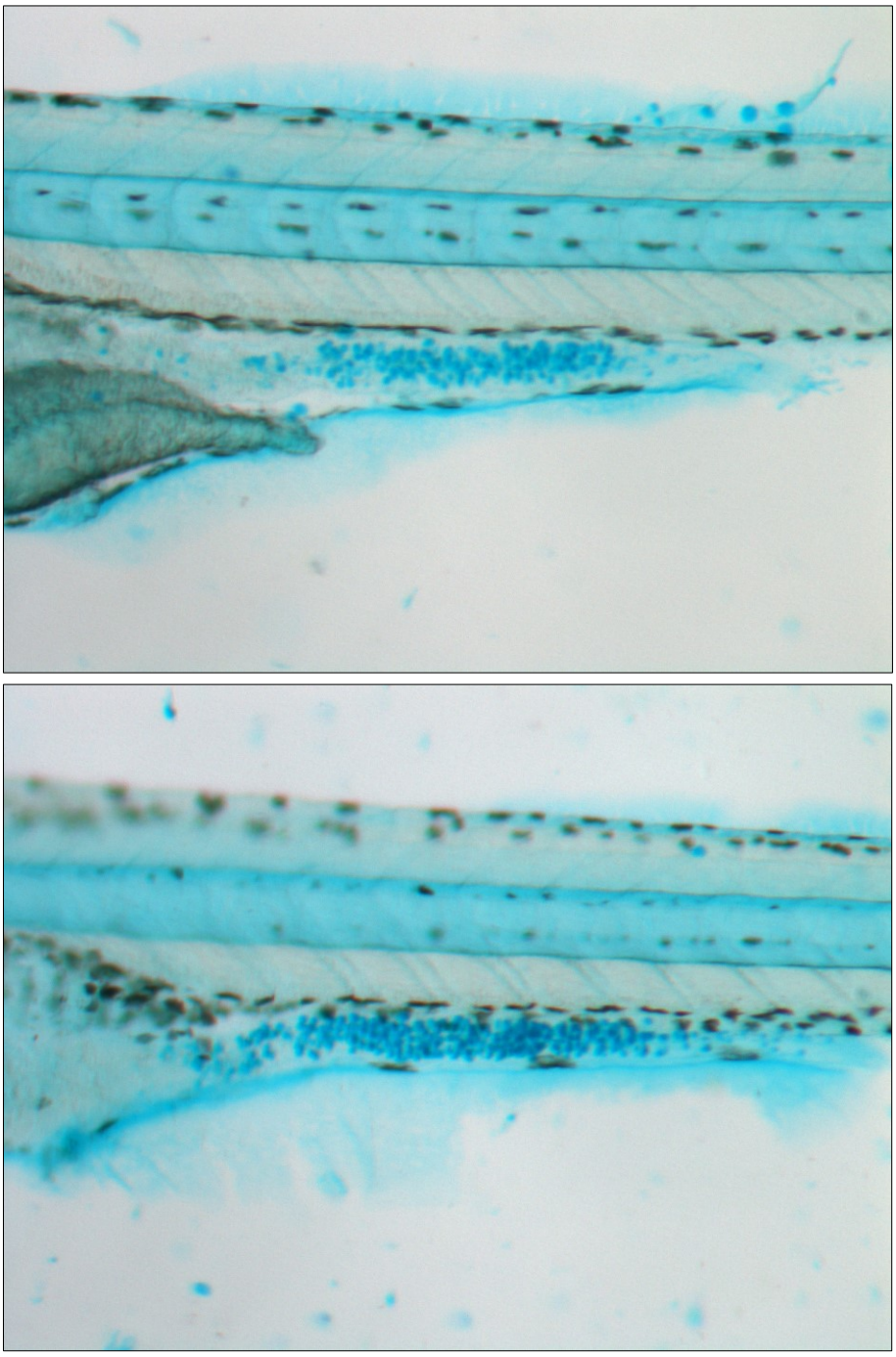

Figure 4. Micrography of the intestinal region of whole mount Alcian blue stained zebrafish larvae. **A.** Negative control. **B.** Larva exposed to 50 µg.mL^{-1} TNBS. The number of stained goblet cells is increased comparing to control.

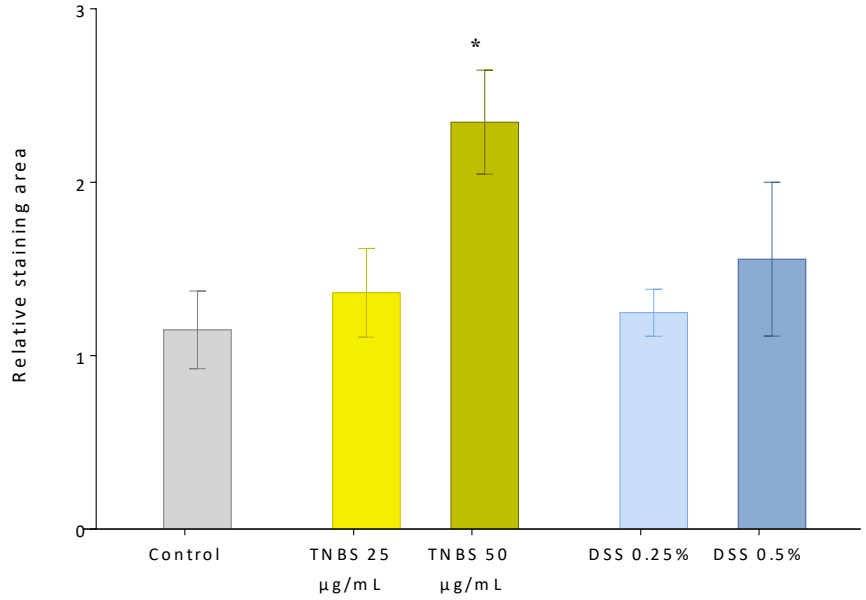

Figure 3. A. Intestinal mucus secretion in larvae exposed to enterocolitis agents. Results shows the mucus staining area of each group relative to the non-treated control staining area. **B**. Intestinal mucus secretion in larvae coexposed to 50 µg.mL^{-1} of TNBS and the evaluated LAB. Results show the mucus staining area of each group relative to the non-treated control staining area. Relative mean ±SD of each group is represented. An asterisk indicates significant differences to the control group (p≤0.05)

3.2 Analysis of neutrophilic inflammation response by live imaging

Live imaging analysis performed on transgenic zebrafish line Tg(mpx::GFP) allowed the evaluation of the neutrophil recruitment in the intestine after exposure to proinflammatory substances (Figure 2). The neutrophil per intestine mean count is listed in Table 2.

Table 2. Mean neutrophil count per intestine of 9-dpf larvae obtained by automatic fluorescent cell quantification. The mean and SEM are represented (n=12).

	Neutrophil per intestine
Control	21.08 (±0.21)
TNBS 25 µg/mL	27.67 (±0.30)
TNBS 50 µg/mL	52.17 (±0.22)
TNBS 75 µg/mL	57.92 (±0.56)
DSS 0.25%	23.33 (±0.23)
DSS 0.5%	44.42 (±0.33)

The quantification of the neutrophil recruitment revealed a dose-dependent effect of the exposure to TNBS. Larvae exposed to 25 µg.ml^{-1} exhibited a small but significant increase in neutrophil count. Exposure to a TNBS dose of 50 µg.ml^{-1} led to a strong neutrophil recruitment with an NRI index of 2.47 (Figure 7).. Remarkably, when the TNBS concentration was increased to 75 µg.ml^{-1}, the resulting NRI value was similar to that obtained with 50 µg.ml^{-1}, although a high variability was detected. The exposure to 0.25 % DSS did not produce a significant increase in the intestinal neutrophil count. In contrast, larvae exposed to 0.5 % DSS exhibited an NRI index of 2.11 (Figure 5).

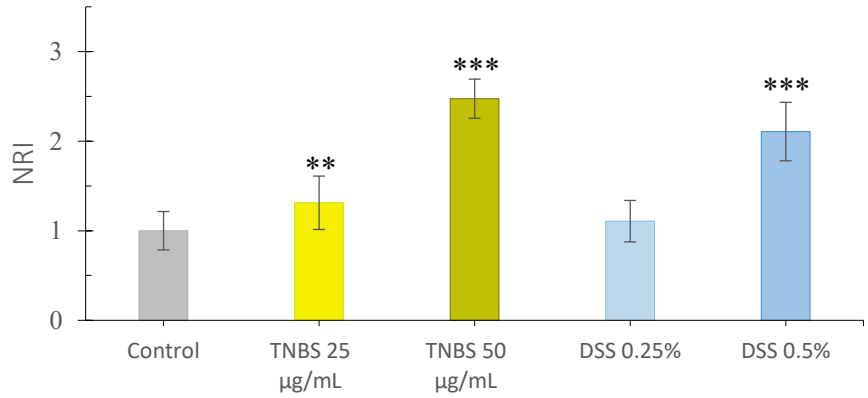

Figure 5. Neutrophil recruitment index values obtained after zebrafish larvae' exposure to inflammatory agents. Highest dose of TNBS and DSS elicited a strong response at neutrophils.

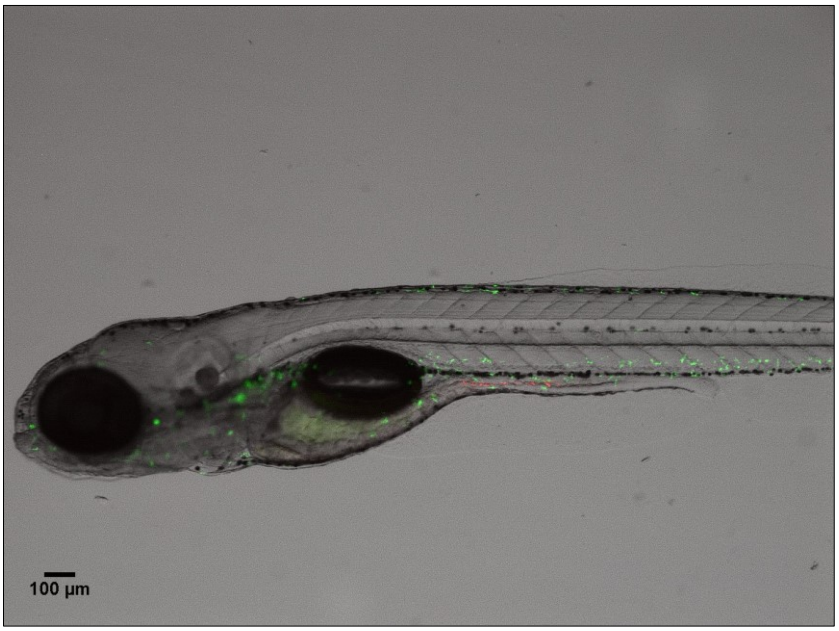

Figure 6. Micrography of Tg(mpo:GFP) zebrafish transgenic line exposed to *L. acidophilus LA5.* Red fluorescence produced by the bacterial is visible on the larva medium intestine.

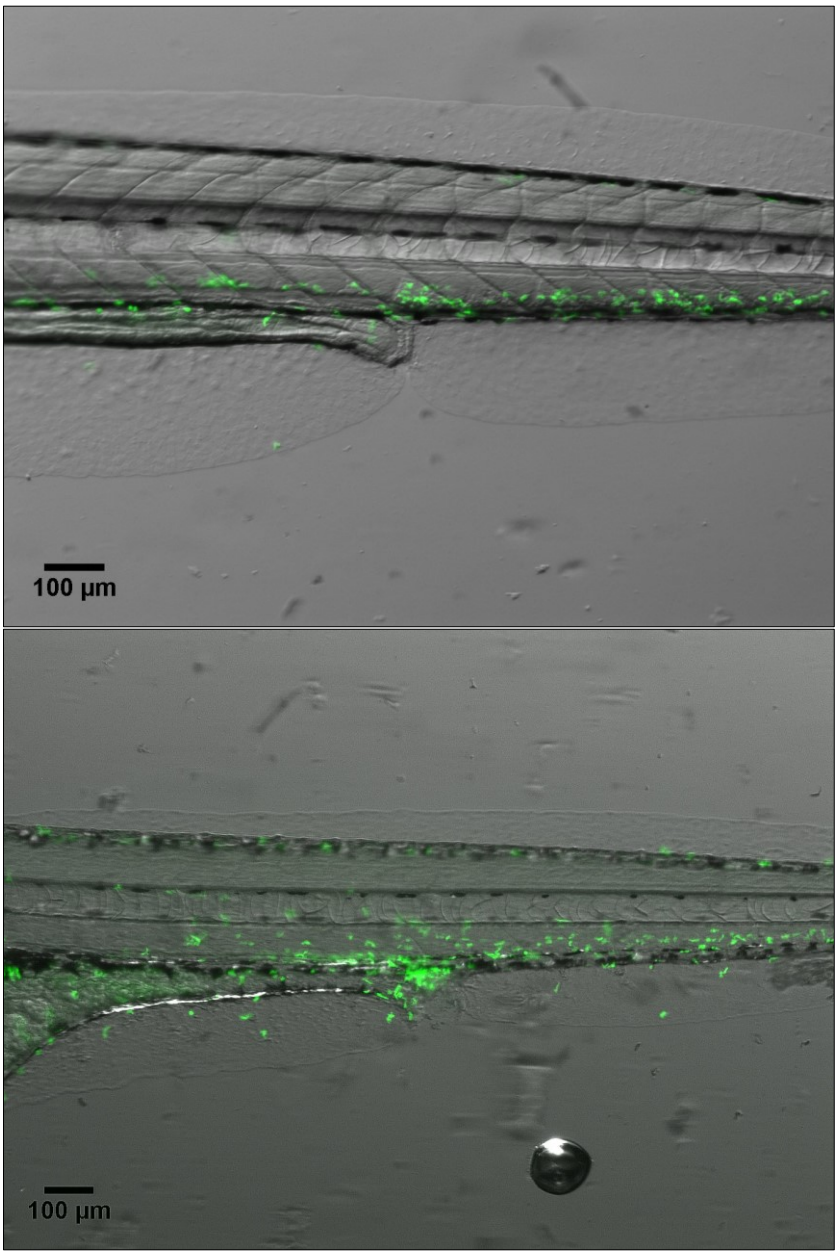

Figure 7. Combined fluorescent/bright-filed micrography of Tg(mpo:GFP) zebrafish transgenic line. **A.** Negative control. GFP^+ neutrophils are located on the caudal hematopoietic tissue. **B.** Larva exposed to 50 $\mu g.mL^{-1}$ of TNBS. Neutrophil recruitment is visible in the posterior intestine.

Embryos were then exposed to the evaluated LAB strains in the presence of TNBS to evaluate the anti-inflammatory effect on the neutrophil recruitment (Figure 6). Interestingly, the neutrophil recruitment was less accentuated when embryos were incubated in the presence of LA5, compared to embryos only exposed to TNBS (Figure 8).

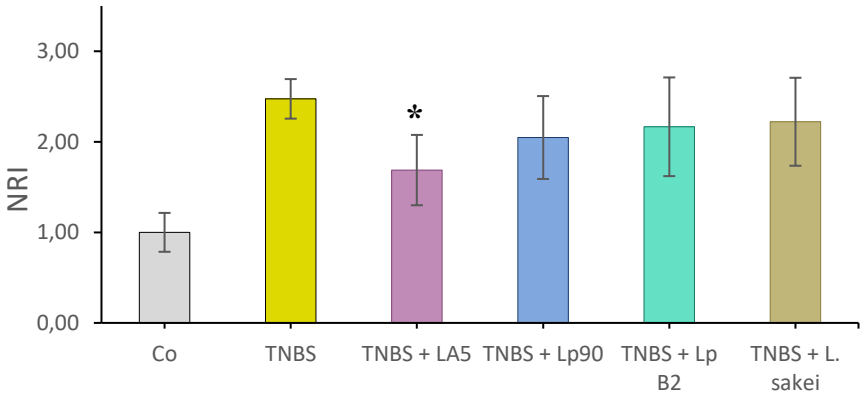

Figure 8. Neutrophil recruitment index values obtained after zebrafish larvae exposure to the probiotic bacteria. An asterisk indicates a statistically significant difference with TNBS only treated larvae. ($p \leq 0.05$)

3.3. rt-qPCR

The expression profile of innate response-related genes was determined in zebrafish larvae exposed to the two evaluated proinflammatory agents. Larvae of 9 dpf were exposed to TNBS and DSS in different concentrations, and the expression of several cytokines and other cellular mediators were monitored by quantitative rt-PCR. The results are shown in Figures 9 and 10. Low doses of TNBS did not significantly alter the expression of the evaluated genes in exposed larvae compared to control larvae. As expected, exposure to 50 µg.mL^{-1} TNBS significantly increased the expression levels of *Il$_{1\beta}$*, *CCl20*, *NF-κβ*, and *Tnfα* genes. In addition, a significant decrease in the transcript levels of the anti-inflammatory cytokine *IL$_{10}$* was also detected. There were not significant differences between larvae exposed to a low dose of DSS and the control samples. In contrast, exposure to 0.5 % DSS resulted in an up-regulation of the Ccl20 gene. It should be noted that a high variability was found in larvae exposed to 0.5 % DSS.

