THE MICROBIOTA-GUT-BRAIN AXIS.

A STUDY IN ZEBRAFISH (*DANIO RERIO*)

by

Luca Borrelli

PhD in

Model Organisms in Biomedical and Veterinary Research

Cycle XXVII

a.a. 2012-2013/2014-2015

A thesis submitted to Università degli Studi di Napoli Federico II for the degree of

Doctor of Philosophy

Napoli, Italy

March, 2015

Department of Veterinary Medicine and Animal Productions

THE MICROBIOTA-GUT-BRAIN AXIS. A STUDY IN ZEBRAFISH (*DANIO RERIO*)

Author: Luca Borrelli

Major Advisor: Professor Alessandro Fioretti

Copyright statement

This copy of the thesis has been supplied on the condition that anyone who consults it is understood to recognize that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the author's prior consent.

Alla curiosità, eterna espressione di liberta e

al silenzio assordante di ogni ispirazione

THE MICROBIOTA-GUT-BRAIN AXIS. A STUDY IN ZEBRAFISH (*DANIO RERIO*)

Abstract

The microbiota is essential in the host's physiology, development, reproduction, immune system, nutrient metabolism, in brain chemistry and behavior. The gut microbiota plays a crucial role in the bidirectional gut–brain axis, a communication that integrates the gut and central nervous system (CNS) activities, and thus, the concept of microbiota–gut–brain axis is emerging where the microbes have considered as signaling components in the gut-brain axis. Animal studies reveals, in particular, that gut bacteria influence the brain-derived neurotrophic factor (BDNF) levels, and behavior specially after probiotic administration. How this alterations in brain chemistry are related to specific behavioral changes is unclear but it will likely be a focus of future research efforts. Among these animal studies, to our knowledge, no studies on the microbiota–gut–brain axis in zebrafish (*Danio rerio*) have been carried out. We hypothesized that a continuous administration of an exogenous probiotic might also influence the host's behavior and neurochemical gene expression. The purpose of this study was to determine whether probiotic strain can modulate gut commensal bacteria influencing brain neurochemistry and behavior in zebrafish. Thus, we treated adult zebrafish for 28 days with *Lactobacillus rhamnosus*, a probiotic strain which is one of the main components of the commensal microflora of human intestinal tract and it is widely used as a probiotic in mammals to adult male and female AB wild tipe zebrafish. We established differences between treated with probiotic strain and control

group in shoaling behavior pattern, using a Video Tracker software; we quantified brain-derived neurotrophic factor (BDNF) gene expression by using RT-qPCR; we at last analyzed the microbiota profiles within two experimental groups by sing the culture-independent methods such as Denaturing Gradient Gel Electrophoresis (DGGE) and Next-Generation Sequencing (NGS). The probiotic treated group, compared to the control group, showed a statistically significant near two-fold increase in BDNF gene expression, different shoaling behavioural pattern and a shift in microbiota composition with a significant increase of Firmicutes and a reduction of Proteobacteria.

The results of each approach may support the existence of a microbiota–gut–brain axis, in adult zebrafish and in line with numerous animal studies we can speculate that microbiota manipulation could influence behavior and brain expression of BDNF.

TABLE OF CONTENTS

LIST of ABBREVIATION

5-HT: norepinephrine

A: Adenine

AD: Average Distance

ANOVA: Analysis of variance

ANS: autonomic nervous system

APS: ammonium persulphate

B: Bacillus

BDNF: Brain-derived neurotrophic factor

BLA: basolateral amygdale

C: Cytosine

CNS: changes in the central nervous system

CP: (Water) column position

CT: threshold cycle

CTRL: control group

D:N: Day:Night light cycle

DA: dopamine

DGGE: denaturing gradient gel electrophoresis

Dl: lateral domains

Dm: medial domains

DNA: Deoxyribonucleic acid

DR: disease resistance

DV: Distance Variance

EDTA: Ethylene diamine tetra acetic acid

ENS: enteric nervous system

F: fecundity/gonadal development/spawning rates etc

FISH: fluorescence in situ hybridization

G: guanine

GABA: gamma-aminobutyric acid

GALT: gut-associated lymphoid tissue

GI: gastrointestinal

GIT: gastrointestinal tract

GM: gut microbiota

GP: growth performance

HPA: hypothalamus–pituitary–adrenal

IBD: inflammatory bowel disease

IBS: irritable bowel syndrome (IBS)

IMS: Industrial Methylated Spirits

LAB: Lactic acid bacteria

Lb: lactobacillus

MCT: micro centrifuge tubes

MDS: multidimensional scaling

MGB: microbiota–gut–brain

MS-222: Ethyl 3-aminobenzoate methanesulfonic acid or “Tricaine” mesylate

mRNA: messenger ribonucleic acid

NaHCO3: sodium bicarbonate

ND: Nearest Distance

NGF: Nerve Growth Factor

NGS: Next-Generation Sequencing

NH3: Ammonia

NH4+: Ammonium

NO2-: Nitrite

NO3-: Nitrate

NT-3: Neurotrophin-3

NT-4: Neurotrophin-4

NT-6: Neurotrophins-6

OA: Occupied Area (or Shoal size area)

OSP: Open Source Physics

OTUs: observed taxonomical units

pH: potential of Hydrogen

PA: pathogen antagonism

PCoA: Principal Coordinates Analysis

PCR: Polymerase Chain Reaction

PGM: Personal Genome Machine®

Pro-BDNF: pro-protein Brain-derived neurotrophic factor

PROBIO: probitic treated group

QIIME: Quantitative Insights Into Microbial Ecology

qPCR: quantitative PCR

RAPD: (PCR) random amplified polymorphic DNA

rER: relative expression ratio

RIN: RNA integrity number

RNA: Ribonucleic acid

rRNA: Ribosomal ribonucleic acid

T: thymine

TAE: Tris-acetate-EDTA

TEMED: Tetramethylethylindiamine

TGGE: temperature gradient gel electrophoresis

Trk: tropomyosin-related kinase

TrkB: receptor tropomyosin-related kinase

UPGMA: Unweighted Pair Group Method with Arithmetic mean

VAN: vagal afferent nerves

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

"The manner in which the secretions of the alimentary canal and of certain other organs … are affected by strong emotions, is another excellent instance of the direct action of the sensorium on these organs, independently of the will or of any serviceable associated habit."

"The Expression of the Emotions in Man and Animals" (Charles Darwin, 1872)

Microorganisms have long been recognised as fundamental to the cause and prevention of human disease, as demonstrated by the early work of Lister, Koch and overall, Louis Pasteur wrote:

"The role of the infinitely small in nature is infinitely great"

This is particularly true of the microbial communities present in the gastrointestinal tract (GIT) of mammals and other vertebrates, termed the gut microbiota, which has received significant interest over the last decade. There is an increasing understanding of the role of the gut microbiota in maintaining health through immunomodulation, protection, nutrition and metabolism, disease and behavior.

1.1 Microbiota–Gut–Brain axis and its Impact

Vertebrates microbiota is a dynamic co-existing microorganism ecosystem, which has evolved in a mutualistic relationship with its host. This micro-ecosystem plays several crucial roles serving the host in development, facilitation and functionality of the innate and adaptative immune response by protecting it against invasive pathogens; in nutrition, metabolizing complex lipids and polysaccharides that otherwise would be inaccessible nutrients. It also neutralizes drugs and carcinogens, modulating intestinal motility; microbiota regulates the intestinal barrier homeostasis and it makes visceral perception possible (Cryan and Dinan, 2012; Montiel-Castro et al., 2013; Wang et al., 2013; Clements et al., 2014). The gut microbiota has played a crucial role in the bidirectional gut–brain axis that integrates the gut and central nervous system (CNS) activities, and thus the concept of microbiota–gut–brain axis is emerging. The microbiota - gut - brain axis represent a bi-directional communication system, comprised of neural pathways, such as the enteric nervous system (ENS), vagus, sympathetic and spinal nerves, and humoral pathways, which include cytokines, hormones, and neuropeptides as signaling molecules. Recent studies from animal models, supports a role of microbes as signaling components in the gut-brain axis. This communication has a bottom-up or top-bottom pathways (Bercick et al., 2012). CNS can influence gut microbiome (the constituent genome, protein and metabolites of the microbiota) through neural and endocrine pathways in both direct and indirect manners. The autonomic nervous system (ANS) and hypothalamus–pituitary–adrenal (HPA) axis that liaise the CNS and viscera can modulate gut physiology such as motility, secretion and epithelial permeability as well as systemic hormones, which in turn

affects the niche environment for microbiota and also host-microbiome interaction at the mucosae (Cryan and Dinan, 2012). The bottom-up regulation of the CNS by microbiome can be achieved through neural, endocrine, metabolic and immunological mechanisms. The neural pathway is operational through the enteric nervous system (ENS), a main division of the ANS that governs the GI functions, and vagal afferent nerves (VAN) that convey sensory information from viscera to the CNS (Wang et al., 2013). Interesting reviews report the recognition that the gut microbiota influences several signaling pathways led to the suggestion of the concept of a microbiota–gut–brain (MGB) axis (Rhee et al., 2009; Cryan and Dinan, 2012; Forsythe et al., 2012). The proposal of a MGB axis suggests that through a dynamic alignment, microbiota inhabiting the intestinal lumen affects its host's CNS activity (including vegetative and cognitive functions), and vice versa brain activity impacts microbiota development and composition.

1.2 Danio rerio: the translational opportunity

The zebrafish (*Danio rerio*; superorder Ostariophysi, order Cypriniformes) native to Southeast Asia is an omnivorous freshwater teleost fish indigenous to the inland waters of Pakistan, India, Bangladesh, Nepal and Burma (Engeszer et al., 2007). Over the last 40 years, the zebrafish has emerged as a pre-eminent vertebrate becoming a popular model organism for biomedical research (Figure 1.1). Historically, it was Dr George Streisinger at the University of Oregon who brought zebrafish into the laboratory setting in the late 1960s to develop the forward

genetic techniques that would ultimately establish zebrafish as a robust research model (Roeselers et al., 2011).

It has multiple advantages in biomedicine research. Zebrafish is an in vivo model and a vertebrate species with common conserved cell types, organs, and physiological systems (e.g., stress endocrine axis), it has sufficient physiological complexity and high physiological homology to humans and other vertebrates, genetically tractable organism with fully sequenced genome and easily manipulated genetically. Zebrafish also has a fast and abundant reproduction (e.g., a single female lays several hundred eggs each week), rapid development (hatching in <3 days and becoming mature by day 90) from 'transparent' eggs and transparent embryos (enables monitoring organ development and manipulating it in vivo – e.g., by injecting drugs or genes) All factors that make it easy of genetic and other experimental manipulations. High space/cost-efficiency and excellent potential for high-throughput screens. Various zebrafish strains are available with over 1000 transgenic and mutant zebrafish strains. As a lower vertebrate, it respects the 3R principles (replacement, refinement, reduction) of the Directive 2010/63/EU of the European parliament and of the council (Kalueff et al., 2014; Stewart et al., 2014).

Zebrafish being physiologically homologous to mammals, it possesses also all major neurotransmitters, including neurotransmitter receptors, transporters, and enzymes of synthesis and metabolism, similar to those observed in humans and rodents and it is a relatively complex vertebrate species (Kalueff et al., 2014a). Zebrafish are currently used to study a wide range of neurobehavioral domains, including anxiety and sociality (Gerlai et al., 2009; Gerlai 2014). Rose et al. (2007),

described fish behavior as simple and stereotyped. Recent studies demonstrate a complex behavioral patterns in zebrafish (Gerlai, 2010; Gerlai, 2014;). For example, affective disorders, such as exposed to stimuli that evoke fear or anxiety, zebrafish display a range of clear-cut quantifiable behaviors, including markedly reduced exploration, increased scototaxis (dark preference), geotaxis (diving/bottom dwelling), thigmotaxis (preference of peripheral areas), freezing (immobility) and erratic movements (sudden bouts of high-velocity darting with rapid successive turns) (Kalueff et al., 2013; Cachat et al., 2010; Wong et al., 2010; Egan et al., 2009;). These behavioral phenotypes are strikingly analogous to those of both rodents and humans. Anxiety is currently one of the most common human brain disorders, affecting millions worldwide. Zebrafish display well-developed functional neuroendocrine systems, generally homologous to those established in mammals. Similar to humans, stress responses in zebrafish are mediated by cortisol activated by the cascade of hypothalamo-putuitary hormones and acting via glucocorticoid receptors.

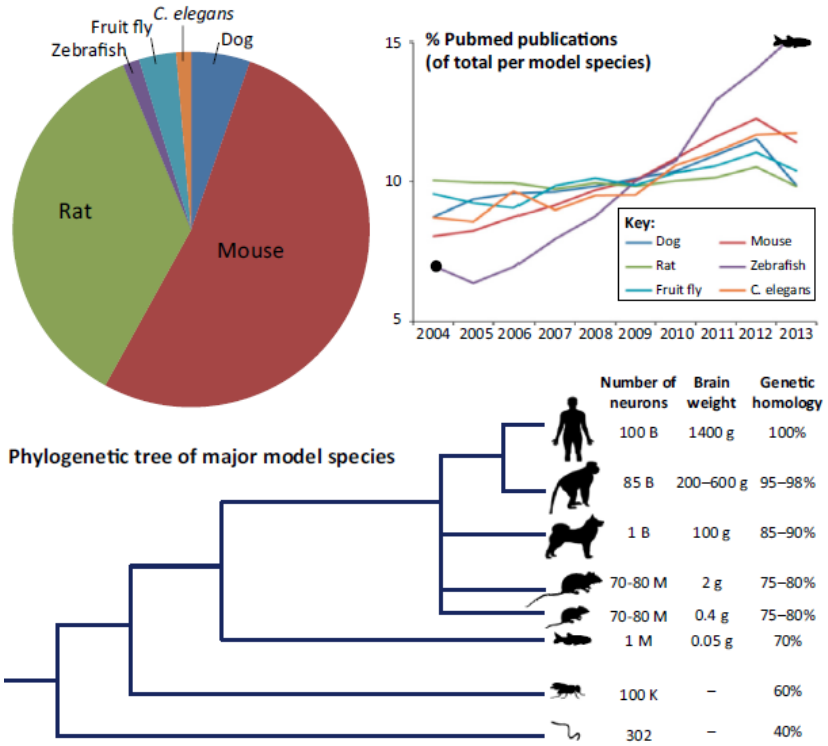


Fig. 1.1 Utility of zebrafish in biomedical research in 2004–2013. The number of PubMed publications (pie diagram) was assessed in December 2013 for various model organisms, yielding more than 532 000 publications for mice, 361 000 for rats, 54 000 for dogs, 34 000 for fruit flies, 15 000 for zebrafish, and 13 000 for nematodes (*Caenorhabditis elegans*). Line diagram shows normalized (expressed as % of total) number of publications per respective species (note that zebrafish publications display the sharpest increase compared with other animal models. Bottom left shows zebrafish in the phylogenetic tree and bottom right shows the comparative analyses of zebrafish brain versus other model organisms; note generally similar brain characteristics in zebrafish and mammals, including humans. (Stewart et al., 2014)

It should be interesting to follow the zebrafish research in a historical perspective. Kalueff et al. (2014) in a recent paper wrote that "The history of Science can be both encouraging and ironic. 110 years ago, Ivan Pavlov won the Nobel Prize for his groundbreaking study of the physiology of digestion. This line of research has later contributed to his theory of conditioned reflexes (Pavlovian conditioning), for which Pavlov remains one of the world's most renowned and influential

physiologists. Back then, all “serious”science was performed in dogs, prompting Pavlov to acknowledge “man's best friend” in his 1904 Nobel lecture. He further expressed his gratitude by commissioning the world's first Monument to the Dog in 1935. One can only imagine what would happen if someone told the fiery Pavlov that “primitive” rats and mice will replace his beloved dogs, becoming the neuroscience's most popular model organisms for decades. Perhaps, Pavlov would have been even more surprised to learn that zebrafish are widely used today to study conditioning and other related complex CNS phenomena” (Kalueff et al., 2014). It is also interesting to know that the reciprocal impact of the gastrointestinal tract on brain function has been recognized since the middle of the nineteenth century just through work of Ivan Pavlov, Claude Bernard, William Beaumont, William James and Carl Lange (Dinan and Cryan 2012).

“Translational” concept is becoming crucial in biomedicine. It links human disorders to animal models and biomarkers using the “bench to bedside” approach (Kalueff et al., 2014).

Animal models, in fact, are revealing how host genes impact the microbiome and how the microbiome regulates host genetic programs. Model systems are revealing roles for the microbiome and its modulation in host physiology ranging from mate selection to skeletal biology (Kostic et al., 2013; Maradonna et al., 2013) lipid metabolism (Wang et al. 2011; Semova et al. 2012) hepatic stress and immunity (Gioacchini et al., 2014) and others studies presented above in this work. Furthermore, increasingly, data are showing that the gut microbiome has played a crucial role in the bidirectional gut–brain axis that integrates the gut and central nervous system (CNS) activities, with psychotropic effects, controlling canonical

aspects of CNS, immunity, neurochemistry and behavior in health and disease (Bercik and Collins, 2014; Wang et al., 2013; Dinan et al., 2013; Dinan and Cryan, 2013; Savignac et al., 2013; O'Manhony et al., 2014;). Furthermore, the main goal of laboratory animal models is to recapitulate the mechanistic features of human diseases and health and to allow the intervention methods that could modify these mechanismsin the desired direction.

The zebrafish (*Danio rerio)* in this case, with its microbiota, still among the simplest vertebrate models, is emerging as a powerful model system for studying the complexities of host–microbiota interactions (Kostic et al., 2013).

1.3 How zebrafish can influence the current understanding of host-microbiota interaction and gut-brain axis

The relationship between gut microbiota and host physiology is an interesting translational area of zebrafish digestive system research. There is a high degree of homology between zebrafish and mammals not only in the adaptive immune system, but also in the digestive system. Zebrafish have a pancreas, gall bladder, liver, and intestine. The cells of the intestinal epithelium include absorptive enterocytes, goblet cells, and enteroendocrine cells similar to mammals. A lot of study support that the zebrafish could be used as an experimentally malleable system for modeling host–microbiota interactions in humans and animals (Rawls et al. 2006;. He et al., 2013; Rawls et al., 2004, 2006; Kanther, 2010; Semova et al., 2012; Brugman et al., 2009). Zebrafish also has innate and adaptive immune

systems similar to higher vertebrates and it is studied for host-bacterial interactions too (Trede et al., 2004).

In the freshwater zebrafish (*Danio rerio*), an experimentally induced lack of microbiota arrests the development of the species' gut at specific points of differentiation, an effect than can, nevertheless, be reversed by the introduction of bacteria (Bates et al., 2006). An experiment by Rawls and collaborators (2006), revealed differences between mammalian and teleost microbiota where a reciprocal gut microbiota transplant was performed between GF zebrafish and mice (Rawls et al., 2006). The gut microbes of the zebrafish microbe-transplanted mice resembled the gut microbes of conventional mice, rather than that of the mouse gut microbe-transplanted zebrafish. Similarly, the gut microbes of conventional zebrafish resembled the gut microbes of mouse gut microbe-transplanted zebrafish. These experiments demonstrate that the gut sculpts the community it has to work with into a predefined shape heavily influenced by the host. This comparative metagenomic profiling of zebrafish and mouse gut microbiota revealed that they share six bacterial divisions, including Proteobacteria, Firmicutes, Bacteroidetes, and in lower abundance, Verrucomicrobia, Actinobacteria and Planctomycetes divisions. The observations of Rawls et al. (2006), raise the question of what host factors perform this sculpting and suggest that zebrafish will be a very useful model system to identify such factors.

Danio rerio, has had their microbiota scrutinized via either culture dependent or independent techniques (Table 1.1). Roeselers et al. (2011) revealed a "core microbiome" among this species, dominated by γ-Proteobacteria and enriched

with a diverse assemblage of Fusobacteria species. Striking similarities were observed between the microbiomes of domesticated and wild individuals, implying a role for host selection on microbiota, and to an extent validating the conclusions of previous laboratory studies. γ-Proteobacteria and Fusobacteria classes were the most dominant constituents of the microbiota and were shared by all fish, despite the relatively large geographical and generational distances that separated them. The selective pressures of the zebrafish intestinal environment appear to favor a highly specific collection of microbes influenced by host anatomy, physiology, nutrient availability, and immunology (referred to as gut habitat effects) much more strongly than the effects of dietary differences or environment which may be expected to be important factors in mammals (Kostic et al., 2013).

The intestinal microbiota dysbiosis in zebrafish with inflammatory bowel disease (IBD)-like colitis was characterized by an increased proportion of Proteobacteria and a decreased of Firmicutes. This condition is present at the same time in human gut microbiota associated with IBD and in chronic inflammatory diseases. (He et al., 2013). There is also increasing evidence that dysbiosis modulates peripheral and central nervous system function, leading to alterations in brain signalling and behaviour (Bercik et al. 2011; Collins et al. 2013;). Inflammatory bowel disease can be modeled in zebrafish also using a chemical called oxazolone, which induces intestinal inflammation (Brugman et al. 2009). In zebrafish treated with the antibiotic vancomycin, Fusobacteria became the dominant phyla in the gut microbiota, and the inflammatory response observed in response to oxazolone was markedly decreased. Treatment with colistin sulfate increased γ-Proteobacteria in the gut microbiota, and these

zebrafish developed intestinal inflammation in response to oxazolone treatment. These results demonstrate that certain members of the microbiota, such as the γ-Proteobacteria, may help drive intestinal inflammation in an experimental model of colitis and may increase propensity for inflammatory responses in the gastrointestinal tract. Animal studies in general, have demonstrated that the early phase of enteric infection is accompanied by anxiety-like behavior, which is mediated through vagal ascending pathways. Chronic infection alters gut function, including motility and visceral sensitivity, as well as feeding patterns, anxiety and depression-like behavior. (Bercik and Collins 2014). The high co-morbidity between stress-related psychiatric symptoms such as anxiety with gastrointestinal (GI) disorders including irritable bowel syndrome (IBS) and inflammatory bowel disorder (IBD) is further evidence of the importance of the gut-brain or brain-gut axis. Thus, modulation of the brain-gut axis is being seen as an attractive target for the development of novel treatments for a wide variety of disorders ranging from obesity, mood, and anxiety disorders to GI disorders such as IBS (Dinan and Cryan, 2013). These studies, associated with those conducted on zebrafish, revealed this widely used cyprinid fish, as a valuable vertebrate developmental model, interesting to study gut microbiota ontogenesis, host-microbiota and host-pathogen interactions by a multidisciplinary approach to the study of both health and disease. In this regard, we can understand the bidirectional signaling between the microbiota, gut and brain, in zebrafish underlie potential and significant impacts on human and animal health, opening new research prospective and preventive and therapeutic opportunities.