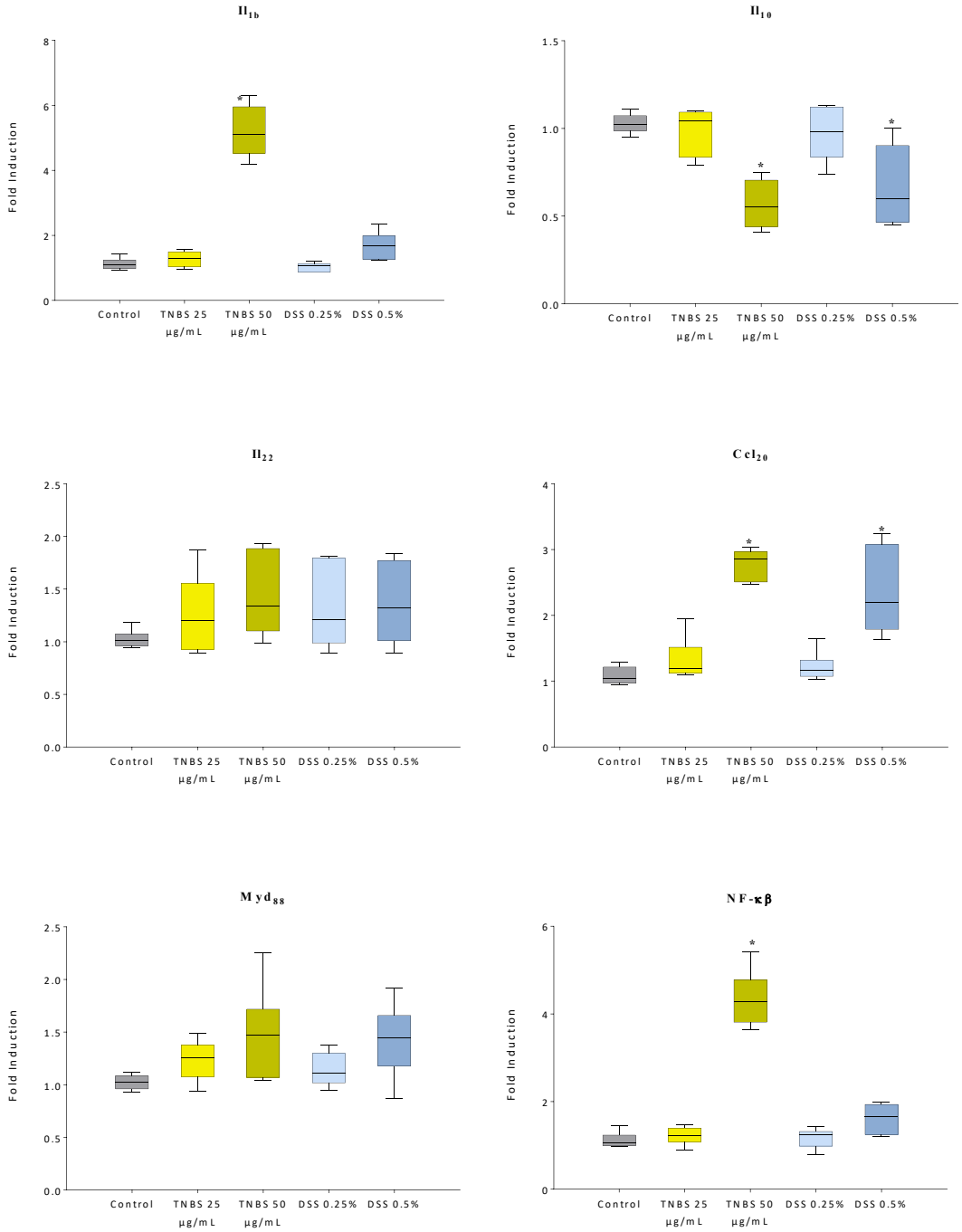

Figure 9. Relative expression levels of $Il_{1\beta}$, Il_{10}, Il_{22}, Ccl_{20}, Myd_{88}, and NF-κβ genes in zebrafish larvae of 9 dpf exposed to the pro-inflammatory substances. Box-plot indicates the values in the percentile range of 25-75. The median is represented by a line inside the box. Whiskers include the values in the percentile range of 5-95. Asterisks indicate a significant difference from the control ($p<0.0001$).

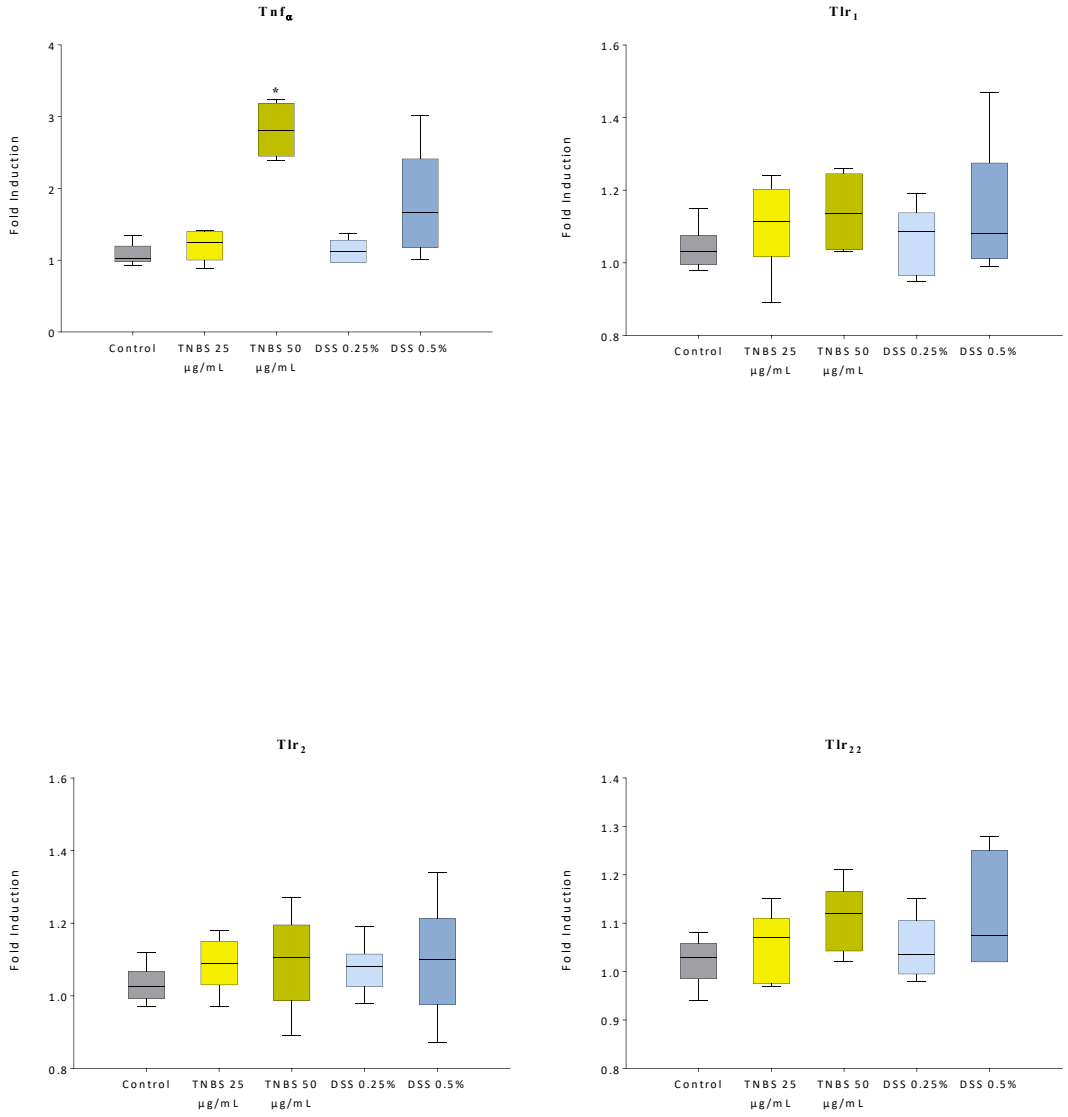

Figure 10. Relative expression levels of Tnfα, Tlr_1, Tlr_2, and Tlr_{22} genes in zebrafish larvae of 9 dpf exposed to the pro-inflammatory substances. Box-plot indicates the values in the percentile range of 25-75. The median is represented by a line inside the box. Whiskers include the values in the percentile range of 5-95. Asterisks indicate a significant difference from the control ($p<0.0001$).

Subsequently, the gene expression profile of larvae co-exposed to TNBS and the evaluated probiotics was determined to evaluate the probiotics immunomodulatory effect on zebrafish immunity (Figure 11). The results revealed an anti-inflammatory effect of strain LA5. Transcript levels of *Il1β* and *NF-κB* genes were significantly lower than in positive controls. Similarly, *Il10* transcript levels were similar to those of non-treated control larvae. Larvae exposed to Lp 90 also exhibited a slight decrease in *Il1β* transcript levels.

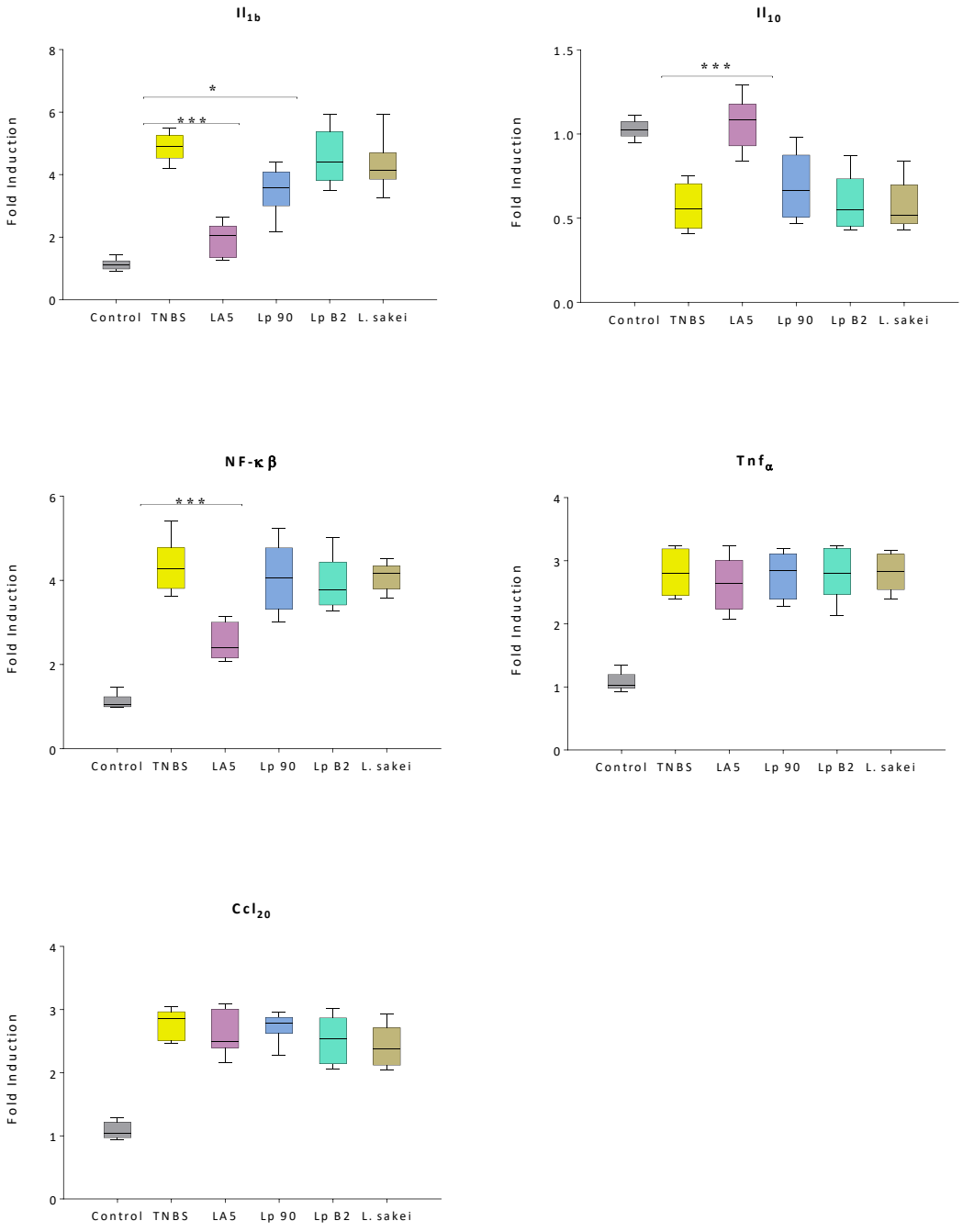

Figure 11. Relative expression levels of *Il1β, Il10, NF-κβ, Tnfα,* and *Ccl20* genes in larvae coexposed to TNBS and the evaluated LAB. Box-plot indicates the values in the percentile range of 25-75. The median is represented by a line inside the box. Whiskers include the values in the percentile range of 5-95. Asterisks indicate a significant difference from the control (* $p<0.05$; *** $p<0.001$)

4. Discussion

Irritable bowel disease represents a group of chronic pathologies that affect the colon and the small intestine and are characterized by abdominal pain, vomiting, diarrhea, muscle clamps, and weight loss. Although no specific disease blood markers have yet been defined, the conditions can be identified by the location and the nature of the inflammatory symptoms[46]. The earliest cases of disease were first described in the 19th century.[47] However the incidence rate has increased exponentially in the last 40 years in the developed world,[48] as well as in developing countries over the past decade.[49] The onset of IBD is caused by a combination of genetic predispositions and environmental factors. Moreover, several susceptibility genes have been identified. Studies with large groups of patients identified that homozygous mutation in the *CARD12* gene (encoding for NOD_2 receptors) was present in 15% of the patients.[50,51] The mutation impairs the recognition of pathogen-associated molecular patterns (PAMPS) by NOD_2 receptors. Studies in rodents have demonstrated that the absence of NOD_2 expression can lead to TLR signaling alteration and a subsequent increase of proinflammatory cytokine production by monocytes and macrophages, as well as defective activation of NF-κβ, contributing to intestinal disbiosis.[52] Recently, genome-wide association studies (GWAS) have identified more than 160 genetic loci containing susceptibility genes for IBD.[53]

In this work, an array of tests to evaluate the immunomodulatory effect of LAB strains in a chemically induced zebrafish larva colitis model was performed. This firstly involved comparing the effect of two chemical enterocolitis agents used in animal models: TNBS and DSS. The extent of the induction of chemical enterocolitis is dependent on the microbiota and is influenced by the specific microbial environment of each laboratory. This meant that the experimental conditions had to be adjusted for each case.[25] To that end, an initial toxicologic test was performed to determine the optimal dose that led to intestinal inflammation while avoiding extra-intestinal damages. It was found that concentrations higher than 50 $\mu g.mL^{-1}$ for TNBS and 0.5 % for DSS led to extensive extra-intestinal damage and 100 % mortality after 72 h exposure. Hence, lower concentrations were selected for the subsequent assays. The selected concentrations are similar to those reported in other works. Oehlers et al. have determined that a dose of up to 50 $\mu g.mL^{-1}$ TNBS produced 30 % mortality after three days, while a dose of 75 $\mu g.mL^{-1}$ led to a rate of mortality of more than 80 %.[56] However, other authors did not register such mortality or extra-intestinal damage with a dose of 75 $\mu g.mL^{-1}$.[19] It should be noted that the research was carried out on germ-free larvae, and that the presence of microbiota is a crucial factor in the inflammation induction[57].

In physiological conditions, the mucus layer isolates the epithelial surface from the luminal content. However, in IBD patients, the epithelial surface is exposed to commensal and pathogenic bacteria, triggering a chronic inflammatory response. An acidic mucus staining was performed in this study to estimate the mucus production

alterations in response to an inflammation. A significant increase in the staining area was observed in response to a dose of 50 µg.mL^{-1} TNBS. The increase in globet cell number was mainly located in the medial intestine. In contrast, DSS exposure did not produce a significant increase in the staining area, albeit exposure to 0.5 % DSS produced highly variable results. Some larvae exhibited an important increase of alcian blue staining area in the intestinal bulb, while the majority did not undergo any alteration. It is here hypothesized that this work's methodology failed to produce a complete staining by underestimating the mucus located on the intestinal lumen. Indeed, other works have described a mucus-phenotype after exposure to DSS. An intestinal lumen mucus accumulation is present in those cases, with no difference in the number of mucus-producing goblet cells.[58]

Neutrophils are the most numerous immune cells in both humans and zebrafish[59] These highly motile phagocytic cells play a critical role in immune response to injury and infections, being the first immune cells to migrate to the site of injury, where they carry out their phagocytic activity and secrete antimicrobial proteins. The GFP^{+}-neutrophil recruitment at GIT level was evaluated on larvae exposed to each proinflammatory agent. The highest evaluated doses of both agents produced a consistent neutrophil relocation from the caudal hematopoietic tissue to GIT. GFP-expressing neutrophils were mainly located at the perianal zone when exposed to TNBS. Finally, neutrophil concentration in the skin and gills was also observed, indicating an extra-intestinal inflammatory effect of TNBS. The results obtained in the present work are in agreement with the study of Oehlers et al.[56], which first described the neutrophil recruitment evaluation in a zebrafish larva colitis model. The authors further observed a strong neutrophil recruitment at GIT when exposing 3-dpf larvae to a dose of 50 µg.mL^{-1} TNBS or 0.5% DSS and no inflammation induction with lower doses. Interestingly, the colitis agent dose had to be increased when small volumes and high larva densities were used in a 48 well plate format, indicating an accumulation or metabolization of enterocolitic compounds by larvae.

Finally, the expression of several genes was monitored by rt-qPCR in 9-dpf larvae to further characterize the host's innate response to the enterocolitic agents. Several innate immune-related genes were assessed: *Tnf-α* and *Il1β* are classical proinflammatory cytokines; *Il10* is an anti-inflammatory cytokine involved in both cell-mediated and humoral immune responses; Il_{22} is a mucosa-associated proinflammatory cytokine related to autoimmunity and tissue regeneration[60]; *Ccl20* is a cytokine with a strong chemotaxis effect on lymphocytes and neutrophils. Two signal transductor proteins (Myd_{88} and NK-κβ), as well as TLR receptors, were also monitored. As expected, a TNBS dose of 50 ug.mL^{-1} was able to elicit a strong inflammatory response, where $Il_{1\beta}$, CCl_{20}, NF-κβ, and Tnf_{α} gene expression was up-regulated. In addition, Il_{10} gene expression was lower than in non-treated larvae. In contrast, exposure to DSS only up-regulated the expression of *Ccl20*. Oehlers et al. have also detected an up-regulation of *Il1β*, *Tnfα*,

CCl_{20}, and *Il_8* genes in TNBS-exposed larvae[56]. Remarkably, the authors demonstrated that the inflammatory state was reverted when antibiotic treatment was administered, demonstrating the influence of the microbiota on the onset of inflammation.

Summarizing previous results, a TNBS dose of 50 ug.mL^{-1} was selected as a enterocolitis model to evaluate the immunomodulatory properties of evaluated LAB strains. To that end, the probiotic strains were administered to TNBS-treated zebrafish larvae. Subsequently, the larvae were subjected to the set of tests described above. This was done to evaluate the immunomodulatory effect of the probiotic strains on the larva immunity.

The LA5 strain, whose probiotic effect is well established[39,61–63], elicited a strong anti-inflammatory effect in the zebrafish larva colitis model. Larvae treated with the probiotic strain LA5 exhibited a decrease in the NRI value, indicating a positive effect on neutrophil recruitment. The neutrophil infiltration at GIT level was reduced compared to TNBS positive controls, albeit the detected value was still higher than that detected in control larvae. In the same way, the gene expression analysis of innate immune-related genes showed an immunomodulatory effect over the host. Transcript levels of $Il_{1\beta}$ and NF-κβ genes in LA5 treated larvae were lower than those found in TNBS positive controls. Transcript levels of the anti-inflammatory cytokine Il_{10} were also increased, indicating an anti-inflammatory effect. Similar results have been demonstrated in enterocolitic models of zebrafish and other animals. Rieu et al. have detected a reduction in Tnf_{α} and IL_{10} transcript levels in TNBS-exposed zebrafish larva after the administration of the *Lactobacillus casei ATCC334* probiotic strain.[19] Administration of the probiotic mixture VSL#3 in a mice colitis model induced a immunoregulatory effect, mediated by an increase of Il_{10} levels and ameliorating the severity of recurrent colitis.[64] Similarly, exposure to strain Lp 90 induced a reduction of $Il_{1\beta}$ transcript levels. The co-exposure to the evaluated LAB failed to produce any modification relative to mucus production. The relative staining area remained similar to larvae only exposed to TNBS. In contrast, other works indicated a reversion of histopathologic changes, with a reduction of epithelial lesions and an absence of dilated goblet cells in TNBS-exposed rats treated with probiotic *L. fermentum 5716*[65] and *L. plantarum*[66] strains.

5. Conclusions

The present work has developed a set of tests using a zebrafish chemical colitis model to evaluate the immunomodulatory effect of probiotic administration. Firstly, the effect of two of the most used chemical colitis agents (TNBS and DSS) have been characterized and compared in the zebrafish animal model. Several tests, including toxicologic evaluation, histologic staining, image-based analysis of neutrophil recruitment, and gene expression analysis have been carried out. TNBS was identified as being the most suitable chemical model. The zebrafish colitis model demonstrated to be a useful screening method for the evaluation of the immunomodulatory profile of new probiotic strains.

6. References

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Chapter III

Evaluation of the protective effect of probiotics against digestive pathogens

1. Introduction

Gastrointestinal infections are a source of significant mortality and economic burden worldwide. According to the WHO, more than two billion cases of diarrheal disease are registered worldwide every year[1], causing the dead of more than 1.9 million children younger than 5 years specially in developing countries. In industrial countries, the mortality caused by those diseases is generally low, but it represents an important cause of morbidity and health care cost. Antibiotic therapy is useful to shorten the duration of bacterial-caused cases, but have no effect on cases of viral or non-infectious etiology. Unfortunately, the massive use of antibiotic during the XX century, including misuse and under-dosing, has contributed significantly to the selection of resistant strains[2]. Many bacterial pathogens related to human diseases have evolved into multi-resistant strains. The most prevalent Gram-negative pathogens, such as *Escherichia coli*, *Salmonella enterica*, and *Klebsiella pneumoniae*, cause a variety of diseases in humans and animals, and a strong correlation between antibiotic use in the treatment of these diseases and antibiotic resistance development has been observed over the past half-century[3]. In this context, probiotic therapy has proved to be an alternative option to overcome a variety of digestive pathologies. Some probiotic strains have demonstrated to be useful reducing the severity and duration of acute diarrhea[4], antibiotic-associated diarrhea[5,6], infection by *Clostridium difficile*[7]. In the same way, a meta-analysis of randomized trials indicated that complementation of *Helicobacter pylori* antibiotic therapy with certain probiotics may also be effective in increasing eradication rates[8,9]. Nevertheless, those health benefits must be linked to specific strains of probiotics. A selected strain should undergo *in vitro* and *in vivo* testing on animal models, and ultimately human clinical trials to demonstrate the claimed benefits.

In this work, an intestinal infection model was developed using gnotobiotic zebrafish as an animal model, to determine the antimicrobial effect of new probiotic strains. The study of the interplay between the host immune system and the digestive microbiota is complex due to the diversity of microorganisms found in the intestinal tract. Those difficulties may be reduced using gnotobiotic animal models, in which the microbiota is either known or absent. In this method, the studied animal models are bred in a sterile environment and can be colonized with the selected bacterial strains. This way the effect of the colonization can be studied avoiding the influence of other microorganisms[10,11]. Furthermore, the use of zebrafish provides a number of advantages that simplify the generation of gnotobiotic organisms as compared to mammalian models. The external fertilization of zebrafish eggs and the embryo development within their protective chorions eases the gnotobiotic embryo generation. In this research, axenic larvae were obtained through a combination of chemical and physical sterilization. Subsequently, axenic larvae were colonized with the studied bacteria to evaluate their effect on the host.

Two bacterial strains were evaluated as digestive pathogens: *Vibrio anguillarum NB10* (VAN), an invasive pathogen of fish causing a septicemia called vibriosis[12]; and *Enterococcus faecalis* OG1RF, a Gram-positive, bacterium which can cause endocarditis septicemia, urinary tract infections, meningitis, and other infections in humans[13]. Both pathogens express the GFP fluorescent protein[14,15], allowing to monitor the progression of the infection by fluorescent microscopy. The bacterial load on infected larvae was also evaluated by direct plate count. Additionally, the immune response to the infection was monitored. After the most suitable pathogen was selected, zebrafish larvae were co-exposed to the pathogen and the LAB strains selected in this work to evaluate the protective effect against the infection and the modulatory effect over the host immune system.

2. Materials and Methods

2.1. Experimental organism

2.1.1. Zebrafish breeding

Adult zebrafish (*Danio rerio*) were raised and maintained at 27 ± 1 °C with a 12 h light/dark cycle in 60 L tanks. Tank water was filtered continuously through biological, chemical, and physical filtration system. Fish were fed with *Artemia nauplii* and commercial dry feed (Gemma Micro 300, Skretting). Embryos were collected through natural breeding and maintained in embryo water (EW) (sterilized deionized water solution containing $CaCl_2$ 294 $mg.L^{-1}$, $MgSO_4$ 123 $mg.L^{-1}$, $NaHCO_3$ 64.7 $mg.L^{-1}$, KCl 5.7 $mg.L^{-1}$) at 27 ± 1°C. At 24 hpf, embryos were inspected under a microscope, and dead or malformed embryos were discarded. All experimental procedures were approved by the regional animal welfare body.

2.1.2. Axenic embryos production

Axenic embryos were obtained following the procedure described in section 2.1.2 of chapter I.

2.2. Bacterial strain and exposure

2.2.1. Bacterial strains

The following fluorescent mCherry-labelled LAB strains were used in the present study: *Lactobacillus plantarum Lp90, Lactobacillus plantarum B2 (CECT8328), Lactobacillus sakei MN1, and Lactobacillus acidophilus 5.* The designated strains were described in section 2.2.1. of chapter II. LAB were routinely grown in MRS containing chloramphenicol (Cm) at 10 $\mu g.mL^{-1}$ at 37 ºC. Two fluorescent GFP-labeled bacterial strains were evaluated as intestinal infection models in zebrafish larva: *Vibrio anguillarum* NB10 serotype O1[14] (VAN) and, *Enterococcus faecalis* OG1RF[15] (EFA). VAN was grown at 25ºC in TSB (Tryptone soya broth, Oxoid) supplemented with 10 $\mu g.mL^{-1}$ chloramphenicol, and 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce GFP expression. EFA was grown at 37ºC in BHI (Brain heart infusion, Oxoid) supplemented with 5 $\mu g.mL^{-1}$ tetracycline.

2.2.2. Bacterial exposure

Infection of zebrafish larva. Overnight cultures of EFA and VAN were washed three times with PBS and resuspended at 10^7 $cfu.mL^{-1}$ in PBS (EFA) or PBS + NaCl 0.5% (VAN). Larvae of 5 dpf were placed in individual wells of a 24 well plate, along with 1 mL of a solution containing the bacterial dilutions. After 24 h, the media was replaced with a sterile PBS (or PBS + NaCl 0.5 %) solution.

Co-exposure procedure. Larvae were co-exposed to the evaluated LAB strains and pathogen bacteria to evaluate the protective effect of probiotic administration against a digestive infection. To that end, the continuous exposure described section 2.2.3 of chapter I, was carried out with some modifications (Figure 1). Twenty embryos of 4 dpf were immersed in 15 mL of a 10^7 cfu.mL^{-1} LAB dilution with 50 µL of sterilized 1 % (w/v) embryo feed dilution and incubated at 27 ±1 ºC with gentle shaking. At 5 dpf, the media was renewed with a dilution containing 10^7 cfu.mL^{-1} of LAB and 10^7 cfu.mL^{-1} of VAN dilution, as well as 50 µL of sterilized 1 % larva feed dilution. After 24 h, the solution was replaced with a dilution containing 10^7 cfu.mL^{-1} LAB and 50 µL of sterilized 1 % (w/v) larva feed dilution. Twenty embryos were placed in sterile PBS with 50 µL of sterilized 1 % embryos feed dilution as a negative control. embryos immersed for 24 h on VAN dilution were used as positive control. At the end of the exposure embryos can be considered larvae.

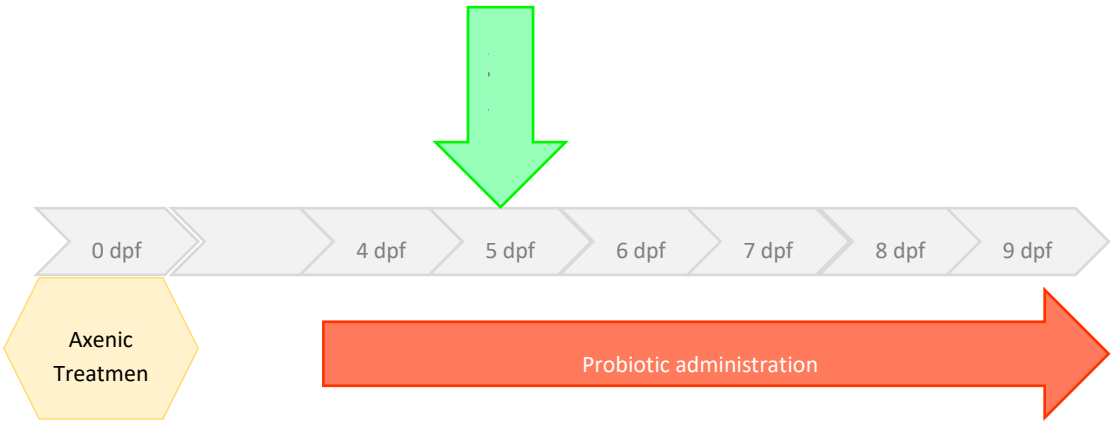

Figure 1. Schematic view of the bacterial exposure setting. Larvae were exposed to the pathogen bacteria for 24 h (Green arrow). The probiotic exposure spanned from 4dpf to the end of the experiment (Red arrow).

2.3. Infection monitorization

The evolution of the infection was monitored with two methods; 1) plate count, and 2) fluorescence quantification.

Plate count. Infected larvae of 3, 24 and 48 hpi (hours post infection) were euthanized by submersion in ice water for 15 min and rinsed several times in sterile PBS with 0.1% (v/v) Tween 20. Subsequently, larvae were individually homogenized in 100 µL of sterile PBS. Finally, serial dilutions of the homogenate were plated on TSB (VAN) or BHI (EFA) agar medium. Fluorescent colonies were counted after 72h of incubation at 25 ºC (VAN) or 37 ºC (EFA).

Fluorescence. Living larvae were observed in lateral view using a Leica M205 FA stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). Larvae were

anesthetized using tricaine (MS- 222) (Sigma-Aldrich, St Louis, MO) at 120 mg.mL^{-1} and placed laterally in 3% (w/v) methylcellulose. Larvae images were captured using a Leica DFC 360FX camera. mCherry fluorescence was detected by exposure to ultraviolet light in the excitation range of 545 nm. GFP fluorescence was detected by exposure to the excitation frequency of 488 nm. All images were captured using a Planapo 1x objective, with the same acquisition. Images were registered in lossless TIF format using the LAS-AF software suite (Leica Microsystems, Germany). The obtained images were subjected to automatic image analysis to quantify the fluorescence intensity. The image analysis was realized following the procedure described in section 2.3.3 of Chapter I.

2.4. Survival rate

Twenty axenic zebrafish larvae were infected with either VAN or EFA as described in the infection procedure in section 2.2.2. Infected larvae were monitored during 72 hpi. Survival and sub-lethal symptoms were registered. Larvae placed in sterile PBS solution were used as negative control. The experiment was repeated twice.

2.5. Acidic mucin production

Alcian blue staining was performed on whole mount larva as described in section 2.4 of chapter II. Fifteen larvae per condition were stained and observed through a Nikon SMZ1000 stereomicroscope (Nikon, Tokyo, Japan). Images were captured with a Nikon DN100 digital camera and stored in lossless TIF 16-bit color format. Manual cropping of GIT area was performed with an ROI selection. A macro was set up for automatic image analysis with ImageJ 1.48 software[20]. The macro performs an initial background subtraction with color correction, according to Landini et al.[21]. Following, color deconvolution tool, based on the Ruifrok and Johnston method[22], was used to determine the staining dye separation. Specific vectors were determined from control stained larvae. Finally, the total stained area was calculated.

2.6. rt-qPCR

Twenty zebrafish larvae were euthanized and homogenized in 500 µL of $_{dd}H_2O$. Total RNA was isolated with TRIzol® following manufacturer instructions (Invitrogen Life Technology, Merelbeke, Belgium). The RNA concentration and integrity were assessed with an Agilent 2100 Bioanalyzer (Agilent Technology, Santa Clara, Ca). RNA samples with a RIN (RNA Integrity Number) lower than 8.5 were discarded. The reverse transcription was carried out with TaqMan® Reverse Transcription following the instructions of the manufacturer. cDNAs were synthesized from the RNA samples in a reverse transcription reaction (RT) containing 20 ng of RNA per assay. RT was performed in a mix containing 1x TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM dNTPs, 2.5 µM oligo-dT, RNase inhibitor (0.4 U/µL), and 1.25 U/µL MultiScribe reverse transcriptase (Applied Biosystems). The mixture was incubated at 25 °C for 10 min and at 48 °C for 30 min, and

the enzyme was inactivated at 95 °C for 10 min. Quantitative PCR was performed with SYBR Green PCR master mix (Roche Diagnostic Rotkreuz, Switzerland.) on a Roche Light Cycler 480. Reaction conditions were as follows: 50 ºC for 2 min and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1min. *β-actin* was used as a housekeeping gene. Each sample was tested in triplicate. The mean Ct of each sample was normalized against the housekeeping gene and the corresponding control. The relative quantification of each gene was calculated by the 2-ΔΔCt method, using the REST 2009 software (Qiagen, Hilden Germany). The following genes were investigated: *$Il_{1\beta}$, Lyz, Mpx, Tlr_4* and *Tlr_{22}*. Primers sequences are listed in table 1.

	Gene	Description	NCBI ID	Sequence
act	Beta actin	Housekeeping gene	NM_131031.1	TGCTGTTTTCCCCTCCATTG
				TTCTGTCCCATGCCAACCA
$Il_{1\beta}$	Interleukin 1β	Pro-inflammatory cytokine	NM_212844.2	CATTTGCAGGCCGTCACA
				GGACATGCTGAAGCGCACTT
Lyz	Lysozyme	Antimicrobial enzyme	NM_139180.1	AGGCTGGCAGTGGTGTTTTT
				CACAGCGTCCCAGTGTCTTG
Mpx	Myeloid-specific peroxidase	Lysosomal protein	NM_212779	CAATGGCCCGCATAATCTG
				GCGAAAAGGATCTCTGGGAACT
Tlr_4	Toll-like receptor 4	Pathogen-associated molecular pattern receptor	NM_001131051.1	GGGAAGTCAATCGCCTCCA
				ACGGCTGCCCATTATTCCT
Tlr_{22}	Toll-like receptor 22	Pathogen-associated molecular pattern receptor	NM_001128675.2	CCAGCTCTCGCCGTACCA
				TTGGGCCAGCGGATGT

Table 1: Primers used in the present study.

2.7. Probiotic administration

Axenic zebrafish larvae were exposed to the selected pathogen and the evaluated LAB strains following the co-exposure procedure described in section 2.2.2. The survival rate and immune response to the co-exposure were characterized with the above-mentioned assays.

2.8. Statistical analysis

Results were expressed as the mean ± SEM. The significance of differences was determined using a one-way ANOVA, followed by Tukey's test for multigroup comparisons, using statistical software, GraphPad Prism 7 (GraphPad Software Inc.). A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Infection monitorization

The progression of the infection by VAN and EFA was evaluated *in vivo* by fluorescent microscopy, and by plate count after larva euthanasia. The bacterial load evaluation by plate count revealed and increasing cfu count during VAN infection (Figure 2). At 3 hpi the registered VAN load per larva was 6.18 (±2.4) 10^5 cfu. The bacterial load increased to 2.76 (±1.8) 10^6 cfu per larva after 48 hpi. Dead larvae presented higher bacterial loads. In contrast, the bacterial load of EFA infected larvae remained nearly constant (from 9.24 (±4.49) 10^4 cfu/larva at 3 hpi, to 1.24 (±2.7) 10^5 cfu/larva after 48 hpi).