Table 1.1 Studies evaluating the diversity of zebrafish associated microbial communities

Study	Organ	Tecnique	Phyla (in order of abundance)
Semova et al., 2012	Hindgut	16S/454 Pyrosequencing	Firmicutes, Proteobatceria, Bacteriodetes + minor phyla
Roeselers et al., 2011	Intestinal mucosa	16S/454 Pyrosequencing, Sanger sequence, TRFLP profiling	Proteobacteria, Fusobacteria, Firmicutes, Actinobateria
Cantas et al., 2012	Intestinal contents	13 16S/Culture + Sanger Sequencing	Gamma-proteobacteria, beta-proteobatceria, alpha-proteobatceria, firmicutes
Merrifield et al., 2013	Hindgut	16S/DGGE + Sanger sequencing	(no order) Fusobacteria, Gammaproteobacteria

1.4 Probiotics manipulate the microbiota

Probiotic studies are among the most commonly carried out to support a relationship between gut microbiota and brain and behavior and data is now emerging using different models to support the contention that a variety of other potential probiotics can exert psychotropic potential (Dinan and Cryan, 2013). Probiotics, from the Greek, meaning "for life", are live organisms that, when ingested in adequate quantities, exert a health benefit on the host. They have been reported to have a widerange of effects in both human and animal studies (Cryan and Dinan, 2012). The first formal description of a probiotic was provided by Elie Metchnikoff in 1908, based on his observation that individuals who lived in a certain region of Bulgaria had a longer life span than those in other parts of the country, a fact that he related to the regular consumption of a fermented milk product. In 1912, in a special contribution to Cosmopolitan, Metchnikoff wrote *"In*

effect, we fight microbe with microbe…there seems hope that we shall in time be able to transform the entire intestinal flora from a harmful to an innocuous one…the beneficent effect of this transformation must be enormous" , (Bested et al., 2013).

The idea of manipulating gut microbiota of fish developed as a consequence of the fact that potentially beneficial bacterial communities such as lactic acid bacteria (LAB) naturally constitute only a minor proportion of intestinal microbiota of fish or shellfish (Ringø et al., 2010). LAB are a group of Gram-positive rods and cocci that are non-sporing, lacking catalase and oxidase (cytochrome c), and are fermentative in Hugh–Leifson medium (Merrifield and Ringø, 2014). The group of LAB represents a large part of the microbiota of vertebrates and their beneficial effects on the immune system, gastrointestinal tract, and reproduction, have been widely reported. (Avella et al., 2012). In a recent study, Lyte (2011) hypothesizes the ability of probiotics to synthesize neuroactive compounds and these probiotics have the potential to act as psychotropic agents. Furthermore the ability of certain probiotic bacteria, such as *Lb. rhamnosus* (JB-1), to influence emotional behavior in mice has been shown to be mediated via GABA receptors (Bravo et al., 2011). Certain strains of *Lactobacillus* and *Bifidobacterium* secrete gamma-aminobutyric acid (GABA). This is the main inhibitory neurotransmitter in the brain regulating many physiological and psychological processes, with dysfunction in the system implicated in anxiety and depression. Other essential neurotransmitters such as serotonin (5-HT), norepinephrine and dopamine (DA) are also produced by microbes. For example, certain *Lactobacillus* and *Bifidobacterium* species produce gamma-aminobutyric acid; *Escherichia*, *Bacillus* and *Saccharomyces* spp. produce noradrenaline; *Candida*, *Streptococcus*, *Escherichia* and *Enterococcus* spp. produce

5-HT; *Bacillus* produces DA; and *Lactobacillus* produces acetylcholine (Dinan et al., 2014). Serotonin functions as a key neurotransmitter at both terminals of the gut-brain axis and emerging data implicates the gut microbiota influence on tryptophan metabolism and the serotonergic system and therefore, on behavioral effects (O'Mahony et al., 2015). A recent studies have shown that fatty acid concentrations in the brain (including arachidonic acid and docosahexaenoic acid) are elevated in mice whose diets were supplemented with the *Bifidobacterium breve* strain NCIMB 702258. Arachadonic acid and docosahexaenoic acid are known to play important roles in neurodevelopmental processes, including neurogenesis, can alter neurotransmission and protect against oxidative stress. Moreover, their concentrations in the brain influence anxiety, depression and learning and memory. Further study present in different reviews showing that certain probiotic strains can modulate various aspects of brain function and behaviour, some of which are vagus dependent (Cryan and Dinan, 2012).

Genetic, nutritional and environmental parameters affect the abundance and diversity of gut microbiota in fish. The manipulation of fish gut microbiota will result in elevation of resistance against pathogens, growth enhancement, improved lipid metabolism, stimulation of immune response and better physiological status for the gut (Llewellyn et al.,2014). Thus, strategies for the manipulation of gut microbiota of fish toward beneficial communities are developing (e.g., lactic acid bacteria) (Ringø et al., 2014). Although the mechanisms by which probiotics exert their beneficial effects on the host are largely unknown, probiotic administration showed promising results on growth performance and health of teleost fish (Llewellyn et al., 2014). The zebrafish has become an important model for

assessing the gut microbiota of vertebrates (Rawls et al. 2004; 2006; Carnevali et al. 2013) and this model has also been used to assess the efficacy of potential probiotic colonization and GI microbial modulation. Two further studies have also verified that *Lb. rhamnosus* (strain IMC 501®) has good capacity to populate the GI tract of zebrafish at multiple life stages (Avella et al. 2012; Gioacchini et al. 2012). The administration of *Lactobacillus* strains in teleosts has shown varying degrees of success. Studies which have successfully modulated the GI microbiota of fish with *Lactobacillus* strains have demonstrated that these changes can often lead to the improvement of general animal welfare in terms of survival, immune status, growth performance and/or stress response (Dimitroglou et al. 2011). Probiotics benefit the host by improving either disease resistance, health status, growth performance, feed utilization, stress response, which is achieved at least in part via improving the hosts or the environmental microbial balance.

The potential consequences of modulation of gut microbiota are here emphasized, considering overall the communication pathways between the gut microbiota and the brain. To our knowledge numerous putative studies were conducted on probiotics and, in general, on microbiota in zebrafish but no one was found to describe the demonstrable impact of modulation of microbiota on behavior and neurochemistry expression. We conclude by providing same prospective considering, zebrafish a potential animal model to understand this bidirectional communication, to continue to provide mechanistic insight and proof-of concept studies. This study could be translated in human and animal in future.

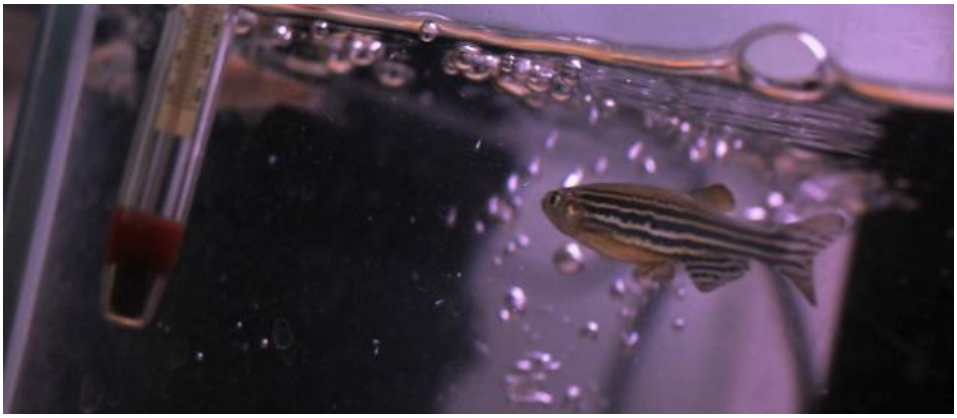
CHAPTER 2

GENERAL METHODS

2.1 Overview

All housing, feeding and behavioural experiments were carried out at the University of Napoli Federico II, Stabulario di pesci rettili ed anfibi, Department of Biology; Real time PCR was conducted in the laboratory of Genetic, Department of Biology University of Napoli Federico II; DGGE and NGS were conducted at Laboratories of the School of Biological Science, Plymouth University, UK. The general procedures and analytical techniques, which were used in the present study, are listed in this chapter. Further methods and techniques specific to individual experiments are described in their respective methodology sections in the relevant experimental chapters. All fishes were treated in accordance with the Directive of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes (directive 2010/63/EU) and in agreement with the Bioethical Committee of University of Napoli Federico II. All experimental works involving fish were conducted in accordance with the Ethic Committee, under authorization with protocol number 47339-2013.

2.2 Experimental fish and husbandry



During the present research project 72 zebrafish (*Danio rerio*) wild-tipe AB were used to conduct the experimental analysis, divided in three biological identical replicates.

Adult 4–6-month-old male and female zebrafish (~ 30:70%) of heterozygous "wild type" strain were obtained from local commercial distributors (Carmar sas, Napoli) (photo 2.1). All fish were given at least 14 days to acclimate to the laboratory environment and housed in groups of 12 fish per 30-L tank. All tanks were filled with deionized water before introducing the fish. Fishes were fed two times daily with commercial food (SERA Vipagran®, Germany). The fish were fed the diets at 1.5%–2% of bodyweight per day automatically using Rondomatic 400 (Grässlin, Germany). Two experimental groups were evaluated: a control group (CTRL), which was fed twice with a commercial diet only and a probiotic-treated group (PROBIO), which was fed twice the commercial diet and twice with the lyophilized probiotic strain *Lactobacillus rham*nosus. The room and water temperatures were maintained at 25–27 °C. Illumination (1010 ± 88 lx) was provided by ceiling-mounted fluorescent light tubes on a 14-h cycle

(D:N=14h:10h) consistent with the standards of zebrafish care (Westerfield, 2000). All fish used in this study were experimentally naïve.

2.3 Probiotic administration

The probiotic strain used was *Lb. rhamnosus* IMC 501, provided by Synbiotec s.r.l. at a final concentration of 10^6 colony-forming units/g (0,01 g/l) for 28 days. The fish were fed twice per day with the lyophilized probiotic strain *Lactobacillus rhamnosus,* automatically, using Rondomatic 400 feeder (Grässlin, Germany). (Figure 2.2)

2.4 Water quality

During the trials, water quality parameters such as temperature, oxygen and pH in the system were measured daily. The water temperature was maintained at a suitable temperature (25 – 27,5 °C) throughout the experiments with a thermostatically controlled chiller (Askoll, 50 Watt). The system pH was adjusted with sodium bicarbonate (NaHCO3) as necessary to maintain the level within the desired range (pH 6.5 - 7.5) and the dissolved oxygen levels were maintained above 80% with additional aeration provided by a side supply of compressed air. Water conducibility was 300-600 microsiemens.

Total ammonia, nitrite and nitrate were measured weekly by using commercial kits and cuvettes for ammonia, nitrite and nitrate (Askoll Test, Italy). The following

levels of nitrogenous compounds were considered acceptable: NH3/NH4+ = 0 mg/L; NO2- = <0.25 mg/L; NO3- = <0.25 mg/L These levels were controlled three times/week with partial changes of water when necessary.

2.5 Fish euthanasia and dissection

Fishes were euthanized by immersion in overdose 500 mg/ L-1 of 3-aminobenzoic acid ethyl ester (MS-222) buffered to pH 7.4 (Sigma–Aldrich, USA) (Photo 2.2). To avoid possible external contamination while removing the intestine, the surface of each fish was cleaned using 70% Industrial Methylated Spirits (IMS). Under aseptic conditions, under a light source, fish were dissected, with sterilized micro surgical blade and forceps, where brain and the GI tract were entirely excised. Each tissue was replace into individual sterile 1.5 mL micro centrifuge tubes (MCT) with 1 ml of RNAlater® sterile solution (Life Technologies, USA) and stored at -80°C until use for analysis. (Fig 2.1)

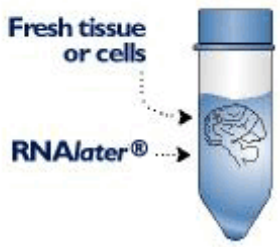


Figure 2.1 Representation of tissue storage at -80°C

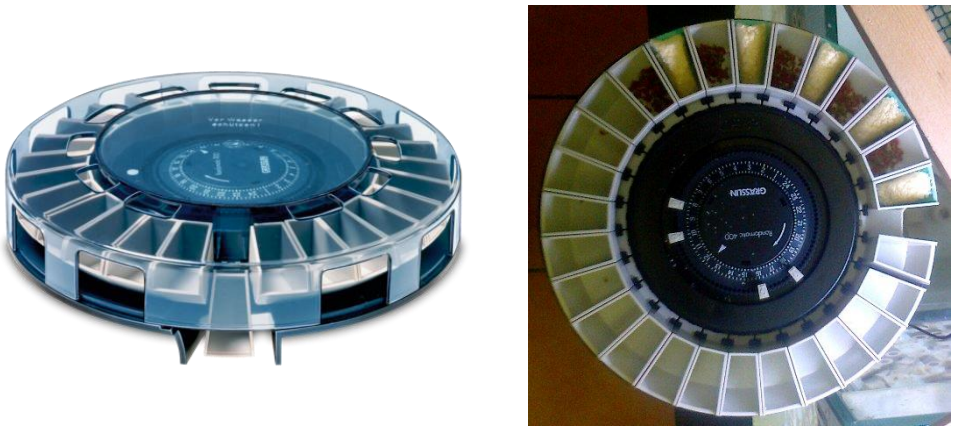


Figure 2.3 Representation of the automatic feeder and probiotic administration

Photo 2.1 Adult zebrafish (*Danio rerio*) AB wild type strain used.