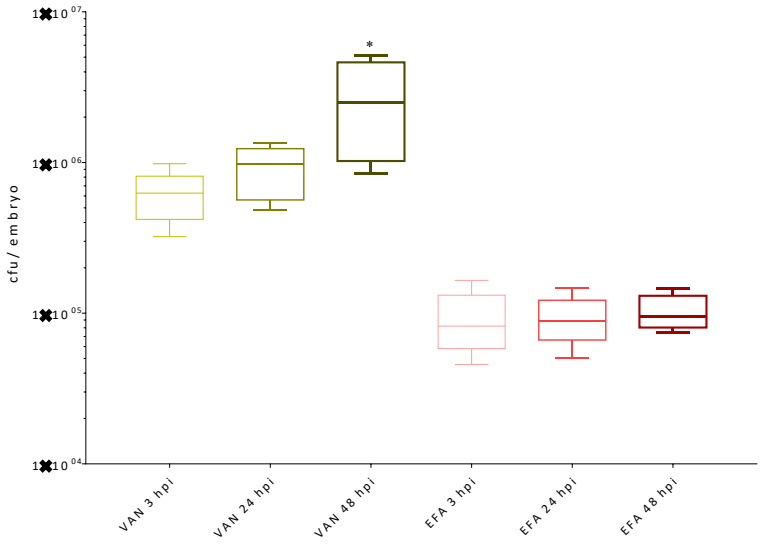

Figure 2. Bacterial enumeration of zebrafish larvae exposed to the bacterial pathogens. Ten larvae were individually homogenated and plated. Multiple comparisons by ANOVA test with Turkey´s post hoc test. * indicates p ≤0.001.

After exposure to both pathogens, fluorescent bacteria could be observed on the GIT starting from 3 hpi (figure 3). The fluorescence was mainly located in the first portion of the digestive tract. After 24 hpi, fluorescence was present throughout the entire GIT. However, fluorescent values observed in VAN-infected larvae presented a high variability at 24 hpi. Fluorescence was limited to specific sections of GIT on some observed larvae, while others presented extended fluorescence levels all over the digestive tract (Figure 3). In contrast, larvae infected with EFA showed a more uniform fluorescence, but the intensity was lower overall. After 48 hpi high fluorescence levels were detected in the full extent of the GIT, as well as in the head and gills in VAN exposed larvae. In contrast, the fluorescence levels found in EFA-infected larvae remained constant (Figure 4).

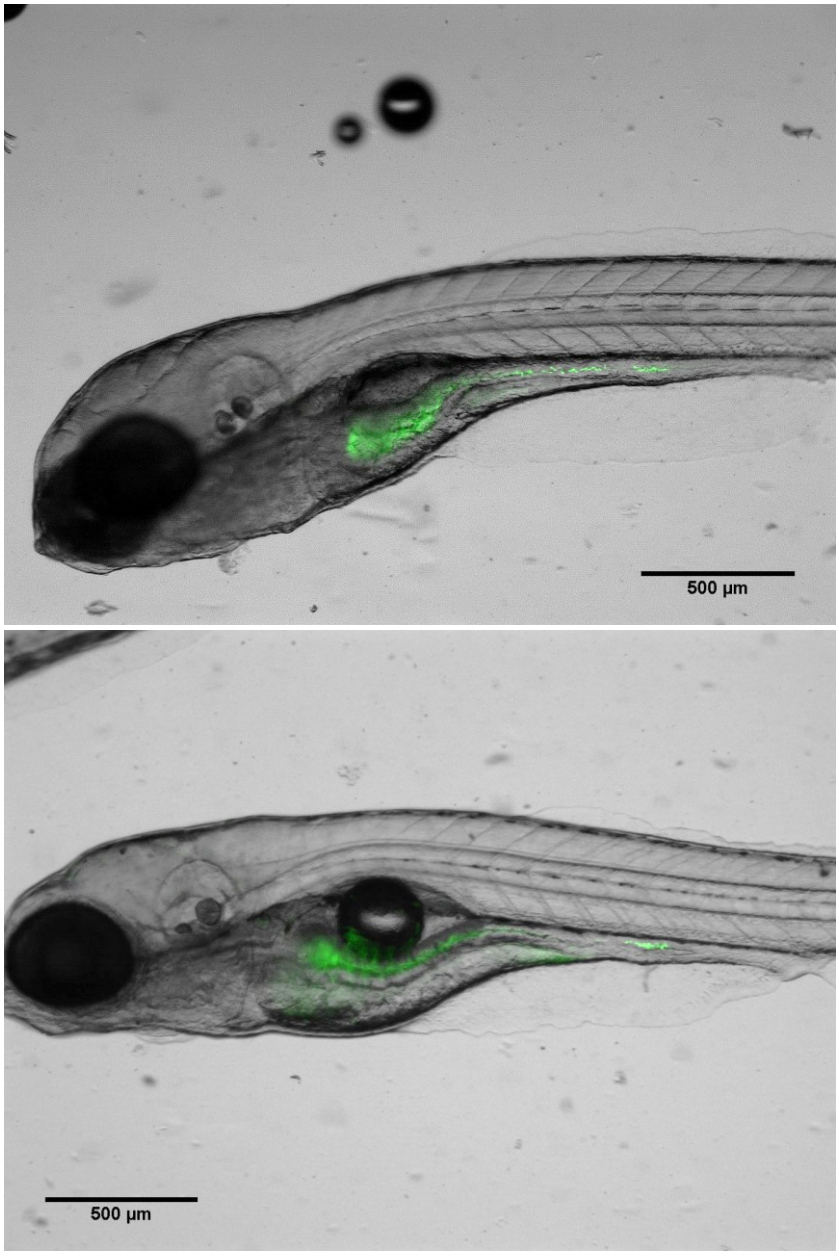

Figure 3. Micrography of zebrafish larvae 24 after the pathogen exposure. **Upper image.** *V. anguillarum NB10* infection. Strong fluorescence is observed all along the digestive track. **Lower image.** *E. faecalis OG1RF* infection. Fluorescence is limited to the intestinal bulb and the mid- intestine.

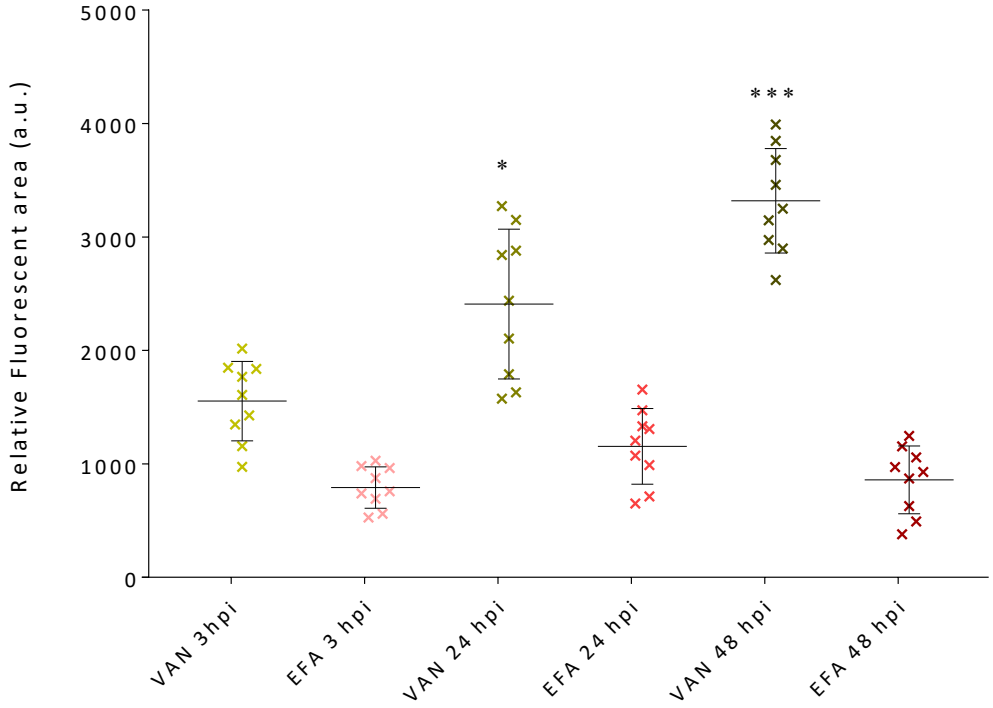

Figure 4. Fluorescence area of larvae exposed to *V. anguillarum NB10* and *E. faecalis OG1RF*. Error bars indicate the standard error of the mean. Multiple comparisons within ANOVA tests were carried out through the post hoc Tukey´s test. (p ≤0.05)

3.2. Survival rate

Infection with VAN resulted in a zebrafish survival rate of 66 % after 24 hpi (Figure 5). The survival rate decreased to 16 % after 48 h. After 96 h, almost all exposed larvae were dead. Lateral swimming and pericardial edema were observed in affected larvae starting from 24 hpi. In contrast, exposure to EFA did not have a significant impact on larvae survival rate when compared to controls. The survival rate detected after 72 hpi remained above 90 %. No malformations were observed among the survivors.

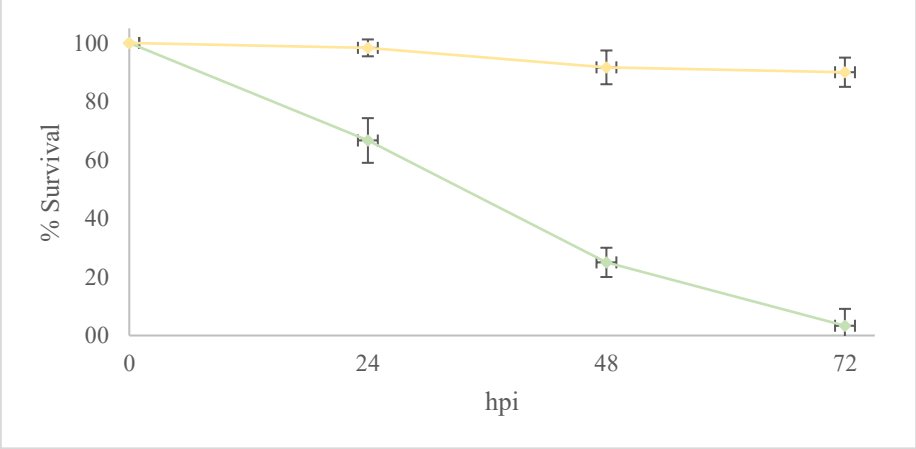

Figure 5. Survival rate of zebrafish larvae exposed to *V. anguillarum NB10* (green line) and *E. faecalis OG1RF* (yellow line). (n=20). The mean of each time point and the S.D. is represented.

3.3. Acidic mucin production

Mucus overproduction in response to an infection was visualized using alcian blue staining on gnotobiotic larvae 48 h after the infection with VAN or EFA. Larvae infected with VAN displayed a statistically significant increase in mucus production when compared to non-infected controls (Figure 6). The stained mucus was mainly located in the mid intestine. A significant increase in goblet cell area, as well as luminal mucus was detected. In contrast, EFA infection did not produce a significant change in stained mucus area even if a small increasing trend was observed.

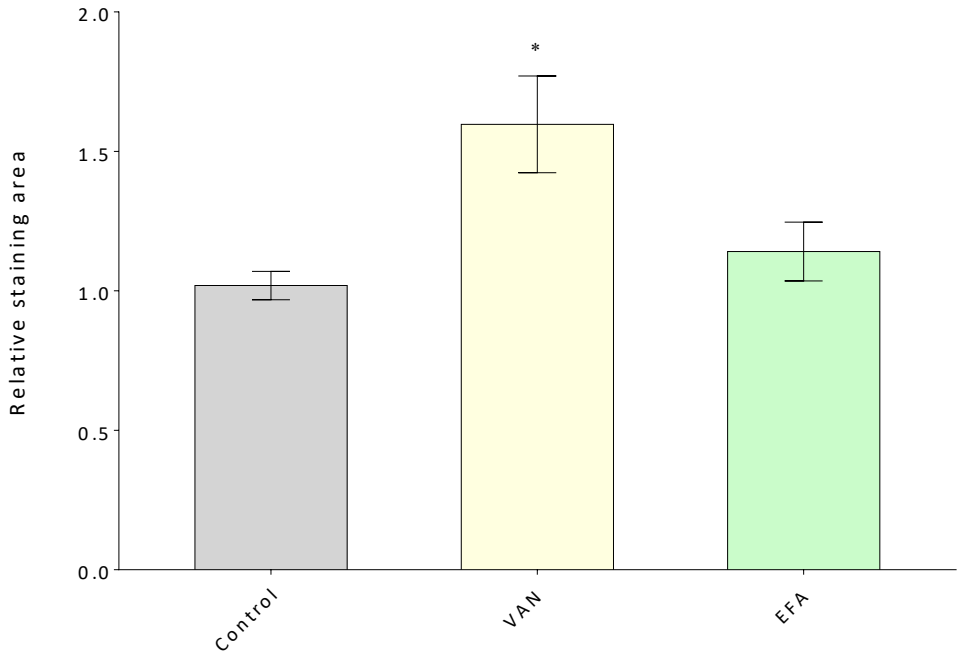

Figure 6. Acidic mucin relative stained area of zebrafish larvae infected with either *V. anguillarum NB10* or *E. faecalis OG1RF*. Total stained area relative to non-infected control larvae is represented.

3.4. rt-qPCR

The gene expression profile of four innate immunity related genes was compared after exposure to VAN, EFA and non-infected axenic larvae as controls (Figure 7). Exposure to VAN produced a strong inflammatory response. *Il$_{1\beta}$, mpx, tlr2* and *tlr22* transcript levels were significantly increased compared to control larvae. On the other hand, EFA exposure resulted in a slight upregulation of *tlr2* and *tlr22* PAMP receptors.

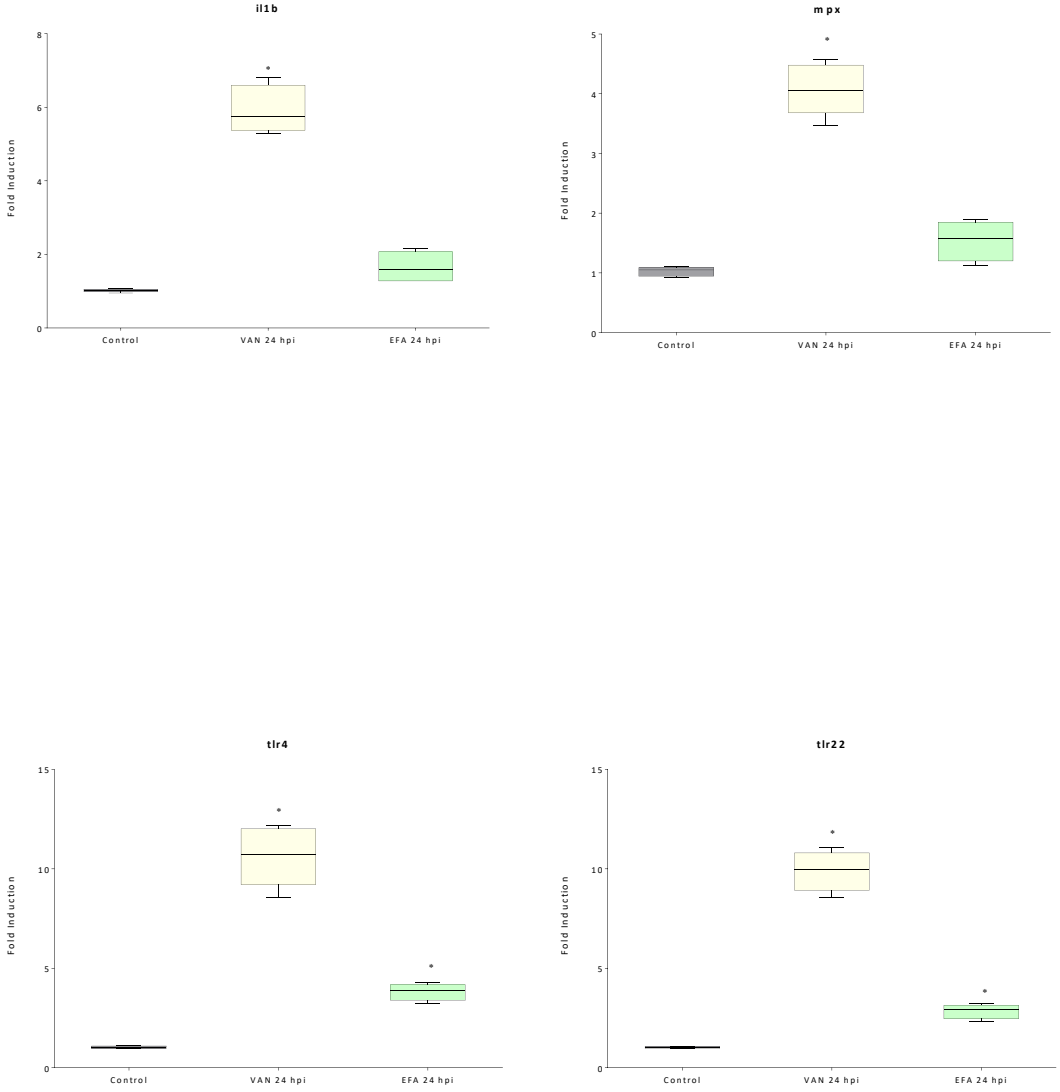

Figure 7. Gene expression profiles of zebrafish larvae infected with *V. anguillarum NB10* and *E. faecalis OG1RF*. Box-plot indicates the values between in the 25-75 percentile. The median is represented by a line inside the box. Whiskers include the values between in the 5-95 percentile. Asterisk indicate a significant difference with the control ($p<0.0001$).

3.5. Probiotic administration

VAN and EFA infection profile was studied in the previous sections. The results showed that exposure to VAN produced a higher mortality rate as well as a more consistent immune response. Therefore, *V. anguillarum NB10* strain was selected as infection model to evaluate the potential protective effect of LAB strains against a digestive pathogen on zebrafish gnotobiotic larvae. Consequently, axenic larvae were co-exposed to the pathogenic bacteria and the evaluated LAB strains and survival rate, as well as immunomodulatory effect, was investigated.

3.5.1. Infection monitorization

No significant differences were observed in probiotic-treated larvae when compared to controls. Nevertheless, a small decreasing trend was observed in *L. sakei*/G pretreated larvae (Figure 8).