Photo. 2.2 Euthanasia by immersion in overdose 500 mg/ L-1 of 3-aminobenzoic acid ethyl ester [MS-222] buffered to pH 7.4

CHAPTER 3

CAN PROBIOTICS MODULATE ZEBRAFISH BEHAVIOR?

3.1 Abstract

The zebrafish (*Danio rerio*) is a well known model organism in translational neuroscience and behavioural research. It is increasingly utilized in biomedical and psychopharmacological research aimed at modeling human brain disorders. Abnormal social behavior represents the core symptom of several neuropsychiatric and neurodevelopmental disorders. The zebrafish is a highly social species and has been proposed for modeling such disorders. Behavioral paradigms that can induce zebrafish social behavior are of importance. It has some advantages over other vertebrate species used in biomedical research that stem from its prolific nature, preference to form tightly packed groups (shoals), and the fact that it has been a preferred subject of geneticists for the past few decades (Gerlai, 2014). A growing body of data supports the hypothesis that probiotics can exert psychotropic effects. Recently, it has been demonstrated that in mice the probiotic *Lactobacillus rhamnosus* impacts behavior and produces neuroactive substances such as GABA and serotonin, which act on the brain-gut axis (Dinan et al., 2013). Here the putative link between the enteric microbiota and brain function was tested by analyzing the effects of *L. rhamnosus* on behavioural swimming pattern (movement in space and time) (Gerlai, 2014) in zebrafish. In this study probiotic fed group and control one shoal differently. These measures was determined by using a 2D video tracking analysis and modeling tool.

3.2 Introduction

Photo 3.1 Zebrafish Shoaling

Shoaling is a typical group forming behavior often seen in cyprinids (*Cyprinidae*), a family of fish to which the zebrafish belongs. Shoaling can also be observed in a wide-variety of marine fish and other organisms as well (Brierley and Cox, 2010). Shoaling is best defined as aggregation behavior that leads to conspecifics being distributed in the given area of space or "body of water", closer to each other than what would be expected in case of stochastic distribution (Photo 3.1). A lot is known about the adaptive function of shoaling. Forming groups among multiple individuals has been shown for example, to reduce the risk of predation confusing predators by the movement of several individuals and thus cannot focus on a single target. Many eyes and other sensory organs in the shoal may be able to detect an approaching predator sooner and more efficiently. Shoaling may also facilitate finding food and may make it easier to find and stay close to potential mates. However, it is notable that the function of shoaling may vary across species as it may be dependent upon the specific evolutionary past and ecological characteristics of the abiotic and biotic environment of the given species. For

example, shoaling has been found to enhance (and not reduce) predation risk in some marine fish. Yet in other species, specific environmental constraints, e.g. oxygen depletion in the middle of large swarms of krills or shoals of sardines, may also influence shoaling behavior while in other species such factors may play no role (Brierley and Cox, 2010). Whatever the actual adaptive function of shoaling may be in zebrafish, it has been observed both in nature (Engeszer et al., 2007) and in the laboratory (Buske and Gerlai, 2011 and Saverino and Gerlai, 2008) as one of the most robust and consistent behavioral features of this species (Gerlai, 2014). Probiotic studies are among the most commonly carried out to support a relationship between gut microbiota and brain and behavior. The impact of *Lactobacillus rhamnosus* on behavior is also evaluated in mice. Animals fed *Lb. rhamnosus* demonstrated reduced anxiety on a variety of behavioral measures. The study provided compelling evidence to indicate that the vagus mediates the behavioral effects of *Lb. rhamnosus*. A growing body of data is emerging using different models to support the contention that a variety of other potential probiotics can exert psychotropic potential (Dinan and Cryan, 2013). To our knowledge this is the first study focused on the correlation among dietary supplementation of probiotics and behavioral pattern changing in zebrafish. This study is focused on zebrafish shoaling behavior with the aim to compare the main differences between the group fed with the probiotic strain, *Lactobacillus rhamnosus* and the control one as described in general chapters 2.2 and 2.3.

3.2.1 Automated behavioral analyses

Zebrafish prefer to swim in shoals and the disruption of this group-forming behavior by various environmental, pharmacological, or genetic factors can be

easily assessed by using video tracking tools. This “part of aggregation behavior” has an oscillating dynamic, and this behavior can be quantified manually or using automated video-tracking systems, assessing several endpoints, including the average inter-fish distance; shoal area size; proximity (time each member of the shoal spent within a specified distance from each other); nearest and farthest neighbor distances; time spent in shoal; time spent away from shoal; number of animals leaving the shoal and polarization (reflecting the uniformity of heading) (Kalueff et al., 2013). Behavioral phenotypes are the most complex product of CNS activity, and the availability of reliable video tracking techniques markedly empowers neurobehavioral analyses in zebrafish (Stewart et al., 2014). Video-tracking has been broadly applied to fish research including zebrafish focusing on swimming mechanics and detection of multiple subjects in shoaling studies (Cachat 2010). For example, both commercial and custom-made video tracking systems are used to assess larval and adult zebrafish behavior. Such automated observations are particularly suitable for measuring loco-motor responses (e.g., distance traveled or speed/velocity, turning, etc.) These software systems often have modular structure and are standardized, user-friendly, and coupled with thoughtfully designed hardware. Although not inexpensive, these packages are also validated by multiple international users, and typically come with regular upgrades and technical support, which becomes especially useful from a practical point of view. Offering a free alternative, the custom-made tracking systems are also available from different laboratories worldwide and can be useful for various specific neurophenotyping tasks and experimental set-ups in zebrafish. (Stewart et al., 2014). Same reports have either applied 2D (one camera) video-tracking methods to assess fish stress-related behaviors or used 3D (two cameras) video-

tracking as well as high sampling rate to characterize fish swimming, including assessment of zebrafish neurotoxic phenotype (Cachat 2010). Gerlai, (2014) dimonstred that three-dimensional presentation of the shoal stimulus is not really required to evaluate shoaling response, making sufficient zebrafish images moving back and forth on a 2D flat surface (the computer monitor).

3.3 Materials And Methods

3.3.1 Animals and housing

Adult 4–6 month-old male and female zebrafish (~ 30:70%) of heterozygous (AB) "wild type" short-fin strain were obtained from local commercial distributors (Carmar sas, Napoli). The AB strain is frequently used in behavioral neuroscience (Nowicki et al., 2014). All fish were given at least 10 days to acclimate to the laboratory environment and housed in groups of 12 fish per 30-L tank. All tanks were filled with deionized water before introducing the fish. The fish were fed at 1.5%–2% of bodyweight per day automatically. Two experimental groups were evaluated: a control group (CTRL), which was fed twice per day with a commercial diet (SERA Vipagran®, Germany) and a probiotic-treated group (PROBIO), which was fed twice per day the commercial diet and twice per day with the lyophilized probiotic at a final concentration of 10^6 colony-forming units/g for 28 days. The probiotic strain used was L. rhamnosus IMC 501, (provided by Synbiotec s.r.l. Camerino, Italy) The room and water temperatures were maintained at 25–27 °C. Illumination (1010 ± 88 lx) was provided by ceiling-mounted fluoreshent light tubes on a 14-h cycle consistent with the standards of zebrafish care (Westerfield

M., 2000). All fish used in this study were experimentally naïve. The experiment was performed in biological duplicates.

3.3.2 Apparatus and behavioral testing

Two identical experimental setups were run in parallel (Figure 3.4). Twelwe zebrafish were evaluated for each tank. Fish swimming behavior was video-recorded between 14:00 and 15:00 h on the first day of probiotic treatment (T0) and successively at 7 days intervals (T1-T4). Recording was performed next the tanks with a Nikon D7000 camera for 6 min, acquaried and analysed with 2D video tracking analysis and modeling tool (Tracker, California, USA) built on the Open Source Physics (OSP) Java framework (www.cabrillo.edu/~dbrown/tracker/) (Figure 3.2). This software allows to analyze a video clip or an image in order to determine multiple variables. The program is designed to be used in physics experiments in order to easily estimate the acceleration and velocity and distance of a certain object. In line with Gerlai (2014), the video the tracking data were used to determine following behavioral measure: **Average Distance (AD); Distance Variance (DV); Nearest Distance (ND); Occupied Area** (or Shoal size area) **(OA) and Water column position (CP).** In particular **AD** represents the Inter-individual distance and it defines and calculates the average of all distances between a focal fish and its shoal members. Each focal fish within a shoal thus will get an inter-individual distance value and this value is calculated for any given moment of time sampled. The disadvantage of this measure, however, is that it is dependent upon the size of the shoal, i.e. the number of individuals that make up the shoal. The larger the number of such individuals and larger the inter-individual distance value will be. **ND** calculates the nearest distance of each single fish from

its neighbor. Thus again, each fish of the shoal will receive a nearest neighbor distance (Gerlai, 2014). Contrary to AD, ND is independent from shoal size. **DV** or the variability of inter-individual distance is the variance of the distances between the focal fish and all of its shoal members. Thus again, each focal fish gets a variance of inter-individual distance value for any given moment of time. Notably, this variability represents the relative position of the given focal fish within a shoal. The mean of variances of inter-individual distances when calculated for the entire shoal represents the homogeneity of the distribution of fish within that shoal. The less uniformly the shoal members are distributed the larger the variance will be. It is important to note that the inter-individual distance takes the position of every fish in the shoal into account and thus it is the most informative measure of shoal cohesion. **OA** is an additional measure in this study and it calculates the occupied area of all animals in a temporal unit on a two-dimensional plane. **CP** is another measure added in this study (after the differences showed by two group. It represents the water column position and indicates the preference of animals to occupy the upper or the lower (part) half of the tank. It was estimated as the % number of animals that stay in the lower part of the tank during the period of observation.

For AD, DV and ND measurement each fish was tracked on six randomly selected 20 frame (2fps) intervals of each video. For OA and CP analysis it was used ImageJ 1.49 software (National Institute of Mental Health, Bethesda, Maryland, USA) to select the areas occupied and the side of water column preferred by each fish in all video collected (Figure 3.3). Ten images were randomly selected from each video.

ImageJ is a public domain Java image processing program inspired by NIH Image for the Macintosh (http://imagej.nih.gov/ij/docs/intro.html).

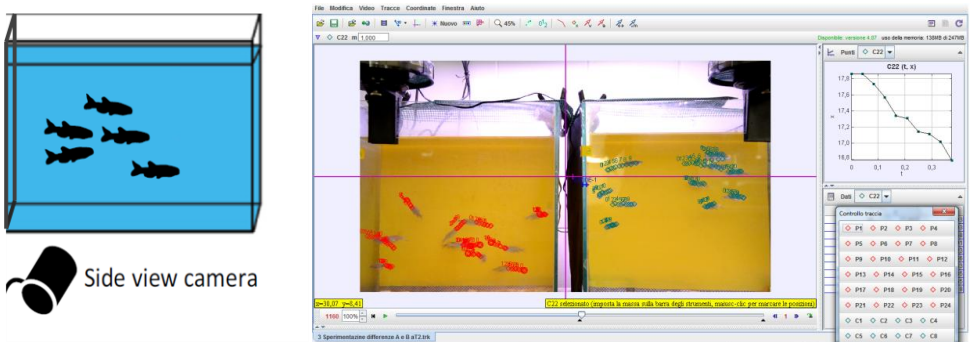


Figure 3.2 representative illustration of the typical set up of the shoaling test (left) and application of video tracking tool (Tracker) (right), to quantify zebrafish behavior.

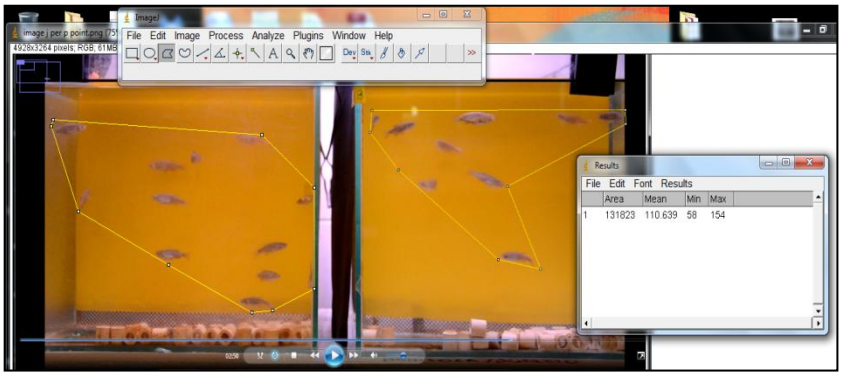


Figure 3.3 illustrates ImageJ softwere used to select the areas occupied and the side of water column preferred by each fish in all video collected.

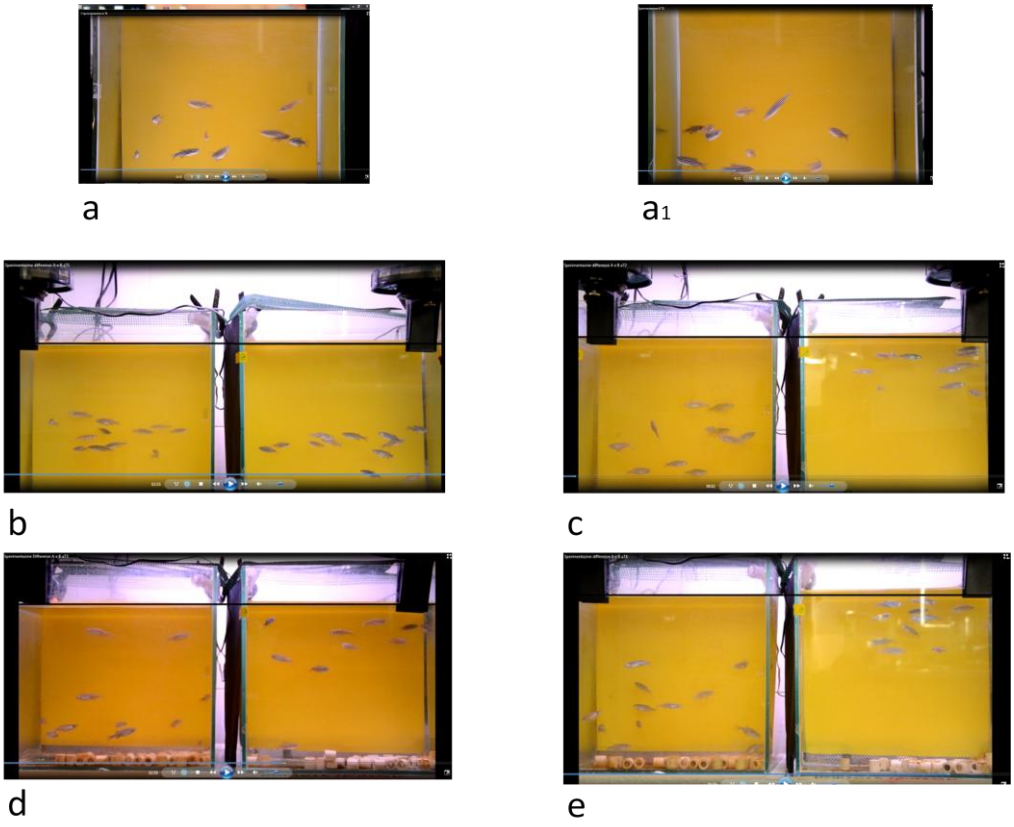


Figure 3.4 Screenshots of a video recordeding at different time of experimentation: a, PROBIO T0; a_1, CTRL T0; b, c, d and e represent respectively T1, T2, T3 and T4 of experimental observation, left tank is PROBIO group and right tank is CTRL group.

3.3.3 Statistical Analysis

Results are reported as means ± SE. Two-way ANOVA with Sidak's post-hoc test was used to evaluate the significance of the effect of the probiotic treatment, using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

3.4 Results

Using the above measures of shoal cohesion we have discovered that zebrafish fed with probiotics shoal differently by the second week (T2) until the end of administration (T4) (Fig. 3.5 and 3.6)

In particular the AD is not significant different between two groups. The inter-individual distance value increased in both CTRL and PROBIO from the second week until the end of experiment (Two-way ANOVA, $p<0.001$).

Noteworthy is the DV that changes from the second week of treatment with a significant difference between the two groups. The probiotic treated group was more uniform and showed a bigger and costant homogeneity of the distribution of fish within the shoal throughout the whole period of treatment. The ND displayed significant dependence from both treatment (two-way ANOVA, $p<0.05$) and time (two-way ANOVA, $p<0.01$). However, despite the tendency of ND to be lower in the CTRL group, a significant difference was evident at T4 only (Sidak's post-hoc test, $p<0.05$). The area occupied or shoal size area (AO) was significantly affected by both tretment ($p<0.01$) and time ($p<0.01$). Except that at T1, the probiotic treated group displayed a larger AO then control group particulary evident during the third and fourth week, when we can see significant differences (Sidak's post-hoc test, $p<0.01$). About the water column preference (CP) the two fish groups showed from the second week a completely different behavioral pattern in term of preferences of location in the water column; this result is correlated with area occupied. From T2 the position for the control group changed completely and fishes spent most of the time in the upper side of the tank while the probiotic

treated group preferred the medium/deeper part of the tank occupying most of area of tank.

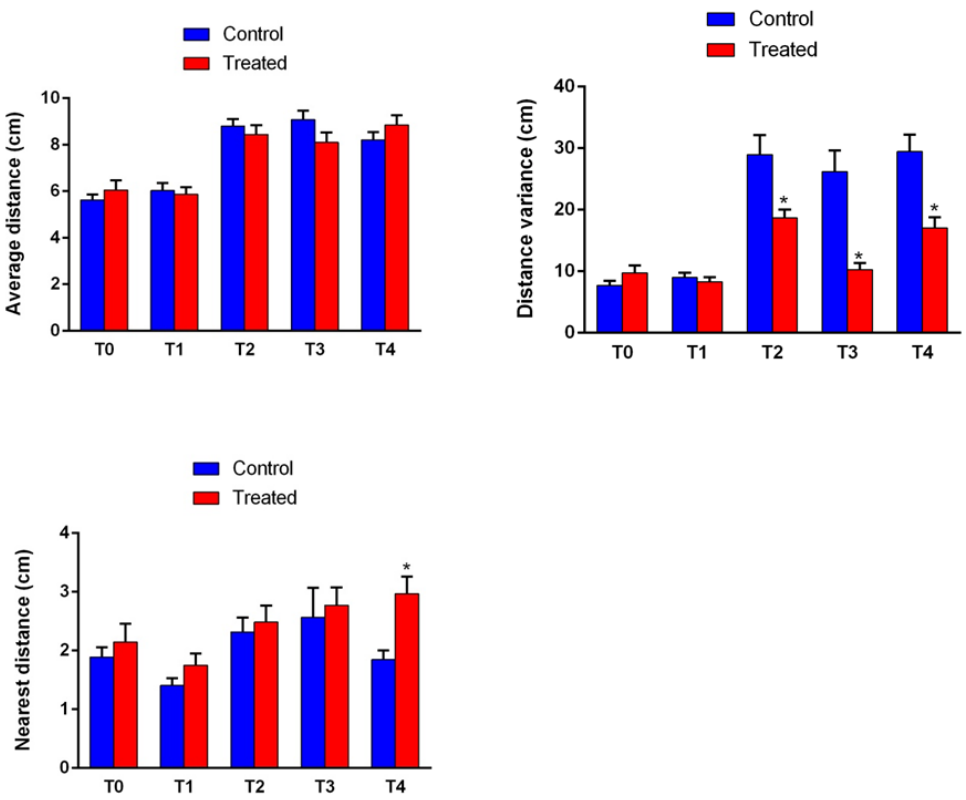


Figure 3.5 AD, DV and ND measurement at different time of experimental observation obtained with Tracker software and analysed statistically. DV The ND displayed significant dependence from both treatment (two-way ANOVA, $p<0.05$) and time (two-way ANOVA, $p<0.01$) and at T4 Sidak's post-hoc test, $p<0.05$

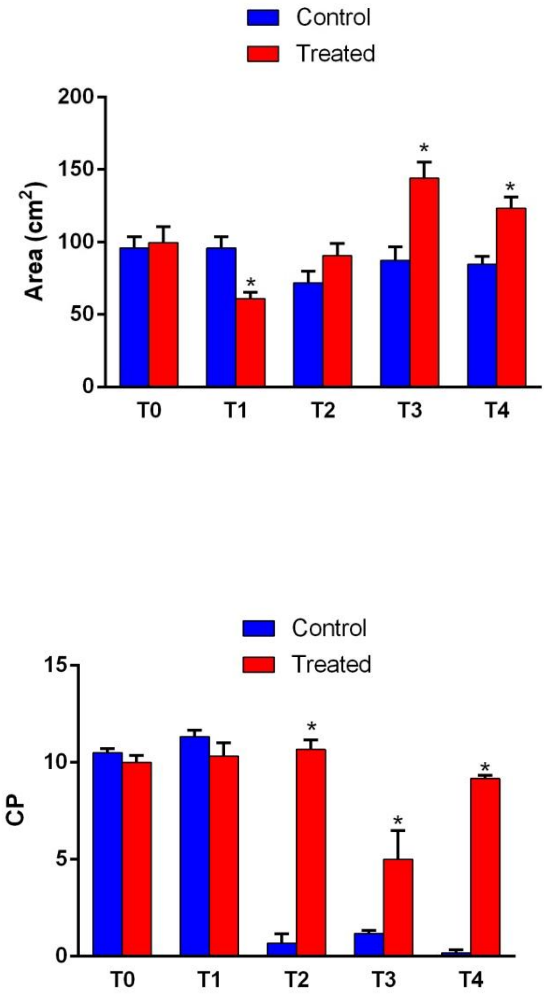


Figure 3.6 OA (up) and CP (down) measurement at different time of experimental observation obtained with ImageJ software and analysed statistically Two-way ANOVA, $p<0.001$ and (Sidak's post-hoc test, $p<0.01$).