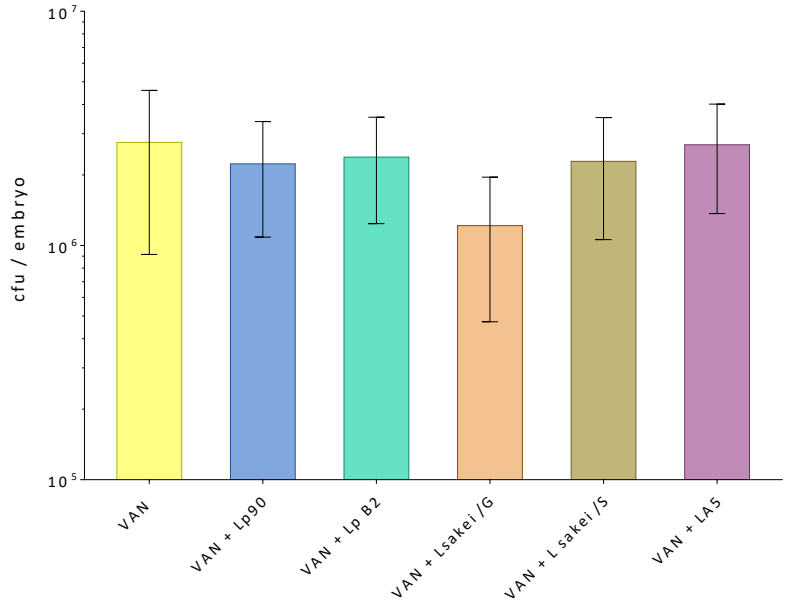

Figure 8. GFP-fluorescence area located on GIT of larvae co-exposed to *V. anguillarum NB10* and the evaluated LAB strains are represented. Error bars indicate the standard error of the mean. Multiple comparisons within ANOVA tests were carried out through the post hoc Tukey´s test.

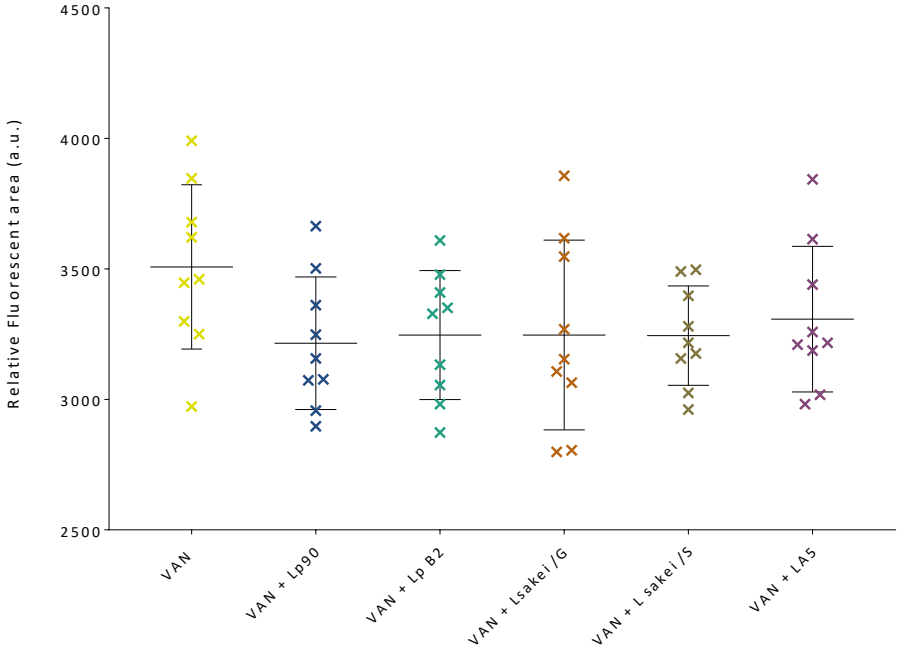

Figure 9. GFP-fluorescence area located on GIT of larvae co-exposed to *V. anguillarum NB10* and the evaluated LAB strains are represented. Error bars indicate the standard error of the mean. Multiple comparisons within ANOVA tests were carried out through the post hoc Tukey´s test.

GFP-fluorescent area, produced by VAN cells located on the GIT of larvae did not display any significant difference when larvae were pretreated with the evaluated LAB strains, although a high variability was observed (Figure 9).

3.5.2. Survival rate

Zebrafish larvae were pretreated with the evaluated LAB strains and infected with VAN (Figure 10). Non-pretreated larvae displayed a survival rate of 25 % after 48 hpi. Pretreatment with *L. sakei* increased the survival rate to 46 % when cultured in presence of sucrose, and 55 % when cultured in presence of glucose. Pretreatment with Lp_{90}, Lp B2 and LA5 strains did not have any influence on survival rate (Figure 11).

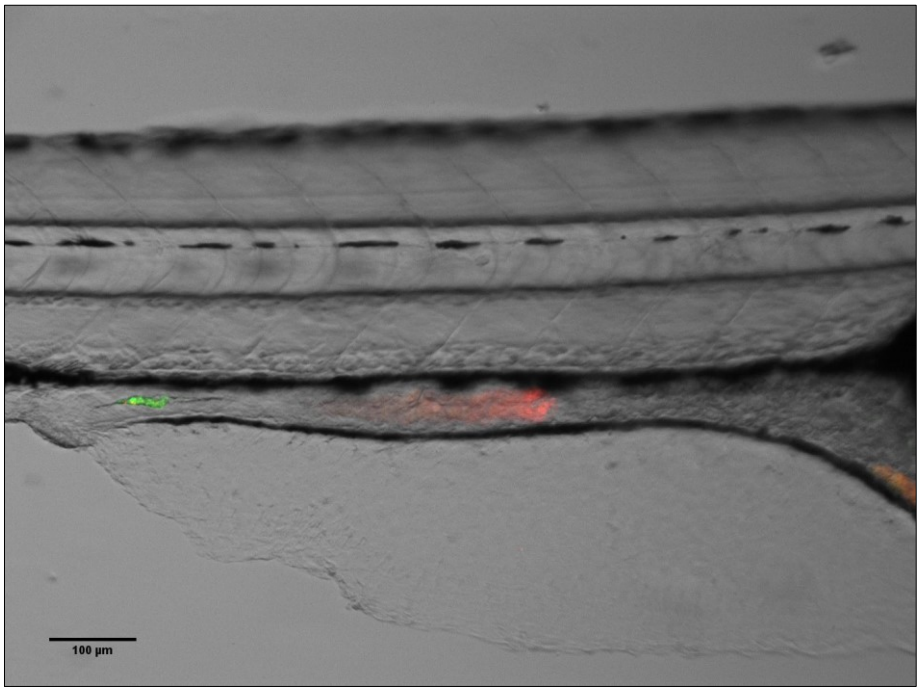

Figure 10. Micrography of the digestive system of a larva co-exposed to *V. anguillarum NB10* and *L. sakei MN1*. Red fluorescence emitted by the probiotic bacteria is located on the medial intestine. Green fluorescence emitted by the pathogen is visible on the posterior intestine.

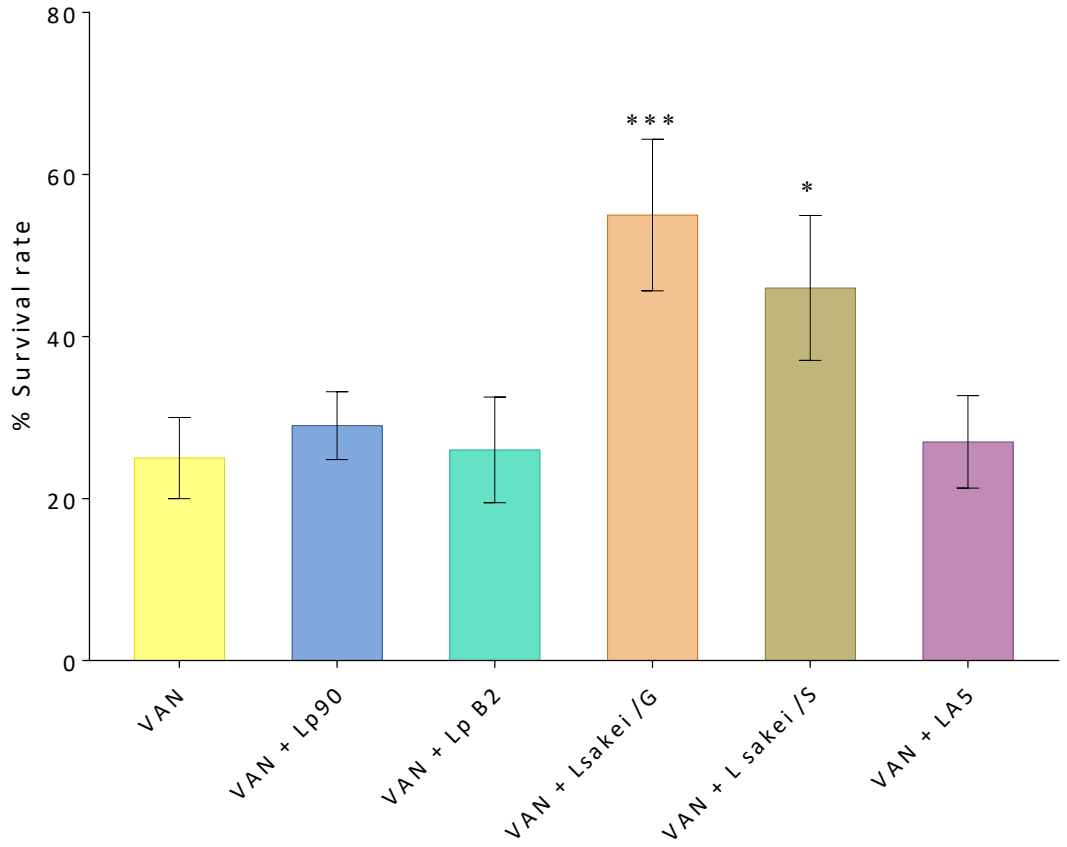

Figure 11. The survival rate of zebrafish larvae pretreated with the evaluated LAB strains and infected with *Vibrio anguillarum NB10*. The mean of 10 samples is represented. Error bars indicate the standard error of the mean. Multiple comparisons within ANOVA tests were carried out through the post hoc Tukey´s test. Asterisk indicate a significant difference with the control (* $p<0.005$; *** $p<0.0001$).

3.5.3. Acidic mucin production

Alcian blue staining performed on co-exposed zebrafish larvae did not reveal any difference after the pretreatment with any LAB strain, comparing to VAN-only exposed larvae (data not shown).

3.5.4. rt-qPCR

The gene expression analysis of co-exposed gnotobiotic larvae did not reveal any significant difference in the transcript levels of the evaluated innate immune-related genes, comparing to VAN-only infected larvae (figure 12).

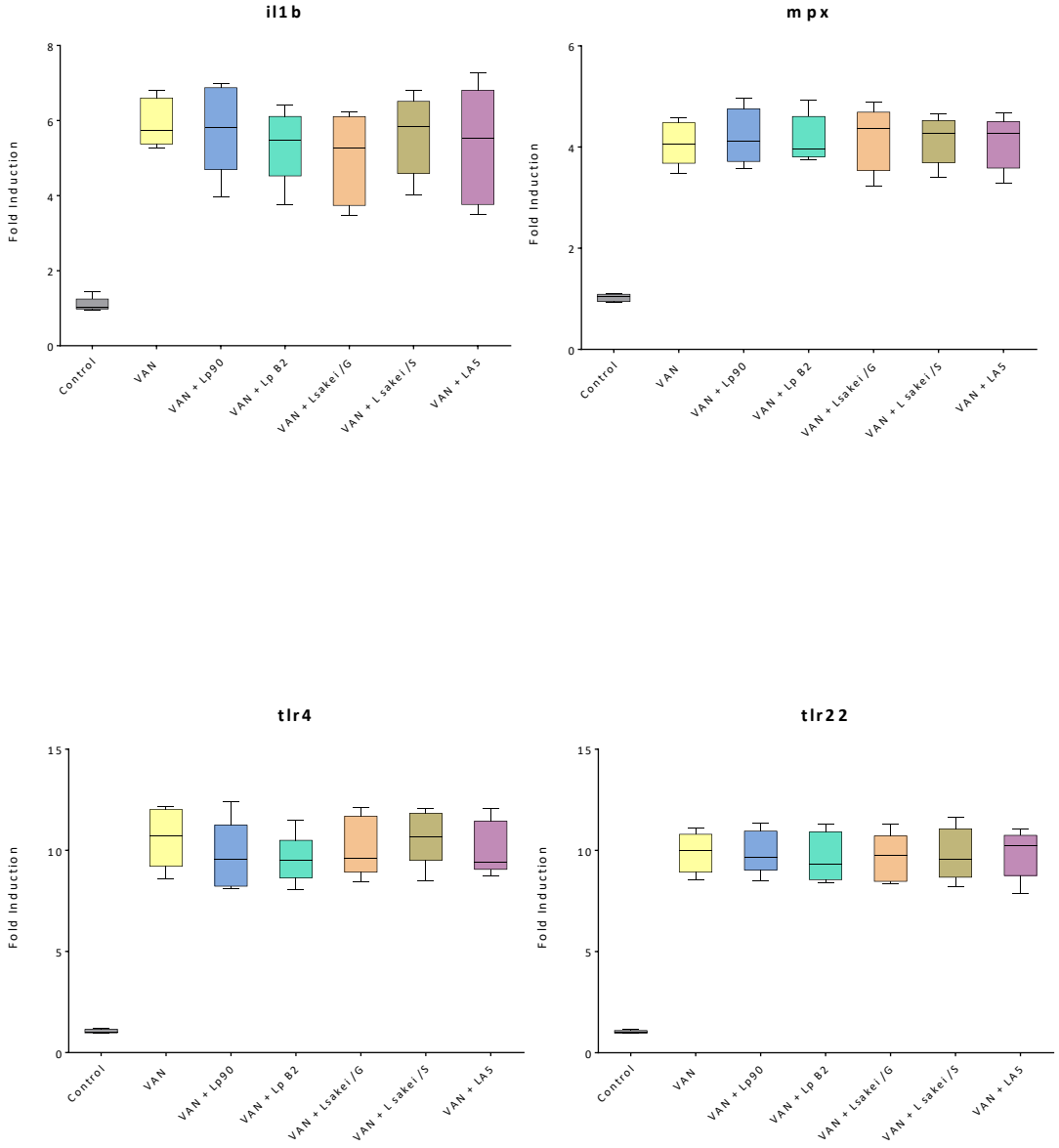

Figure 12. Gene expression profiles of zebrafish larvae co-exposed to *V. anguillarum NB10* and the evaluated larvae. Box-plot indicates the values between in the 25-75 percentile. The median is represented by a line inside the box. Whiskers include the values between in the 5-95 percentile.

4. Discussion

The infective profile of two digestive pathogens were evaluated in zebrafish larvae; *V. anguillarum* and *E. faecalis*. Such infective profiles were then used to develop an assay for the evaluation of the protective effect of LAB on pathogens. Zebrafish is a rapid and cost-effective infection vertebrate model that presents many advantages in comparison with other models[23] such as rodents, among those, the small size allows to carry out a large number of experiments simultaneously. Several studies have described the infection process of zebrafish larvae by *Salmonella enterica*[24], *Listeria monocytogenes*[25] or *E. coli*[26] and other pathogens. This way several protective treatments, such as probiotics or antimicrobial compounds can be evaluated, allowing for large throughput screening assays[27]. According to the results obtained in this work, *in vivo* observation and plate count evaluation of infected zebrafish larvae exhibited a distinct outcome after the exposure to the evaluated pathogenic strains. The bacterial load of larvae exposed *V. anguillarum NB10* increased gradually over the time, whereas bacterial levels of *E. faecalis OG1RF* remained nearly constant. The detected mortality was correlated to the bacterial load. Exposure to *V. anguillarum NB10* resulted in a survival rate of 25 % after 48h, while exposure to *E. faecalis OG1RF* did not significantly affect larva survival. In contrast, Prajsnar *et al*. reported a mortality of 100% 24 hpi, when *E. faecalis OG1RF* strain was injected in the bloodstream of 30 hpf zebrafish embryos[15]. Hence, the pathogen is able to infect embryos only by direct injection, and not external contact.*Vibrio anguillarum* infection elicited a strong immune response, with a 1.5-fold increase in mucus secretion, and an upregulation of $Il_{1\beta}$, *mpx*, tlr_2, and *tlr22*. The survival rate and immune response after infection with *V. anguillarum* is consistent with the results of other works[17,28,29]. Caruffo *et al.* found a consistent upregulation of *Tnfα*, $Il_{1\beta}$ and *mpx* genes and a mortality rate of nearly 95 % after 96 hpi. In the same way, Oyarbide *et al.* observed a mortality of 100 % after 72h.

In the view of those results, *V. anguillarum NB10* was selected to assess the protective effect of probiotics in the zebrafish animal model. The probiotic pretreatment did not reduce the bacterial load per larva with any of the evaluated LAB strains. Nevertheless, the exposure to *L. sakei MN1* strain had a protective effect over mortality. The pretreatment with the mentioned strain increased significantly the survival rate, from 25 % in the negative control, to 46 % in presence of sucrose, and 55 % in presence of glucose. As discussed in Chapter I, this strains presents a distinct phenotype depending on the carbon source[30]. The bacteria produced dextran in presence of sucrose, but not when glucose was available. The results obtained in Chapter I demonstrated the influence of dextran production over the colonization capacity. Previous works established that this strain is able to protect against two types of viruses in salmonids, correlated to the immunomodulatory effect of dextran in the host innate and acquired immunity, although no immunomodulatory effect has been evidenced over zebrafish in

the present work[31]. Given so, more research should be carried out to further characterize the immune response of zebrafish to both the pathogen and probiotic strains.

5. Conclusions

In the present study, two zebrafish infection models were characterized and evaluated. *Vibrio anguillarum* infection model was selected for the evaluation of the protective effect of probiotic strains. Co-exposure assays were carried out with *V. anguillarum* and the evaluated probiotic strains. One LAB strain isolated from meat products, *L. sakei MN1,* displayed a promising antimicrobial effect, increasing the survival rate of larvae exposed to the pathogen. The extent of the protective effect was correlated to the differential dextran production of the strain.

6. References

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CHAPTER IV

Evaluation of probiotic candidates combining *in vitro* and *in vivo* methods

1. Introduction

The microbial community present in the gastrointestinal (GI) track is a highly complex ecosystem that is composed of more than 10^{14} organisms. These are comprised mainly of *Bacteroides*, *Porphyromonas*, *Bifidobacterium*, *Lactobacillus*, and *Clostridium* genera[1]. Gut microbiota plays an important role in the digestive homeostasis. Indeed, the commensal bacteria represent the first barrier against pathogenic microorganisms and provide enzymes which aid the host in nutrient assimilation[2]. Lactic acid bacteria (LAB) are an important bacterial group, indigenous of the GI microbiota of humans and animals. Lactic acid bacteria are present in many fermented foods, playing an important technological role in the fermentation and ripening process[3]. The beneficial effect of LAB ingestion has been known for over a century[4] but the underlying mechanisms of their probiotic action have only recently begun to be understood. The World Health Organization (WHO) defines probiotics as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host."[5] Examples of these benefits are the following: inhibition of main digestive pathogens growth and colonization through the production of antimicrobial compounds and competition over the binding site of the digestive epithelium; enhancement of immune response; increase of nutrient bioavailability; cholesterol lowering effect; and relief of lactose intolerance symptoms[6].

Following this trend, there has been a rising interest in isolating and characterizing novel probiotic strains from fermented foods. Certainly, a bacterial strain must exhibit some technological and functional properties in order to be used as a probiotic[7]. Even if several LAB strains are part of the indigenous microbiota, it is necessary to determine the safety of every evaluated strain. Given the alarming increase of bacterial antibiotic resistance, the European Food Safety Agency (EFSA) requires the determination of the absence of acquired resistance to the most common antimicrobials for any new strain used as a probiotic[8]. Most LAB do not present a risk of infection in humans. However, they can be a source of horizontal transfer of antibiotic resistance coding genes towards pathogenic species[9]. Similarly, the presence of biogenic amines (BA), produced during the fermentation process, can lead to toxicological issues when ingested and should thus be avoided[10]. A potentially probiotic bacterial strain should also exhibit some phenotypic traits to be able to exert a beneficial effect on the health of the host. An adequate number of the bacteria must be able to survive the passage through the digestive system. The main stressors are the low pH and enzymes of the stomach, and the bile and high pH found in the duodenum. The ability of a strain to adhere to the gut epithelium is another important criterion in enabling the colonization of the mucosa. Several studies have pointed out the importance of biofilm formation in enhancing the colonization process and forming a stable microbial community[61]. Another desirable trait of a potentially probiotic strain is the immunomodulatory and anti-inflammatory

properties exerted over the immunity of the host. Several *in vitro* studies have highlighted the anti-inflammatory effect of some selected probiotic strains. Fujie *et al.*[11] have evaluated the immunoregulatory effect of *Bifidobacterium breve MCC117* on an inflammation model using porcine intestine epithelial cell cultures. Perez-Cano *et al.*[12] have determined the modulatory effect of two LAB strains, *L. salivarius CECT5713, and L. fermentum CECT5716,* on cytokine activity over peripheral blood mononuclear cell subset (PBMC). Other researchers have conducted *in vivo* studies to further characterize probiotic effects. For example, Zoumpopoulou *et al.*[13] have demonstrated the increase of Il_{10} anti-inflammatory cytokine in a TNBS-induced colitis murine model after administration of *L. fermentum* strain *ACA-DC179*. Moreover, this strain showed an antimicrobial activity in a *Salmonella*-infection mouse model. Probiotics can also protect the epithelial barrier integrity, a factor involved in the onset of IBD, as shown by Menninger *et al.* using DSS-induced colitis in mice.[14]

Over the last years, zebrafish (*Danio rerio*) has been widely used in many biomedical research fields, such as vertebrate development studies[15], toxicity evaluations[16], screening of new drugs or validation of molecular targets[17]. In addition, several studies have been focused on the study of microbial infections[18,19], interactions between microbiota and host immunity[20–22], and the influence of probiotic administration over the health of the host[23–27], as well as their influence on pathological processes such as dysbiosis and intestinal bowel disease (IBD)[28,29]. In this work, after screening with *in vitro* methods a group of 20 different LAB strains we applied a battery of *in vivo* test specifically designed for the zebrafish animal model to determine in a comprehensive and effective way their probiotic properties.

2. Materials and Methods

2.1. Lactic acid bacteria strain and media

A total of 20 LAB strains from the AZTI research center microbial collection were included in this study (Table 1). Strains were isolated from sourdough bread, milk used in cheese manufacturing and artisanal kefir production. All strains were previously identified by phenotypic and molecular techniques (unpublished results). *Lactobacillus acidophilus 5* was used as reference probiotic strain [30, 31]. Strains were stored at -80 ºC in MRS broth with 20 % (w/v) glycerol. Bacteria were routinely cultured in MRS broth (Oxoid) at 37 ºC.

Table 1. Source of isolation and identification code of LAB evaluated in this study.

CODE	SPECIES	SOURCE
LA5	*L. casei*	CHR Hansen
LBR002	*L. brevis*	Sourdough Bread
LBR004	*L. brevis*	Sourdough Bread
LBR006	*L. brevis*	Sourdough Bread
LCA005	*L. casei*	Kefir
LCA006	*L. casei*	Kefir
LCA007	*L. casei*	Kefir
LCM001	*L. mesenteroides*	Kefir
LCM002	*L. mesenteroides*	Kefir
LLA006	*L. lactis*	Kefir
LLA007	*L. lactis*	Kefir
LLA030	*L. lactis*	Cheese Production
LLA035	*L. lactis*	Cheese Production
LPL002	*L. plantarum*	Cheese Production
LPL003	*L. plantarum*	Cheese Production
LPL004	*L. plantarum*	Cheese Production
LPL005	*L. plantarum*	Sourdough Bread
LPL008	*L. plantarum*	Sourdough Bread
LPL010	*L. plantarum*	Sourdough Bread
LPL011	*L. plantarum*	Sourdough Bread

2.2. Safety properties

2.2.1. Antimicrobial susceptibility test

Strains were tested for their susceptibility to the following antimicrobials: ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol. Several concentrations of each antimicrobial were evaluated (1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 mg/L), and EFSA cut-off values for each species were used to distinguish resistant from susceptible strains[8]. Strains were incubated in MRS broth for 24 h at 37 ºC. Cultures were washed, diluted and inoculated into 200µL fresh MRS broth, supplemented with the evaluated antimicrobial substance. Each treatment was analyzed in four replicated wells. MRS broth was used as a positive control, while sterile MRS broth was used as a negative control. The experiment was performed in 96 wells microtiter plates (Corning Costar, Sigma). The optical density (OD) of cell cultures at 600 nm was evaluated using a Varioskan Flash Spectral Scanning Multimode Reader (Thermo Scientific). The experiment was carried out three times.

2.2.2. Biogenic amines

A multiplex PCR was carried out to simultaneously detect the presence of four genes related to the production of BA, histamine (*Hdc*), tyramine (*Tyrdc*) and putrescine (via either *Odc* or *Agdi* gene), according to the method described by Coton *et al*.[32]. Universal 16S rRNA gene was used as a PCR internal control. *Lactobacillus brevis IOEB 9809* strain [33](*Tyrdc* and *Agdi* positive) was used as a positive control. All strains were incubated in MRS broth for 24 h at 37 ºC. Bacterial DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen), following the instructions of the manufacturer. Each PCR reaction was carried out in a final volume of 25 µL containing 20 ng of bacterial DNA, 200 µM of dNTP, 10 $\mu g.ml^{-1}$ of BSA and 0.25 µL of Ex Taq DNA Polymerase (Takara Clontech). The final concentrations of primers were as follows: 0.8 µM for Odc1, Odc2, Agd1 and Agd2; 0.2 µM for Td2 and Td5; 0.12 µM for Hdc3; and Hdc4 and 0.05 µM for Bsf8 and Bsr1541. The PCR amplification program was as follows: 95 ºC 5 min, 35 cycles of 95 ºC 1 min, 52 ºC 1 min, and 72 ºC 1 min 30 s with a final extension at 72 ºC for 5 min. Obtained PCR products were analyzed on a 0.8 % (w/v) agarose gel in 1x TAE buffer at 80 V for 45 min and revealed using RedSafe Nucleic Acid Staining Solution (Intron Biotech).

2.3. Phenotypic tests

2.3.1. Resistance to gastrointestinal Stress.

The tolerance of bacterial strains to simulated gastric and intestinal transit was determined by means of an *in vitro* model, following the method described in Vizoso Pinto *et al*. [34] The simulated gastric fluid contains 3 $g.L^{-1}$ of pepsin (Sigma-Aldrich), 6.2 $g.L^{-1}$ of NaCl, 2.2 $g.L^{-1}$ of KCl, 0.22 $g.L^{-1}$ of $CaCl_2$ and 1.2 $g.L^{-1}$ of $NaHCO_3$ and the pH was adjusted to 3. The simulated intestinal fluid is composed of 1 $g.L^{-1}$ of pancreatin (Sigma-

Aldrich), 1.28 $g.L^{-1}$ of NaCl, 0.24 $g.L^{-1}$ of KCl, 6.4 $g.L^{-1}$ of NaHCO3 and 4 mL of a 10 % Bile Salt dilution and the pH was adjusted to 8. The solutions were filter-sterilized and used fresh the same day. An aliquot of each bacterial dilution was added to 9 mL of gastric fluid to reach a final concentration of 10^7 $CFU.mL^{-1}$. The dilutions were then incubated in a shaking bath at 37 ºC. After 90 min, 17.5 mL of the intestinal fluid was added to each dilution. The solutions were then homogenized and incubated in a shaking bath at 37 ºC. Samples were taken at 0, 90 and 180 min of incubation, and ten-fold dilutions were plated on MRS agar. The experiment was repeated three times.

2.3.2. Biofilm production

Biofilm production assays were performed according to Vergara-Irigaray *et al*.[35] with some modifications. 200 µL of a 1:100 dilution of overnight grown cultures was added in sterile 96 wells polystyrene plates (Corning Costar, Sigma). Four replicated wells per strain were inoculated. Four replicated wells were inoculated with sterile MRS as a negative control. The plates were incubated for 48 h and washed with sterile PBS. Subsequently, 225 µL of crystal violet (0.5 % w/v) was added. After 15 min, the plates were washed with PBS and 250 µL of ethanol (97 %) was added. The absorbance of each well was assessed at 590 nm using a Varioskan Flash Reader (Thermo-Scientific). Cut-off values were estimated to categorize the biofilm production capacity[36]. Cut-off values were calculated as the mean OD of the negative controls (OD_{nc}) plus three standard deviations of the negative control (SD_{nc}). The following categories were established: OD ≤ Odc = non-biofilm producer; ODc < OD ≤ 2 × Odc = weak biofilm producer, 2× Odc < OD ≤ 4 × Odc = moderate biofilm producer and OD > 4×Odc = strong biofilm producer.

2.3.3. Autoaggregation assay

Autoaggregation assays were carried out as described in Del Re *et al*.[37]. Overnight cultures were centrifuged and resuspended in PBS to an OD= 1. 1.6 mL of each bacterial suspension was transferred to a tube and vortexed vigorously. Every hour over a period of 5 h, an aliquot of 200 µL was taken from the upper section of the tube and transferred to another tube containing 1.8 mL PBS. Absorbance was measured at 600 nm. The autoaggregation percentage was determined as $1-(A_t/A_0)$ *100. A_0 represents the absorbance at t=0h. A_t represents the final absorbance value after 5 h.

2.4. *In* vivo Zebrafish assays

2.4.1. Zebrafish maintenance and breeding

Zebrafish embryos were obtained from wild-type adult zebrafish bred in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain) following standard conditions. Adult zebrafish were maintained at 27 ±1 °C with a 12 h light/dark cycle in 60 L tanks containing aerated freshwater. Each tank was fitted with an external filtration system (biological, chemical, and physical filtration). Zebrafish were maintained

according to standard protocols[38]. Twice a day, fish were fed with a pellet-formulated diet (Gemma Micro 300, Skretting) to achieve a total daily feed input of 5 % (w/w) of body weight. Embryos were collected and maintained in embryo water (EW) ($CaCl_2$ 294 mg/L, $MgSO_4$ 123 mg/L, $NaHCO_3$ 64.7 mg/L, KCl 5.7 mg/L) at 27 °C. All experimental procedures were approved by the regional animal welfare body.

2.4.2. Zebrafish exposure to bacteria

Zebrafish embryos of 4 dpf were exposed to the formerly selected probiotic strains to evaluate the immunomodulatory and antimicrobial effect *in vivo*. To that end, overnight cultures were centrifuged at 3700 rpm for 5 min. The pellet was suspended in embryo water and diluted to 10^7 CFU.mL^{-1}. Fifty mL of each bacterial dilution were placed into Petri dishes, along with 50 larvae of 4 dpf. As an untreated control, fifty larvae were placed in a dish with 50 mL PBS. 100 µL of a sterilized solution containing 1 % (w/v) of embryo feed (ZF Biolabs) in embryo water was added to each plate. The plates were incubated at 27 ºC with gentle shaking. The medium was renewed every 24 h with a fresh bacterial dilution, as well as 100 µL of embryo feed dilution.

2.4.3. *Vibrio* anguillarum challenge test

Zebrafish larvae were challenged against *V. anguillarum* (VAN), a pathogenic bacteria, following the procedure described in Oyarbide et.al [39]. Briefly, 4 dpf larvae were individually co-exposed to the pathogen and one LAB strain. One larva per well was immersed in 1mL of embryo water containing 10^7 CFU of VAN and 10^7 CFU of LAB strain. 24 larvae per treatment were monitored on a 24 wells plate. Larvae immersed in a VAN dilution were used as a positive control. Larvae immersed in a LAB dilution were used as a negative control. Mortality and malformations were determined every 24 h between 5 and 9 dpf. Malformations were determined by visual inspection with a Leica MZFL III stereomicroscope (Leica Microsystems GmbH, Wetzlar, Germany). Mortality was identified by heartbeat absence or embryo coagulation.

2.4.4. Chemical induction of enterocolitis

Chemical enterocolitis was induced by adding a freshly prepared dilution of 2,4,6-Trinitrobenzenesulfonic acid (TNBS; Sigma-Aldrich, St. Louis, MO) to embryo water from 4 dpf until the end of the experiment. The larvae were exposed to a final concentration of 50 µg.mL^{-1} [40].

2.4.5. Gene expression analysis by rt-qPCR.

The differential expression of selected genes was carried out in zebrafish larvae maintained under different conditions. Pools of 25 larvae of 9 dpf were euthanized with Tricaine (Sigma-Aldrich, USA) and homogenized. Total RNA was extracted with Trizol following manufacturer indications (Sigma-Aldrich). The quantity and quality of extracted RNA were assessed with an Agilent 2100 Bioanalyzer (Agilent Technology,

USA). RNA samples with a RIN (RNA Integrity Number) of at least 8.5 were used. The reverse transcription was carried out with TaqMan® Reverse Transcription following the instructions of the manufacturer. Quantitative PCR was performed with SYBR Green PCR master mix (Roche Diagnostic Rotkreuz, Switzerland.) on a Light Cycler 480 (Roche Diagnostics, Switzerland). Reaction conditions were as follows: 50 ºC for 2 min and 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. *β-actin* was used as a housekeeping gene. Each sample was tested in triplicate. The mean C_t of each sample was normalized against the housekeeping gene. The relative quantification of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method, using the REST 2009 software (Qiagen, Hilden Germany). The following genes were evaluated: Il_{10}, $Il_{1\beta}$ and *NF-κβ*. Primers sequences are listed in Table 2.

Table 2: PCR primer sequences list used in this study.

Gene	Code	NCBI accession	Forward primer	Reverse primer	Ref.
β-actin	Act	NM_131031.1	TGCTGTTTTCCCCTCCATTG	TTCTGTCCCATGCCAACCA	41
Interleukin 1β	$Il_{1\beta}$	NM_212844.2	CATTTGCAGGCCGTCACA	GGACATGCTGAAGCGCACTT	41
Nuclear factor κβ	Nf-κβ	NM_001003414	AGAGAGCGCTTGCGTCCTT	TTGCCTTTGGTTTTTCGGTAA	39
Interleukin 10	Il_{10}	NM_001020785	ATATTTCAGGAACTCAAGCGGG	ACTTCAAAGGGATTTTGGCAAG	42

2.4.6. Statistical Analysis

Results were expressed as the mean ± SEM. The significance of differences was determined using a one-way ANOVA, followed by Tukey's test for multigroup comparisons, using the statistical software GraphPad Prism 7 (GraphPad Software Inc.). A $p < 0.05$ value was considered statistically significant.

3. Results

3.1. Safety properties

The results of antimicrobial susceptibility test are shown in table 3. A strain was considered to be resistant to an antimicrobial when the minimal inhibitory concentration (MIC) value obtained was superior to the breakpoint value established for this species by the EFSA Panel (EFSA-FEEDAP)[8]. Five of the strains (LBr006, LCA005, LCA007, LLA006 and LLA007) were found to be resistant to tetracycline. Two strains (LCA007 and LcM001*)* exhibited resistance to kanamycin. One strain (LBr006) was found to be resistant to three antimicrobials (gentamycin, streptomycin, and tetracycline). The *Lactobacillus* and *Leuconostoc* strains were not susceptible to vancomycin; due to an intrinsic resistance caused by the absence of D-Alc-D-lactate[9,43]. The plate setup is illustrated in Figure 1.

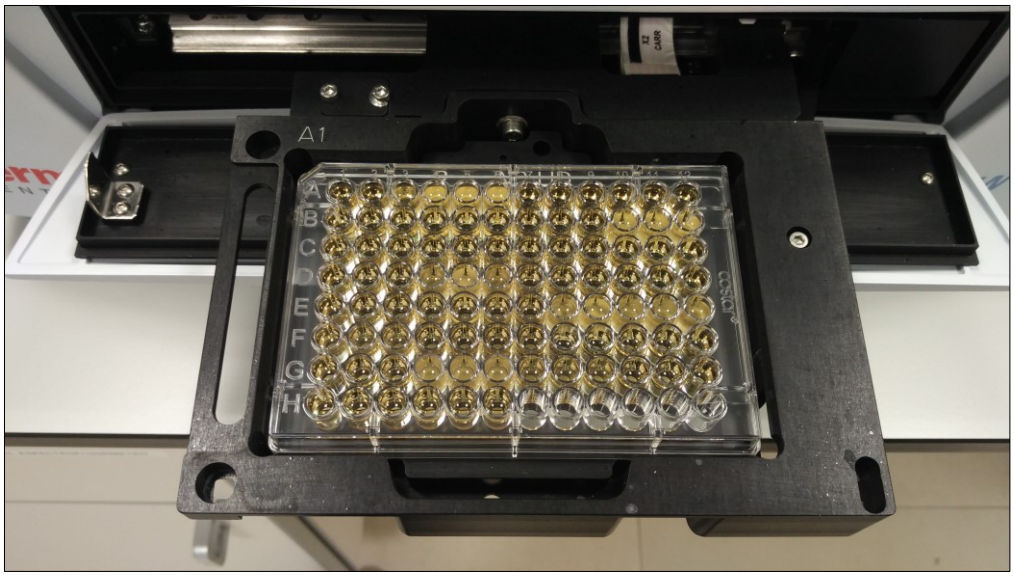

Figure 1. View of antimicrobial susceptibility test performed on microtiter plate.