3.5 Discussion and Conclusion

Video-tracking of zebrafish yields objective analysis of behavioral endpoints and therefore provides researchers with an important tool for the investigation of behavior in this animal model. Furthermore, such standardization promotes reproducibility in experimental design, strengthening the investigator's ability to draw valid conclusions from zebrafish study data and results. Research on the

molecular biology and genomics of probiotics has focused on the interaction of gut microbiota with the immune system, brain development, potential as an anticancer agent and potential as a biotherapeutic agent in many diseases. In a study rats fed *Lb. rhamnosus* demonstrated reduced anxiety based on a variety of behavioral measures. The study provided compelling evidence to indicate that the vagus mediates the behavioral effects of *Lb. rhamnosus* (Dinan and Cryan 2013). Here in our study probiotic fed group and control one shoal differently from the second week until the end of observation. In particular the lower value of distance variance (DV) in the probiotic treated group, meaning more homogeneity of the distribution of fish within that shoal (Gerlai et al.,2014), suggest us a different signal in the shoal behavior. With regard to the nearest distance (ND), the fishes in control group swam closer together then probiotic treated group. In this case the shoal pattern may be associated to more anxiety/fear that causes the shoal to "tighten" (the fish swim closer together) (Kalueff et al.,2013b). The larger occupied shoal area (OA) by probiotic treated group suggests an increased exploration area with a preference in the middle/deeper part of the water column of the tank. In contrast, the control group showed a reduced shoaling area occupying most of time to the top of the tank. This different behavior might be explained with an increasing of attention or possibly alert in the probiotic treated group. Blaser and Goldsteinholm (2012) supposed that aerial predation provides selective pressure on defensive behavior in zebrafish, making avoidance of the water surface more adaptive than seeking cover near the bottom. Further study will be needed to determine the mechanisms as well as the ontogeny of this behavioral differences. A lot of individual study on zebrafish were performed and individual differences in activity were found in zebrafish behavior (Tran and Gerlai, 2013) and although

there is currently a relatively small group of highly trained zebrafish neuroscientists and pathologists, the field is expanding rapidly (Kalueff et al., 2014). Testing grouped zebrafish produces more homogeneous data and preserves their behavioral repertoire, but requires more laborious or sophisticated data acquisition and analysis. Such conditions may favor the study of more complex behaviors such as those involved in sophisticated cognitive and social interactions that are often challenging to investigate in rodents (Pagnussat et al., 2013).

Overall this study represents the first video tracking shoaling analysis of adult zebrafish group treated with a probiotic strain such as *Lb. rhamnosus*. This study has shown how a probiotic strain can modulate the behavior of zebrafish in term of shoaling. Therefore it provides a basis for further studies on the gut-brain axis in *Danio rerio*. Although human behavior will never be similar to fish responses (and vice versa), the evolutionarily conserved nature of complex CNS traits suggests that many human and zebrafish phenotypes share common genetic and physiological factors, representing an exciting emerging field for further translational studies in neuroscience (Stewart et al., 2014). The same concept is valid for other vertebrates. Taken together, these results confirm zebrafish as a valid, reliable, and efficacious model for basic translational research to understand with further studies the microbiota-gut-brain axis.

CHAPTER 4

CULTURE-INDEPENDENT METHODS FOR MICROBIOTA EVALUATION IN ZEBRAFISH (*DANIO RERIO*) TREATED WITH *L. RHAMNOSUS* AND CONTROL GROUP: DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) AND NEXT-GENERATION SEQUENCING (NGS)

4.1 Abstract

Methods of measuring bacterial communities are rapidly improving. The earliest and most traditional technique is the culture-dependent method. In recent decades, microbiologists have developed new culture-independent techniques to obtain a better representation of bacterial communities present in host organisms, for example denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) (Sevellac et al., 2014), PCR-random amplified polymorphic DNA (RAPD) (Spanggaard et al., 2000), fluorescence in situ hybridization (FISH) (Huber et al., 2004) and clone libraries (Kim et al., 2007). These approaches are useful in that they offer new opportunities for detection and identification of the microbiota, leading to a broader understanding of the microbial composition in the gastrointestinal tract (GIT) of fish. In contrast few studies have applied Next-Generation Sequencing (NGS) methods to investigate the microbiome of vertebrates in their natural environment and in freshwater fishes in particularly (Sevellec et al., 2014). The capability of high through-put sequencing of 16S rRNA gene sequences by means of Next Generation Sequencing (NGS) technologies has been pivotal in facilitating the discovery of gut microbiota biodiversity. The Ion Torrent PGM instrument represents a recently commercialized bench-top NGS platform and is marketed as being less costly and

with a faster turnaround as compared to other NGS techniques such as the 454 and Illumina platforms (Milani et al., 2013). Therefore, the aim of this study was to gain a better overall understanding about differences in the GIT microbiota between a group of zebrafish (*Danio rerio*) treated with *L. rhamnosus* IMC 501 and control groups by using DGGE and NGS technologies. Here we use the Ion Torrent PGM (Personal Genome Machine) technology to allow a more complete description of complex bacterial communities and biodiversity of the zebrafish (*Danio rerio*) gut. The average of observed taxonomical units (OTUs) detectable in the probiotic group increased compared with control group, therefore this study indicates that dietary supplementation of *Lb. rhamnosus* modulates intestinal microbial communities of zebrafish. Feeding zebrafish probiotic *Lb. rhamnosus* showed a significant increase of Firmicutes phylum and, although not significant, a reduction of Proteobacteria the greather microbiota present in dysbiosis, supporting the antagonistic activity role of this probiotic strain.

4.2 Introduction

Vertebrate species host a considerable bacterial diversity, which may influence their development, physiology, immune system and nutrition. The relationships between bacteria and their hosts consists in four types. The first two types are commensal bacteria, which may either have beneficial or neutral effects on the host. The second type has a symbiotic obligatory relationship with the host, thus allowing a mutual benefit between symbiotic bacteria and host. The third type is opportunistic bacteria, which are facultative pathogenic bacteria that may become

actively pathogenic when the host immune system is impaired and unable to fight off infection. The fourth type of relationship pertains to pathogenic bacteria which are responsible for infectious diseases (Sevellec et al., 2014). The group of Lactic acid bacteria (LAB) represents a large part of the microbiota of vertebrates and their beneficial effects on the immune system, gastrointestinal tract and reproduction, have been widely reported. *Lactobacillus rhamnosus*, is one of the main LAB components of the commensal microflora of human intestinal tract and it is widely used as a probiotic in mammals (Avella et al., 2012). A number of recent studies have evidenced the positive role of *Lb. rhamnosus* on zebrafish gamete quality, spawning rates, oocyte growth and maturation, larval development, fecundity, backbone calcification and the expression of genes which regulate growth, development and immunity (Table 4.1) (Carnevali et al., 2014a).

Tab. 4.1: Parameters investigated of administration of *Lb. rhamnosus* on zebrafish

Potential probiont	Parameters investigated
Lb. rhamnosus IMC 501	F
Lb. rhamnosus IMC 501	F
Lb. rhamnosus IMC 501	F
Lb. rhamnosus IMC 501	GM , F
Lb. rhamnosus IMC 501	GM , F
Lb. rhamnosus IMC 501	F
Lb. rhamnosus IMC 501	F
Lb. rhamnosus IMC 501	GP
Lb. rhamnosus IMC 501 and *Lb. casei*	F, GH, GM, IR
37 commensal or probiotic Gram-positive and Gram-negative bacteria often used as probiotic strains in the food industry and/or aquaculture	DR, IR
Lactobacilli (multiple species)	DR, GM, PA
B. coagulans	DR, IR, PA

Genera abbreviations: *B*. = *Bacillus*, *Lb*. = *Lactobacillus*.
Parameters investigated: DR = disease resistance, GM = gut microbiota (inclusive of GI probiont recovery), F= fecundity/gonadal development/spawning rates etc.,
GP = growth performance,
IR =immunological/haematological response,
PA = pathogen antagonism
(Carnevali et al., 2014 10 Probiotic Applications in Temperate and Warm Water Fish Species.)

The intestinal microbial communities and their metabolites play an integral role in the ontogeny of teleosts. (Cerf-Bensussan and Gaboriau-Routhiau, 2010; Merrifield et al., 2010; Sekirov et al., 2010; Llewellyn et al., 2014). Intestinal microbial communities consist of allochthonous (digesta-associated, transient) and autochthonous (mucosa-associated, indigenous) microbiota (Ringø and Birkbeck, 1999; Ringø et al., 2003). The microbiota play important roles such as assembling of the gut-associated lymphoid tissue (GALT), it helps the immune system, influences the integrity of the intestinal mucosal barrier, modulates proliferation and differentiation of its epithelial lineages, regulates angiogenesis, modifies the

activity of the enteric nervous system and plays a key role in extracting and processing nutrients consumed in the diet (Rawls et al., 2004). The mechanisms by which the mammalian gut microbial community influences host biology remain almost, despite these important effects, entirely unknown. Deciphering the pathways through which microbial signals operate promises to provide new chemical entities and host targets for enforcing health, and perhaps treating diseases affecting both the intestine and extra-intestinal tissues. The zebrafish, has several unique features that make it an attractive model organism for analyzing these pathways (Rawls et al., 2004).

Here we sought to determine whether gut microbiota composition varies between zebrafish treated with dietary supplementation of *Lb. rhamnosus* and a control group. We used the DGGE analysis at first and Next Generation Sequencing with Ion Torrent PGM profiles to assess individual variation in gut microbial communities.

4.3 Materials and Methods for DGGE

4.3.1 Animal Housing

The experiment was conducted as described under section 2.2 in Stabulario of fish anphybians and reptiles a of the University of Napoli Federico II and water quality was monitored accordingly the section described in 2.3

4.3.2 Experimental fish and feeding

24 (twelve PROBIO and twelve CTRL) of 72 zebrafish (Danio rerio) were randomly selected, in this experiment. All fishes was carefully acclimatized and feeded as described under the section 2.2 and 2.3. In particular four zebrafish treated and four zebrafish control for each experimentation were euthanized and dissected as described in 2.4 of general methods.

4.3.3 DNA extraction

DNA was extracted from the zebrafish (*Danio rerio*) gut samples using a combination of QIAamp® Stool Mini Kit (QUIAGEN, West Sussex, UK) with minor modifications to the manufacturer's instructions, as described in Appendix 1. and phenol-chloroform method. Gut samples were prepared in a sterilized Eppendorf tube, and DNA extracted by the following five phases:

1. **Lysis**: 60-80 mg of samples were macerate with sterile macerators and mixed with 500 µl of fresh lysozyme solution (50mg/ml TE buffer). Then, the samples were incubated at 37 ºC for 30 minutes. 700 µl of buffer ASL was added and mixed for 1 minute. The mixture was placed on a hot plate at 90 ºC for 10 minutes and vortexed for 5 seconds with centrifugation for 1 min at 14000 rcf.

2. **Inhibitor removal**: Half an inhibitor tablet was added to 800 µl of the supernatant and vortexed for 1 min immediately, then, centrifuged for 3 min at 14000 rcf. All of supernatant was pipette into a new Eppendorf tube. The supernatant was centrifuged for other 3 minutes.

3. **Protein removal**: 400µl of the supernatant was mixed with 20 µl of proteinase K and 400µl of buffer AL was added and mixed for 15 seconds, then incubated at 70ºC for one hour.

4. **Phenol Chloroform Clean-up**: The entire samples were poured into a 15 ml falcon tubes carefully, and added an equal volume of ice cold Tris-buffered phenol solution. The samples were mixed by hand and left on ice for 10 minutes. An equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed, then centrifuged for 5 minutes at 6000 rcf. The aqueous layer was pipette off carefully and placed in new 1.5 ml Eppendorf tube.

5. **Precipitation**: 400 µl of ice-cold isopropanol was added. The samples were vortexed and placed in -20 °C freezer for overnight. Then, samples were centrifuged at 14000 rcf for 30 minutes at 4 °C. The supernatant were pipette carefully and discarded. 500 µl of 70% molecular grade ethanol was added slowly, and discarded. The addition of 70% ethanol was repeated and discarded again. The pellets were dried for 5 minutes maximum. Finally, the DNA extracted was resuspended overnight at 4 °C by adding 30 µl of molecular grade water. The concentration of DNA and purity were determined using a Nanodrop-100 Spectrophotometer.