Table 3. The bacterial strains were classified as resistant to an antimicrobial when the MIC value attained was above the cut-off value defined by the FEEDAP Panel (EFSA 2012) for that species; (S) susceptible; (R) resistant. Antimicrobials: (A) ampicillin; (V) vancomycin; (G) gentamicin; (K) kanamycin; (St) streptomycin; (E) erythromycin; (C) clindamycin; (T) tetracycline; (Cm) Chloramphenicol.

STRAIN	A	V	G	K	ST	E	C	T	CM
LA5	S	R	S	S	S	S	S	S	S
LBR002	S	R	S	S	S	S	S	S	S
LBR004	S	R	S	S	S	S	S	S	S
LBR006	S	R	R	S	R	S	S	R	S
LCA005	S	R	S	S	S	S	S	R	S
LCA006	S	R	S	S	S	S	S	S	S
LCA007	S	R	S	R	S	S	S	R	S
LCM001	S	R	S	R	S	S	S	S	S
LCM002	S	R	S	S	S	S	S	S	S
LLA006	S	S	S	S	S	S	S	R	S
LLA007	S	S	S	S	S	S	S	R	S
LLA030	S	S	S	S	S	S	S	S	S
LLA035	S	S	S	S	S	S	S	S	S
LPL002	S	R	S	S	S	S	S	S	S
LPL003	S	R	S	S	S	S	S	S	S
LPL004	S	R	S	S	S	S	S	S	S
LPL005	S	R	S	S	S	S	S	S	S
LPL008	S	R	S	S	S	S	S	S	S
LPL010	S	R	S	S	S	S	S	S	S
LPL011	S	R	S	S	S	S	S	S	S

Regarding the BA metabolism, the multiplex PCR exposed six positive strains for at least one of the targeted genes. As can be seen in figure 2, the Tyramine-producing gene (*Tyrdc*) was detected in LBr002 strain. *Odc* gene, involved in one of the putrescine producing metabolic route, was found in the LLA006, LLA007 and LLA030 strains. Finally, *Tgdi* gene, involved in another putrescine metabolic route, was detected in LCA005, LCA007, and LLA030.

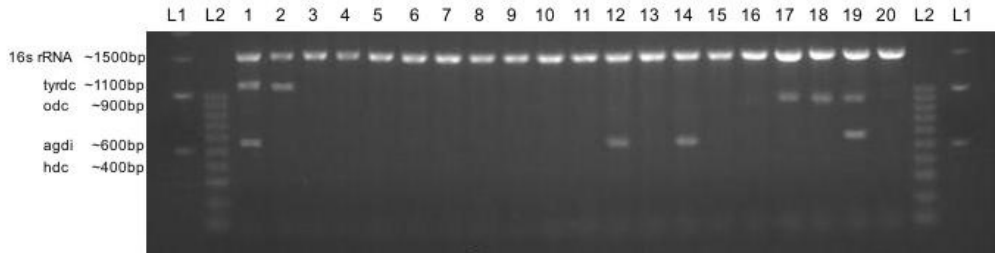

Figure 2. Agarose gel of the simultaneous detection of biogenic amines coding genes. L1: 1kbp DNA ladder (Invitrogen); L2 100bp DNA ladder (Invitrogen); 1: *L. brevis IOEB 9809* Positive control; 2: *L. brevis 002; 3. L. brevis 004; 4: L. brevis 006 5: L. plantarum 002; 6: L. plantarum003; 7. L. plantarum 004; 8. L. plantarum 005; 9. L. plantarum008; 10: L. plantarum 010; 11: L. plantarum 011; 12: L. casei 005; 13: L. casei 006; 14: L. casei 007; 15: Lc. Mesenteroides 001 ; 16 : Lc. Mesenteroides 002 ; 17 : L. lactis 006 ; 18 : L. lactis 007 ; 19 : L. lactis 030 ; 20 : L. lactis 035*

3.2. Phenotypic tests

3.2.1. Resistance to gastrointestinal stress

All evaluated strains retained a high viability during the simulated gastric transit (Figure 3). The reduction in viable count was negligible for all strains (less than 0.1 log). When confronted with the simulated intestinal passage, the strains showed a variable degree of resistance. The viability reduction after 180 min ranged from less than 1 log (LBr002, LBr004, LBr006, LPL003, LPL005, LPL008, LPL010 and LPL011), between 1 and 2 log reduction (LCA006, LcM001, LcM002, LLA035, LPL002 and LPL004), to more than 2 logs *(*LCA005, LCA007, LLA006, LLA007 and LLA030). The LLA006 and LLA030 strains exhibited the lowest survival rates, with a 3.64 and 3.65 log reduction in viability respectively after 180 min.

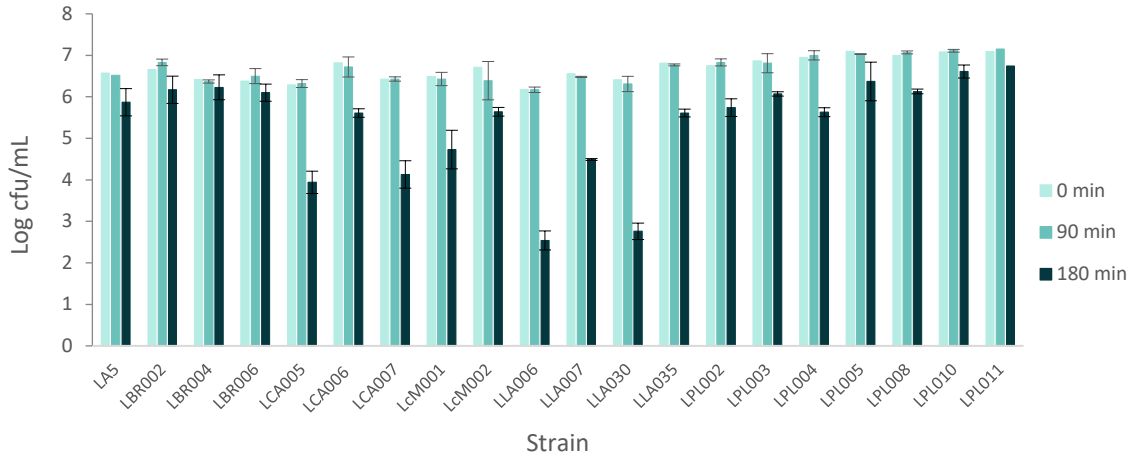

Figure 3. Survival rate of selected strains after exposure to simulated gastric (90 min) and intestinal fluid (180 min). (Error bars indicate standard deviation from three replicates).

3.2.2. Biofilm production

Biofilm production was evaluated measuring the absorbance of crystal violet staining. after 24 h incubation (Figure 5). Six strains (LBr006, LCA006, LcM002, LLA035, LPL003 and LPL004) were found to be strong biofilm producers. Five strains (LBr002, LBr004, LcM001, LLA006 and LLA030) were classified as moderate biofilm producers. Six strains were classified as weak biofilm producers (LCA007, LLA007, LPL005, LPL008, LPL010 and LPL011). Two strains were unable to produce biofilm (LCA005 and LPL002). Results are shown in figure 4.

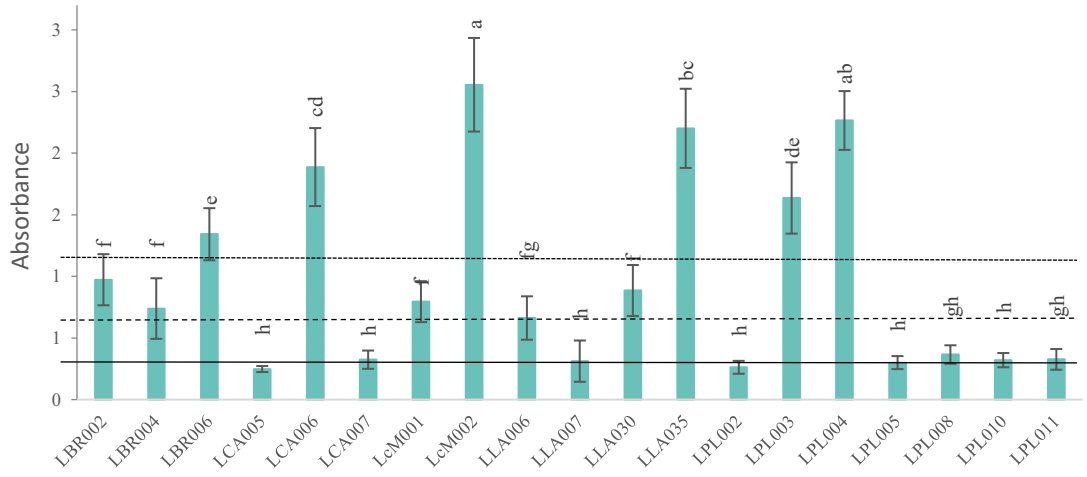

Figure 4. Biofilm formation on polystyrene plates after 24h incubation. Mean value and standard deviation of the measured absorbance at 590 nm of three experiments are represented. Values marked with the same letter do not differ significantly ($p > 0.05$ per Fisher's least significant difference). Cut-off line (ODc): (___); 2 × ODc:(__ __ __); 4 × ODc:(.....)

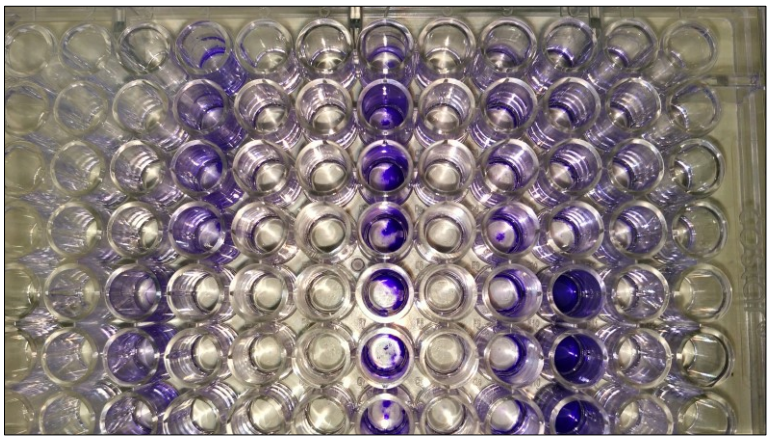

Figure 5. Evaluation of biofilm production of LAB on microtiter plates. Crystal violet staining revealed a strain-dependent biofilm formation.

3.2.3. Autoaggregation assay

The autoaggregation index of the evaluated strains ranged from 34 % to 66 % (Figure 6). The LLA6, LLA7 and LLA30 strains proved to have the lowest autoaggregation capacity (34.3 %, 36.5 % and 38.4 % AA index, respectively). The strains with the uppermost AA index were LBR6, LCA 5 and LPL10, with 66.3 %, 65.9 % and 63.3 % respectively. Figure 7 illustrates the autoaggregation results of the different strains.

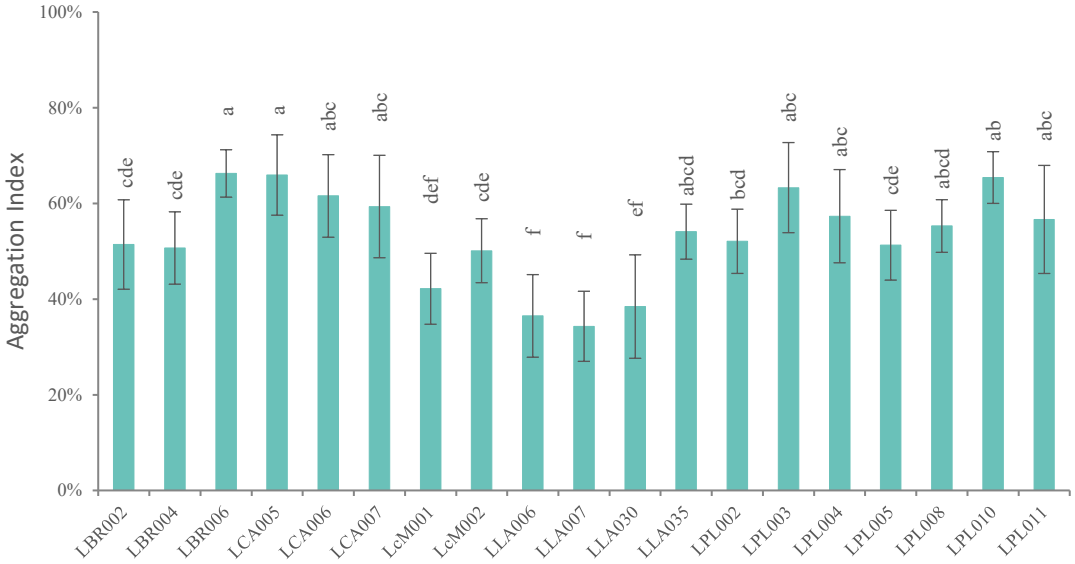

Figure 6. Autoaggregation index of selected strains after 5h. Mean value and standard deviation of three experiments are represented. Values marked with the same letter do not differ significantly ($p > 0.05$ per Fisher's least significant difference)

Figure 7. Autoaggregation assay of LAB strains. Important differences are visible between bacterial strains.

3.2.4. Strain selection

The ensemble of *in vitro* test results was compiled to select the strain with the best overall probiotic score (Table 4). Initially, the strains that did not meet the safety criteria were discarded. Secondly, the results of the phenotypic characterizations were averaged to select the strains with the highest resulting combined score. The criteria used to select the candidate strains were the following: GI stress resistance greater than 80 %, strong biofilm producers and minimal aggregation index of 50 %. Subsequently, the following LAB strains were selected for their *in vivo* screening evaluation: Lca006, LcM002, LLA035, LPL003 and LPL004. The selected probiotic candidates were then tested to evaluate their anti-inflammatory and antimicrobial properties *in vivo*, using the zebrafish animal model.

Table 4. Summary of evaluation criteria considered during the *in vitro* screening of potentially probiotic Lactic acid bacteria strains. Susceptibility to antimicrobials column displays the antimicrobial resistant strains. (A) ampicillin; (V) vancomycin; (G) gentamicin; (K) kanamycin; (St) streptomycin; (E) erythromycin; (C) clindamycin; (T) tetracycline; (Cm) chloramphenicol. BA column indicates the presence of genes relates to the metabolism of biogenic amines in the evaluated strain. (Ty) tyramine, (Pt) putrescine. GI stress shows the fitness to withstand adverse conditions such as gastric and duodenal medium. Data is expressed as viability loss percentage according to the following equation: $\left(1-\frac{(\log C_0-\log C_{180})}{\log C_0}\right)\times 100$. Biofilm indicates the biofilm formation capacity after 24h on a glass surface. Represented categories are described above. – No Biofilm formation capacity; + Low biofilm capacity; ++ Medium biofilm formation capacity; +++ High biofilm formation capacity. Red-colored lines indicate discarded strains due to safety concerns. Green-colored lines highlight the selected strains further evaluated *in vivo.*

	SAFETY RESULTS		PHENOTYPIC RESULTS		
Strain	**Antimicrobial susceptibility**	**Biogenic Amines**	**GI Stress**	**Biofilm**	**Autoaggregation**
LBR002		**Ty**	**92,6 %**	**++**	**51,4 %**
LBR004			97,1 %	++	50,7 %
LBR006	**G, S, T**		**95,9 %**	**+++**	**66,3 %**
LCA005	**T**	**Pt**	**62,7 %**	**-**	**65,9 %**
LCA006			**82,6 %**	**+++**	**61,6 %**
LCA007	**K, T**	**Pt**	**64,9 %**	**+**	**59,3 %**
LcM001	**K**		**71,9 %**	**++**	**42,2 %**
LcM002			**84,5 %**	**+++**	**50,1 %**
LLA006	**T**	**Pt**	**41,1 %**	**++**	**36,5 %**
LLA007	**T**	**Pt**	**68,6 %**	**+**	**34,3 %**
LLA030		**Pt**	**43,1 %**	**++**	**38,4 %**
LLA035			**82,6 %**	**+++**	**54,1 %**
LPL002			88,3 %	-	52,1 %
LPL003			**85,0 %**	**+++**	**63,3 %**
LPL004			**90,4 %**	**+++**	**57,3 %**
LPL005			89,7 %	+	51,3 %
LPL008			87,6 %	+	55,3 %
LPL010			93,4 %	+	65,4 %
LPL011			95,0 %	+	56,6 %

3.3. *In vivo* assays

3.3.1. Protective effect of probiotic candidates against *VAN* infection

The protective effect of the evaluated probiotic candidates was evaluated in the VAN infection model described in Chapter III (Figure 8). Larvae exposed to the pathogen with no probiotic pretreatment exhibited a survival rate of 15 % after 48 h. The results show that the LAB strains were able to protect larvae from infection to a variable degree. The survival rate when larvae were pretreated was between 22 % and 41 %. Among the evaluated bacteria, LCA6 and LLA35 induced the highest protection (41 %). The pretreatment with LA5 and LPL3 strains resulted in a survival rate of 30 %. Finally, two strains (LcM2 and LPL4) did not generate any statistically significant difference in survival rate when compared to non-pretreated larvae.