4.3.4 16S rRNA amplification, Polymerase Chain Reaction (PCR)

PCR was conducted to amplify the V3 region of the 16S rRNA gene using PCR with the forward primer P3 with a GC clamp on its 5'-end (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and the reverse primer P2 (5'- ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993). Each

single PCR reaction consisted of 25 µl ReadyMixTM Taq PCR Reaction Mix with MgCl2 (Sigma-Aldrich Company Ltd., Gillingham, England), 1 µl each of primer P2 and P3 (50 pmol/µl Eurofins MWG Operon, Ebersberg, Germany), 1 µl of DNA template and sterile, molecular grade water to adjust the final volume of the reaction to 50 µl. Touchdown thermal cycling was conducted using a GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA), under the following conditions: 94 °C for 10 min, then 30 cycles starting at 94 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min as described by Muyzer et al. (1993). The annealing temperature decreased by 1 °C every second cycle until 55 °C and then remained at 55 °C for the remaining cycles. In order to check the purity and molecular weight characteristics of PCR products, PCR products (6 µL) were loaded onto a 1.5% agarose gel (Lonza, Rockland ME, USA), made with 1x Tris-acetate-EDTA (TAE) buffer prestained with 4 µL of SYBR® Safe™ DNA Gel Stain (Life TechnologiesTM UK) per 100 mL of agarose (Fisher Scientific) and run with 1x TAE buffer in a Pharmacia electrophoresis tank at 90 volts for 60 min. Five µL of Hyper Ladder IV (Bioline) was run alongside the PCR products to assess the size of DNA products. Viewing of agarose gels was achieved under UV light using a Bio-Rad universal hood 11 (Bio-Rad laboratories, Italy). 18 positive PCR products samples (9 belonging to PROBIO and 9 to CTRL) were chosen for DGGE and stored at 4 °C until use (Fig 4.1).

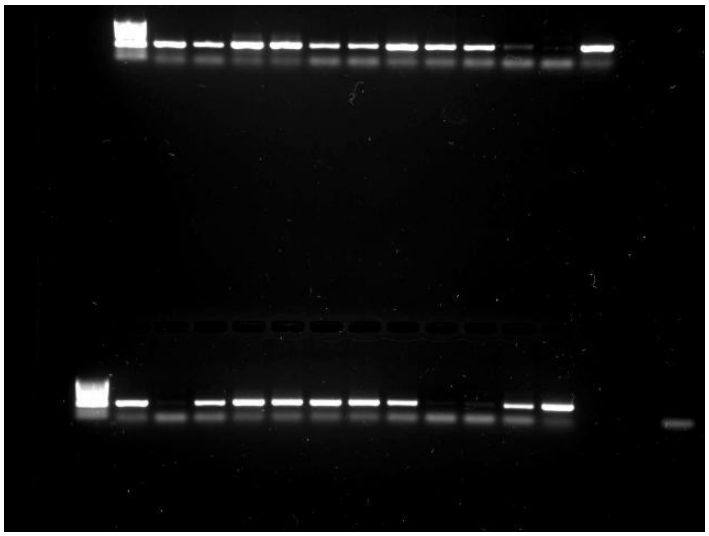


Fig. 4.1 Touchdown thermal cycling PCR of DNA samples extraction (1-24). Eighteen samples positive were chosen (9 PROBIO in the upper line and 9 CTRL down).

4.3.5 Denaturing gradient gel electrophoresis (DGGE)

The resulting 18 PCR products were used to obtain DNA fingerprints of the bacterial community present in the gut by DGGE using a Bio-Rad DGGE system (DCode™ System, Italy). DGGE was carried out by loading 15 µL of PCR products onto 10% acrylamide gels with a denaturing gradient of 40 - 60% (where the denaturants were 5.6M urea (Sigma, UK) and 40% formamide (Sigma, UK).

Made using the following stock solutions; an 80% denaturant polyacrylamide solution consisted of 25 mL of 40% acrylamide mix (high purity acrylamide), 2mL of 50x TAE buffer (pH 8.3), 32 mL of molecular grade formamide (Sigma, UK), 34 g of 5.6M ultrapure urea (Sigma, UK) and volume of MilliQ H2O yielding a total volume of 100 mL. Stock 0% denaturant polyacrylamide solution consisted of 25 mL of 40% acrylamide mix (high purity acrylamide), 2mL of 50x TAE buffer (pH 8.3) and 73 mL of MilliQ H2O. One-hundred and fifty µL of 10% ammonium

persulphate (APS, electrophoresis grade, Sigma, UK) and 17.5 mL of Tetramethylethylindiamine (TEMED) were added to the high and low denaturant solutions. Twenty one mL of each acrylamide solution was added to separate 30 mL syringes and these were mounted onto a Bio-Rad gradient delivery system (model 475, Bio-Rad laboratories). The major steps of DGGE are presented in Figure 4.2. This was then used to pour the gel between gel plates and the gel was left to polymerize for two hours. Additionally, PCR products from *L.rhamnosus* pure colonies were loaded to the gel as a reference species to aid probiotic identification. The gel was run at 65 V for 17h at 60 °C in 1 x TAE buffer. Viewing of the DGGE bands was accomplished after SYBR® gold staining. Briefly, the gel was incubated for 20 min at room temperature in 200 mL tank buffer containing 20 µL of 10000x SYBR® gold nucleic acid gel stain (Invitrogen™, UK) with shaking on an IKAO VIBRAX VXR basic shaking platform at 100 rpm/ min. The gel was scanned in a Bio-Rad universal hood 11 (Bio-Rad Laboratories, Italy) and optimized for analyses by enhancing contrast and greyscale.

4.3.6 Excision of DGGE bands, for sequence analysis

After DGGE, bands (or 'operational taxonomic units', OTU) of interest (those showing clear and consistent specialization either to intestinal regions or dietary treatments, or those clearly unaffected) were excised from the gel using sterile pipette tips and DNA was eluted overnight at 4 °C in 1.5 mL Tube containing 20 µL Molecular Grade Water (Photo 4.1)

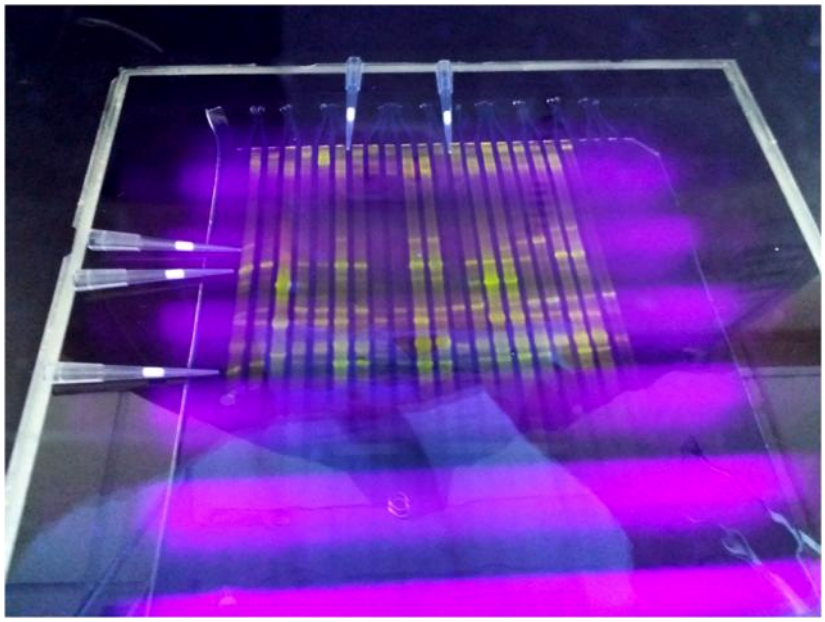


Photo 4.1 Excision of DGGE bands (or 'operational taxonomic units', OTU), for sequence analysis

4.3.7 16S rRNA amplification of excised DGGE bands

From the eluate, 5 µL was used as the template for reamplification using the forward primers P1 (5-CCTACGGGAGGCAGCAG-3; essentially P3 without the GC clamp at its 5' end) and the reverse primer P2 under the same conditions as previously described (Section 4.2). Six µL was loaded onto a pre-stained agarose gel (1.5%) to check the PCR product size.

4.3.8 Purification of the PCR products and sequence analysis

The PCR products were cleaned using a QIAquick PCR Purification Kit (Qiagen), according to the manufacturer's instructions and PCR yields (the concentration and purity of DNA) were checked using a Nanodrop® 1000 spectrophotometer. Protein purity (A260/A280) and humic acid purity (A260/ A230) were checked.

The PCR products were sequenced by GATC Biotech Ltd. (Germany) and sequenced by GATC laboratories (GATC-biotech laboratories, Germany). Nucleotide sequences were then submitted to a BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to retrieve the closest known alignment identities for the partial 16 S rRNA sequences. (Figure 4.3)

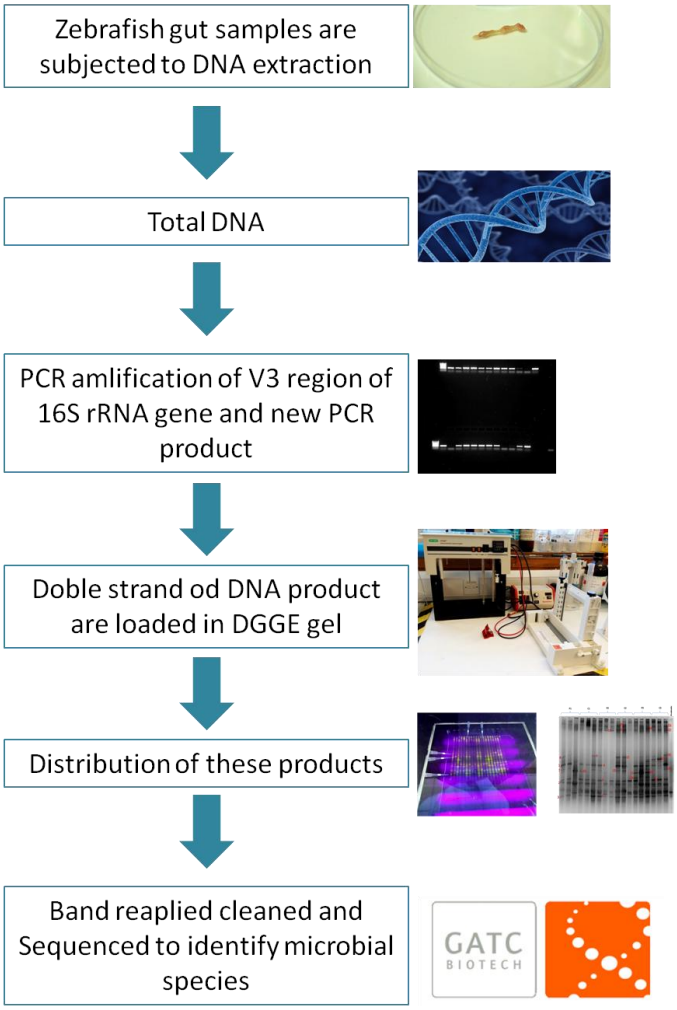


Fig. 4.2 Schematic representation of the principle steps of the denaturation gradient gel electrophoresis (DGGE) process.