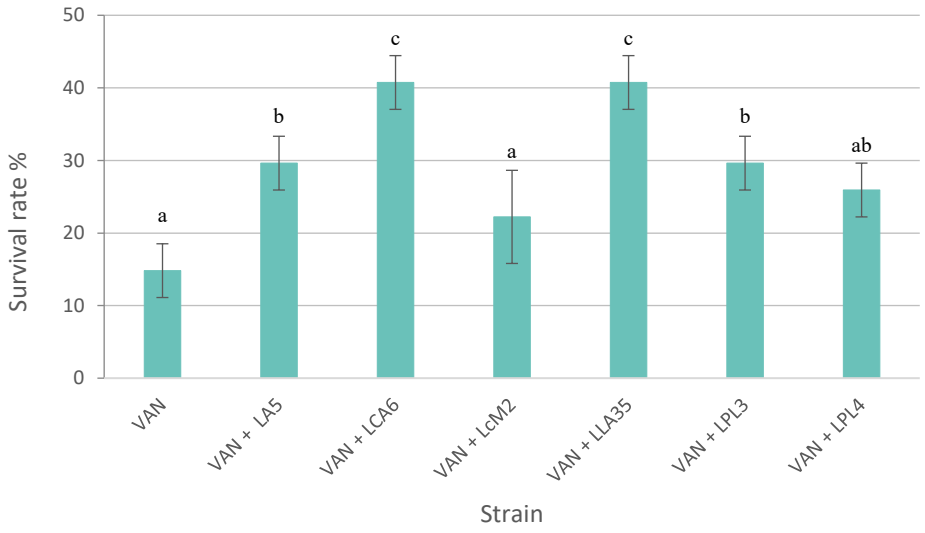

Figure 8: Survival rate of zebrafish larvae co-exposed to VAN and the evaluated LAB strains for 48h. Larvae exposed to VAN only were used as positive control. This test was carried out in triplicate and repeated three times. Survival rate and SEM are represented. Values marked with the same letter do not differ significantly ($p > 0.05$ per Fisher's least significant difference).

3.3.2. Immunomodulatory effect of probiotic candidates in a chemical enterocolitis model

Larvae exposed to a chemical enterocolitis agent (TNBS) displayed an induction of the expression of two pro-inflammatory immune-related genes, *NF-κB* and *Il1β*, when compared with control (Figure 9). The expression of the anti-inflammatory cytokine Il_{10} was repressed. When larvae were co-exposed to the evaluated bacteria, a strain-specific immunomodulatory effect was observed. Larvae brought into contact with LA5

exhibited a lower transcript level of *NF-κB* and *Il1β*, as well as an increase of Il_{10} transcript levels over the TNBS-only treated larvae. When larvae were co-exposed to the LPL4 isolate, the pro-inflammatory transcript level were significantly lower than in TNBS-only treated larvae. Also, Il_{10} transcript levels increased. No statistically relevant differences were found between larvae exposed to LCA5, LcM2 and LLA35 and the control larvae.

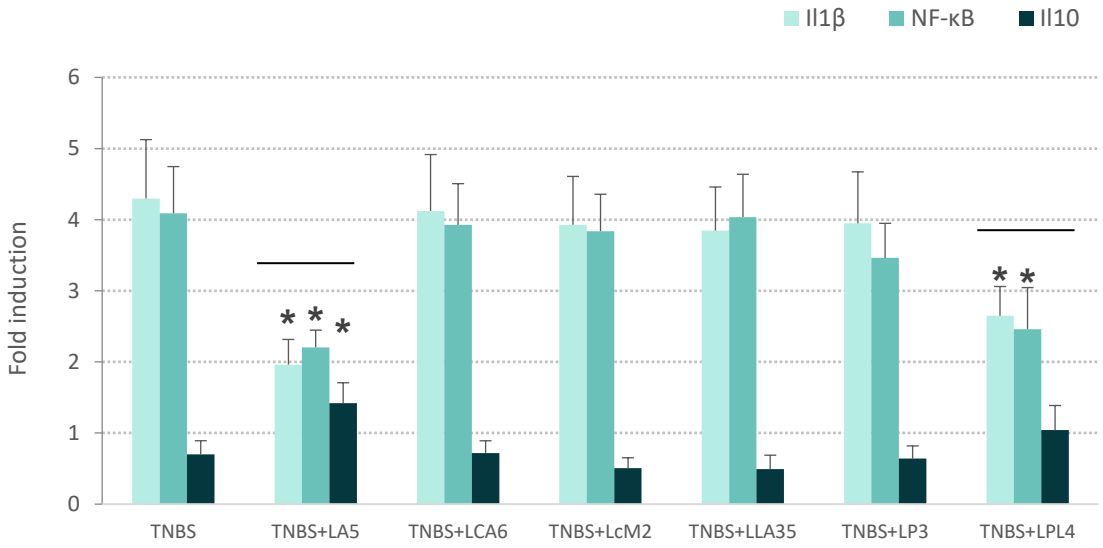

Figure 9: Characterization of zebrafish larva innate immune response to LAB colonization in a chemical enterocolitis model. The mean value ± SEM are represented.

4. Discussion

In this work, a combination of *in vitro* and *in vivo* assays was performed in order to evaluate the probiotic potential of LAB isolated from diverse sources.

In the first place, two *in vitro* assays were carried out to ensure the safety of selected strains. Lactic acid bacteria can be found in almost every fermented food and are generally considered to be safe for human consumption. Nevertheless, in order to grant the "Qualified presumption of safety" (QPS) status, EFSA requires the ensuring of the absence of acquired resistance determinants to antimicrobials of clinical importance[8]. Commensal bacteria harboring antimicrobial resistance genes can be a source of horizontal transfer towards pathogenic species[44]. Moreover, some reports of infections of immunocompromised patients due to LAB have been pubished[45]. Therefore, the strains were examined to determine the susceptibility to nine antimicrobials chosen to detect a wide range of determinants for resistance. The results found in this study are concordant with previous works. The *Lactobacillus* and *Leuconostoc* strains were resistant to vancomycin, a cell-wall inhibiting antimicrobial agent. Nevertheless, this is an intrinsic resistance, due to the synthesis of a modified cell wall peptide precursor D-Ala-D-Lac instead of D-Ala-D-Ala[46]. Moreover, Klein *et al*. have demonstrated that the vancomycin resistance in *Lactobacillus* strains is not genetically transmissible[47]. Tetracycline resistance was found in 21% of tested strains (1/3 *L. brevis*; 2/3 *L. casei*; 2/4 *L. lactis*). Previous studies have illustrated a high prevalence of tetracycline resistance in *Lactobacillus* strains isolated from dairy products[48,49], as well as other sources[50]. Furthermore, several plasmid-borne genes coding for tetracycline resistance have been identified[51]. Ten percent of strains demonstrated kanamycin resistance (1/3 *L. casei*; 1/2 *L. mesenteroides*), although other researchers have reported a higher prevalence of kanamycin resistance in strains contained on commercial probiotic products[46] or fermented olives[52]. Interestingly, one strain (*L. brevis*) exhibited a resistance towards three antimicrobials (tetracycline, gentamicin, and streptomycin). In summary, six bacterial strains exhibited a phenotypic resistance to some antimicrobials and thus require further molecular investigations to confirm that the resistance is non-transmissible. Therefore, we concluded that these strains should not be used as potential probiotics for human consumption.

Biogenic amines (BA) are nitrogenous compounds formed by microbial decarboxylation of amino acids. They can be found naturally in a wide range of food products containing proteins. Fermented foods may contain large doses of these compounds and lead to toxicological effects[53]. Histamine, putrescine, and tyramine are the most common BA found in fermented foods. While there is no legislation concerning BA content in fermented products, their presence is to be avoided, since it can have a negative impact on human health (hyper-sensibility, hypotension, flushing, migraine and tachycardia)[54]. Given that the ability to decarboxylate amino acids is strain-dependent[55], the isolated

bacteria were screened for their potential biogenic amine production using molecular methods[32]. Four strains were found to harbor genes coding for putrescine and one strain for tyrosine. These results agree with those reported in previous works. Coton *et al*. have screened 810 LAB isolated from wine and cider, and they have found that the *Agdi* gene, coding for putrescine metabolism, was present in 14 % of tested strains. This was followed by *Tyrd*, coding for tyramine, found in 8 % of strains. Other researchers have found a higher prevalence of tyramine-producing strains isolated from dairy products[56] and other fermented foods[57].

Bacteria are confronted with stressful conditions in the stomach (low pH, digestives enzymes) and the intestines (high pH, digestives enzymes, bile salts). Consequently, to produce their probiotic effect in the intestine, the bacteria must be able to survive these conditions in a sufficient concentration. All evaluated strains exhibited high resistance to gastric conditions, with a loss of viability of less than 1 log cfu.mL^{-1} after 90 min. Exposure to simulated intestinal conditions caused a greater reduction in viability in some tested bacteria. While *L. plantarum* and *L. brevis* strains showed around 1 log loss in viable count, strains of *L. casei*, *L. mesenteroides* and *L. lactis* displayed a reduction in viable count to 3 logs after the passage through the simulated intestinal tract. The results of this work are consistent with previous studies[52,58]. Furthermore, the administration conditions may have an influence on metabolic conditions and thereby modulate the fitness and survival rates of probiotic bacteria in the stomach and duodenum. Pure culture dilutions are fully exposed to the harsh GI conditions, whereas probiotics administered within a food matrix benefit from a buffering effect. However, the optimal growing conditions of culture media (high nutrient availability, optimal incubation temp and O_2 levels) are not representative of those found in food-contained probiotics. Low pH, the absence of nutrients and a lower conservation temperature may lead probiotics bacteria to a non-optimal metabolic state. Thereupon, further clinical trials should be conducted in humans to assess the surviving rate *in vivo*.

The biofilm formation represents a defense mechanism against predatory microorganisms, as well as toxins or antimicrobials present in the medium[60]. Some *Lactobacillus* strains exhibited an enhanced immunomodulatory effect on the host when growing in a biofilm disposition[61,62]. Biofilm formation is a multifactorial process related to aggregation properties, and it is a prerequisite for epithelial adhesion and colonization[63]. Actually, biofilm formation and auto-aggregation capacity are good predictors of epithelial adhesion capacity and can be used for a preliminary identification of potentially probiotic bacteria[37,64]. The results of this study are in agreement with previous works,[61,76,77]. Bacterial strains with a high biofilm production capacity (*L. plantarum 003, L. plantarum 004, L. brevis 006, Lc. Mesenteroides 002, L. lactis 030 and L. lactis 035)* also demonstrated a substantial auto-aggregation ability.

An *in vitro* screening procedure, based on safety and probiotic potential parameters, was initially carried out. Five strains (*L. casei 006, Lc. mesenteroides 002, L. lactis 035, L. plantarum 003* and *L. plantarum 004)* were selected, based on their overall score. The selected strains were then evaluated for their *in vivo* probiotic potential on the zebrafish animal model.

Nevertheless, the use of zebrafish as a model for human GI infection faces a potential limitation: the difference between the optimal growth temperature of mammalian pathogens (around 37 ºC) and the optimal temperature for the animal model (28 ºC). The lower temperature may attenuate the virulence of the infectious agent. Given that, most studies make use of fish pathogens that are closely related to mammal pathogen strains or mammalian strains with lower permissive temperature ranges. In the present study, the probiotic bacteria candidates were evaluated in the *V. anguillarum* infection model. This is a marine pathogen closely related to the human pathogen *V. cholerae*. The results obtained in this study show a decrease in mortality of over 40 % when larvae are pretreated with a selected probiotic strain. In the same way, Rendueles *et al*. developed a zebrafish model for the screening of probiotic bacteria with a protective effect on *E. ictaluri* infection, closely related to the *E. tarda* human pathogen[84]

Several immunity-related cellular mediator's transcript levels were monitored to evaluate the inflammatory state of zebrafish larvae. *$IL_{1\beta}$* is a cytokine secreted by activated macrophages and acts as a mediator of the inflammatory response. *NF-κB* is a protein complex related to the fast response to stress and is activated by other cellular mediators, such as *Tnfα* and *$IL_{1\beta}$*, and stressful cellular stimuli, such as reactive oxygen species, lipopolysaccharides or other bacterial components. Finally, *IL_{10}* is an anti-inflammatory cytokine, produced by monocytes. Commensal microbiota is required to stimulate *IL_{10}* production[93], causing a demise of inflammatory state[94] Several works have found anti-inflammatory effect of probiotic administration using zebrafish, as well as other animal models. Rieu *et al*. have studied the immunomodulatory effect of an *L. casei* probiotic strain in an enterocolitis zebrafish larva model[61]. Their findings demonstrate that the biofilm formation enhances the anti-inflammatory effect of the evaluated LAB. Other works have also found an Il_{10} mediated anti-inflammatory effect of probiotic administration in murine[95] and rat[96] colitis models. Zoumpopoulou *et al*. isolated an *L. fermentum* strain which prevented and reduced colitis in a TNBS-induced colitis mouse model and in a human peripheral blood mononuclear cells culture. This bacterial strain also showed a promising effect against Salmonella infection[97]. The results in this work illustrate a decrease in pro-inflammatory mediators and an increase in anti-inflammatory cytokines after an exposure to the probiotic strain *L. acidophilus 5*. Interestingly, the exposure to one of the probiotic candidates (*L. plantarum 004)* resulted in a similar transcript profile, suggesting partial inhibition of the inflammatory response caused by TNBS.

5. Conclusions

In the present work, the probiotic ability of 20 LAB strains, isolated from diverse fermented food was screened. To that end, the array of *in vivo* test developed in the previous Chapters was complemented by a set of *in vitro* assays. The collection of *in vitro* assays represents a fast and effective way to discard bacterial strains presenting safety hazards, such as antibiotic resistance and biogenic amines production. In addition, the results of the *in vitro* tests on adhesion capacity induced a preselection of LAB strains to be tested in a *in vivo* model. This methodology allowed to identify the strain L. *plantarum* 4, isolated from a cheese-production process, as a promising candidate for probiotic use. The selected strain should undergo further evaluation on mammal models and clinical trials to be used as a probiotic in humans.

6. References

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III. CONCLUSIONS

1. Conclusions

The results obtained during this investigation demonstrates that zebrafish is an appropriate animal model for the evaluation of the probiotic efficacy of LAB strains.

The main conclusions derived from the current study are the following:

I. The zebrafish animal model at its earliest developmental stages is a suitable model for studying the intestinal colonization ability of lactic-acid bacteria. This model confirmed that the colonization dynamics are dependent on the phenotype that is specific to the strain.

II. The colonization of zebrafish's larvae digestive system by lactic-acid bacteria was optimized by developing a specific probiotic administration procedure. This consisted of 120 h exposure to lactic-acid bacteria by larva immersion, with daily feeding and bacterial dilution renewal to ensure proper colonization of the GIT.

III. Fluorescently marked bacteria allowed monitoring in real time the evolution of the colonization and the persistence of LAB in the GIT of the zebrafish larvae. The visualization of fluorescence allowed detection and quantification of the strain-specific adhesion capacity of bacterial cells to the intestinal epithelium.

IV. The anti-inflammatory effect observed in the zebrafish chemical colitis model after the administration of the probiotic strain *L. acidophilus LA5* confirmed the usefulness of this system in evaluating the immunomodulatory effect of new probiotic strains on a vertebrate IBD model.

V. The bacterial strain *L. sakei MN1* exhibited a promising antimicrobial effect in the *V. anguillarum*-infection model. The probiotic treatment increased the survival rate of larvae exposed to the pathogen. The extent of the protective effect was correlated to the differential dextran production of the strain.

VI. The battery of *in vivo* test specifically designed for the zebrafish animal model in this work complemented by an array of *in vitro* tests allows the comprehensive screening for the probiotic properties of large group of different lactic-acid bacteria strains in a fast and reliable manner. Using this approach L. *plantarum* strain 4, isolated from a cheese-production process, exhibited promising probiotic potential. The selected strain should undergo further evaluation on mammal models and clinical trials to be used as a probiotic in humans.

2. General conclusion

The current study demonstrated the usefulness of the zebrafish animal model to evaluate the probiotic efficacy of LAB strains. A screening methodology for the *in vivo* evaluation of the efficacy of new probiotic strains was developed using the zebrafish animal model. Such method includes assays for evaluating the colonization potential, immunomodulatory effect, and antimicrobial characteristics of LAB strains. This methodology complemented by a group of in vitro test aimed at establishing the safety of the potential probiotics represents a rapid and cost-effective system for the preliminary screening of new probiotic strains in an alternative vertebrate model. The use of zebrafish larvae for the evaluation of new probiotic strains can help in reducing the number of strains to be tested on rodent models and subsequent human clinical trials.

3. Future research

In the current study, a screening methodology for the evaluation of the probiotic activity of lactic-acid bacteria through *in vitro* and *in vivo* assays was developed. This screening method could be further developed by evaluating additional immune-related genes, such as those belonging to the *defb* gene family, related to the defensine production, or NOD_x genes, coding for pathogen-associated molecular-pattern receptors. The recruitment of immune cells in response to an inflammation could be further investigated using other transgenic zebrafish lines expressing fluorescence in immune cells, such as Tg(mpeg1:mCherry) expressing mCherry fluorescence in macrophages. In addition, the Fluorescence-Activated Cell Sorting (FACS) technique would allow the study of the immune cell proliferation in response to an inflammation on whole larvae.

Another issue that needs to be addressed in future research is the interaction between the digestive microbiota and the probiotic administration. On one hand, the role of indigenous microbiota in probiotic-colonization dynamics could be further investigated to increase its colonization aptitude. On the other hand, the influence of the probiotic strains on microbiota composition and its metabolomic profile represents an exciting line of work. This research could improve the efficacy of probiotics on microbiota-related diseases, such as antibiotic-associated diarrhea, and recurrent *Clostridium difficile* infection.

The range of evaluated beneficial probiotic properties could also be extended, including assays to evaluate the antimutagenic activity for cancer prevention; the production of defensins and other antimicrobial compounds to prevent infectious diseases; bile-salt-hydrolyzing activity and its cholesterol-lowering effect, which is useful for the prevention of metabolic disorders, as well as other health benefits.

Finally, the study of prebiotic administration alongside probiotic bacteria (symbiotics treatment) is another step that should be taken. The synergic effect of pre- and pro-biotics could be tested on a zebrafish model that evaluates the influence of concomitant administration on the colonization, immunomodulatory effect and production of short-chain fatty acids (SCFA) at the intestinal epithelial level.