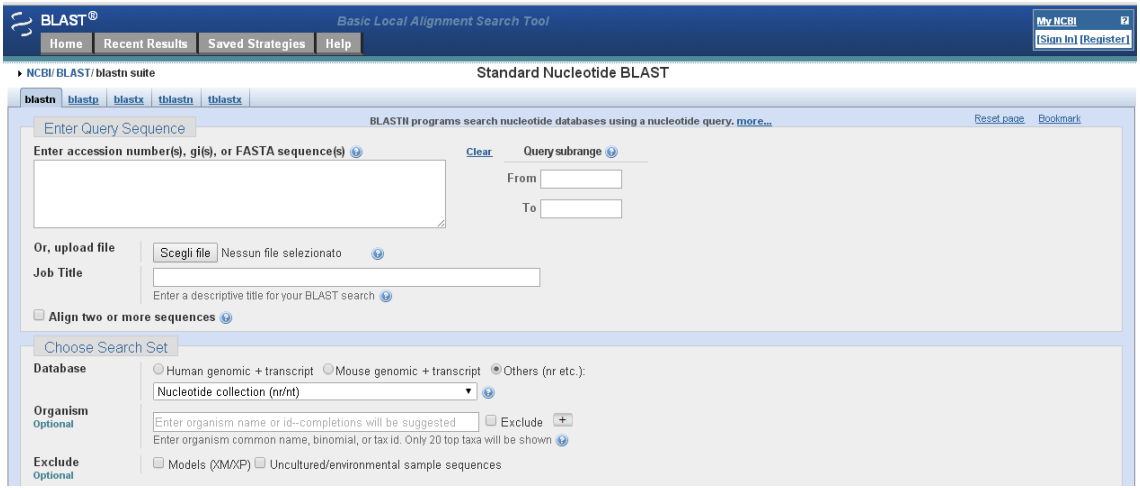


Fig. 4.3 BLAST searching in GenBank screenshot

4.3.9 Statistical analysis

Data was transformed where necessary and statistical analysis was carried out using a One-Way ANOVA Minitab v.16 statistical software (Minitab, Plymouth, UK). Tukey's Multiple Comparison test was used to determine significant differences between means and significance was accepted at the $P < 0.05$ level. Primer V6 (Clarke and Gorley, 2006) was used to calculate species richness, evenness and diversity of the PCR-DGGE fingerprints according to the following formulae: Margalef's species richness: $d = (S - 1)/\log(N)$; and Shannons diversity index: $H' = -\Sigma(pi(\ln pi))$. Where N = total number of individuals (total intensity units),S = number of observed taxonomical units (presumed species) and pi = the proportion of the total number of individuals in the ith species.

4.4 Materials and Methods for Ion Torrent PGM technology NGS

4.4.1 Samples

With regard to the facility conditions, experimental fishes and DNA extraction the same conditions apply as described in 4.3.1, 4.3.2 and 4.3.3. PROBIO and CTRL DNA extraction were analyzed as follow

4.4.2 16S rRNA Gene Amplification

Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair 27F (5'-AGA GTT TGA TCM TGG CTC AG-3'), 338RI (5'-GCW GCC TCC CGT AGG AGT-3') and 338RII (5'-GCW GCC ACC CGT AGG TGT -3') (Roeselers et al 2011). which targets the V1-V2 region of the 16S rRNA gene sequences. The PCR conditions used were 7 min at 95°C, 10 Cycles of 30 sec at 94 °C, 30 sec at 63 ° C, 30 sec at 72 °C and 25 Cycles of 30sec at 94°C, 30 sec at 53 °C and 30sec at 72 °C followed by 10 min at 72 °C. Amplification was carried out by using a Verity Thermocycler (Applied Biosystems). The integrity of the PCR amplicons was analyzed by electrophoresis

4.4.3 Ion Torrent PGM Sequencing of 16S rRNA Gene-based Amplicons

Twenty PCR products derived from amplification of specific 16S rRNA gene hypervariable regions were purified by electrophoretic separation on an 1.5% agarose gel and the use of QIAquick PCR Purification Kit (Qiagen) in order to remove primer dimers. Libraries for sequencing were prepared using the Ion Plus Fragment Library Kit (Life Technologies) and quantified using the Ion Library Quantitation Kit (Life Technologies). A proportion of the amplicon libraries were

also run on the Agilent 2100 Bioanalyzer using the High Sensitivity DNA Kit (Agilent Technologies) in order to confirm adapter ligation and average size of each library. Libraries for each run were diluted to 26pM and pooled, then emulsion PCR was carried out using the Ion PGM Template OT2 400 Template Kit (Life Technologies) according to the manufacturer's instructions. Sequencing of the amplicon libraries was carried out on a 318 chip using the Ion Torrent PGM system and employing the Ion Sequencing 400 kit (Life Technologies) according to the supplier's instructions. After sequencing, the individual sequence reads were filtered by the PGM software to remove low quality and polyclonal sequences. Sequences matching the PGM A and P1 adapters were also automatically trimmed. All PGM quality-approved, trimmed and filtered data were exported as FASTQ files.

4.4.4 Sequence-based Microbiota Analysis

The FASTQ files were converted to FASTA format and processed using QIIME (Quantitative Insights Into Microbial Ecology). Quality control retained sequences with a length between 250 and 400 bp, mean sequence quality score >25, with truncation of a sequence at the first base if a low quality rolling 10 bp window was found. Presence of homopolymers >6 bp, and sequences with mismatched primers were omitted. In order to calculate downstream diversity measures (alpha and beta diversity indices, Unifrac analysis), 16S rRNA Operational Taxonomic Units (OTUs) were defined at >97% sequence homology. All reads were classified to the lowest possible taxonomic rank using QIIME and a reference dataset from the Ribosomal Database Project. OTUs were assigned using uclust. The hierarchical clustering based on population profiles of most common and abundant taxa was performed using UPGMA clustering (Unweighted Pair Group Method with

Arithmetic mean, also known as average linkage) on the distance matrix of OTU abundance.

4.4.5 Statistical Analysis

Ecological metrics for richness (Chao 1) and diversity (Shannon) were calculated in accordance with the bioinformatics of Plymouth University, UK, using Mothur Microbial community profiles generated from amplicon sequence data were compared using Quantitative Insights into Microbial Ecology (QIIME) v1.8.0 using total read counts for all assembled OTU sequences as input. Taxonomy of intestinal bacterial communities data are presented as means ± standard error of the mean. Statistical analysis was performed using the two-tailed t test. A *p* value of less than 0.05 was considered significant.

4.5 Results

4.5.1. DGGE analysis of gut bacterial community

Figure 4.4 shows the PCR–DGGE bacterial profiles from the gut of zebrafish. Many different bands are shown in the DGGE image and the gel bands which are called operative taxonomy units (OTU) in each sample.

The similarity of bacterial population within and between the treatments were measured by nonmetric multidimensional scaling (MDS) and cluster analyses of DGGE fingerprints as shown in Figure 4.5. The half matrix similarity showed in Table 4.2.

The both analyses of gut bacteria populations showed more similarity within samples from same treatments than those from other groups. The half matrix similarity of gut DGGE fingerprints is shown in Table 2 indicates the average similarity within the control ad treatment: 70.54% in the 1st probiotics treated group (P1), 68.70% in the respective control group (C1); 65.95% in the 2nd probiotic treated group (P2) and 51.43% in the respective control group (C2); 82.52% in the 3rd probiotic treated group (P3) and 74.90% in the respective control group (C3). The average DNA bands detectable in the probiotic group increased compared with control group, being 16.88% and 14.77% respectively (Fig 4.6), but it was not significant. However significant differences was observd in P3 (probiotic group) compared with the control group C3 (Table 4.3).

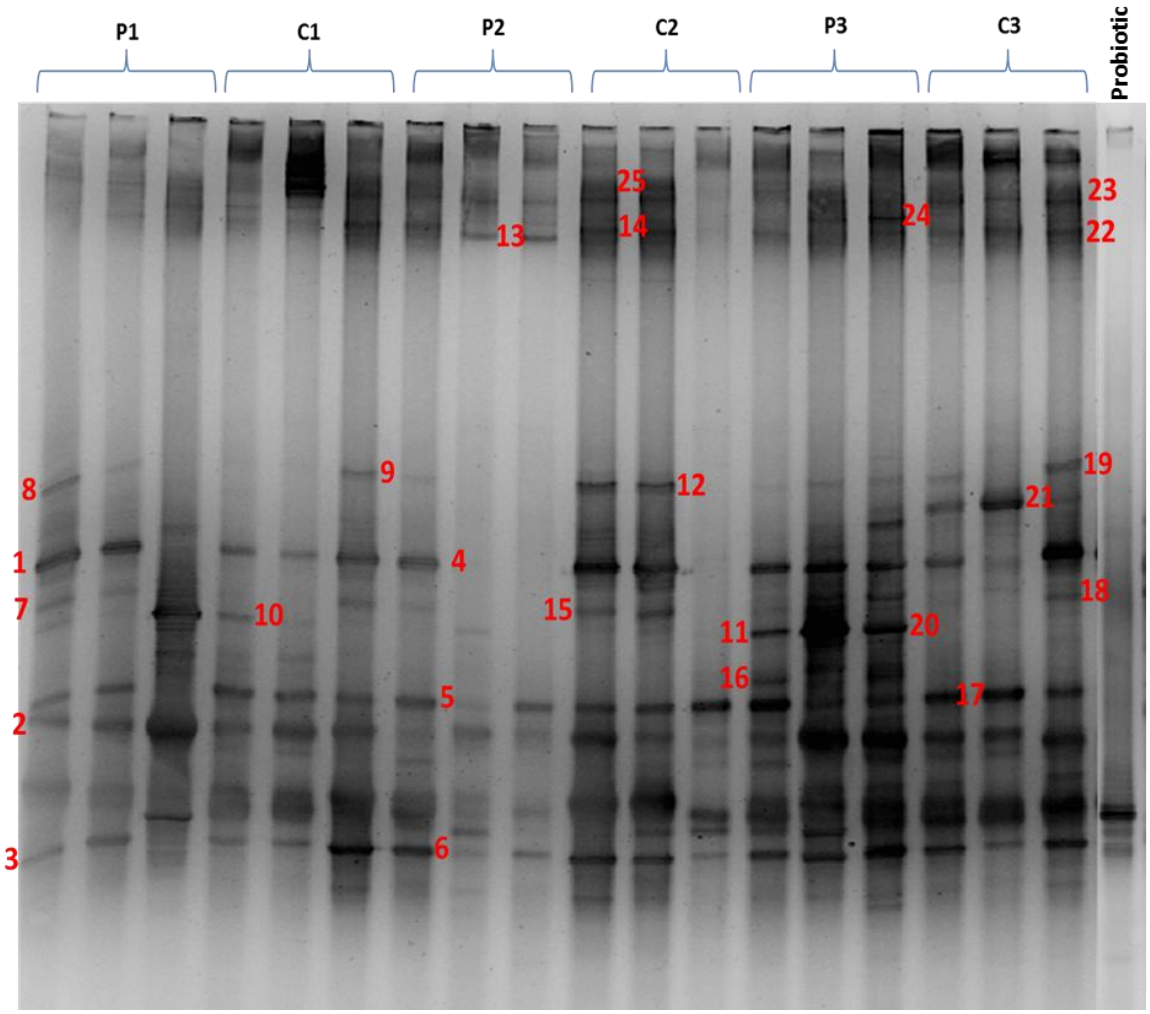


Figure 4.4 DGGE fingerprints of whole intestine of treated and control group of zebrafish. Numbers are represents the bands or operative taxonomy unite (OTU) in each sample which refers to richness in the samples.

Table 4.2 The half matrix similarity of bacterial population of DGGE fingerprints of gut showing the similarities between the replicates treatment.

Group	P11	P12	P13	C11	C12	C13	P21	P22	P23	C21	C22	C23	P31	P32	P33	C31	C32	C33
P11	100																	
P12	78.57	100																
P13	70.97	62.07	100															
C11	59.26	72.00	64.29	100														
C12	75.86	66.67	60.00	76.92	100													
C13	60.61	70.97	58.82	66.67	62.50	100												
P21	64.29	76.92	55.17	64.00	59.26	70.97	100											
P22	64.29	53.85	55.17	64.00	66.67	64.52	53.85	100										
P23	51.85	64.00	50.00	75.00	61.54	66.67	64.00	80.00	100									
C21	66.67	58.06	64.71	53.33	62.50	77.78	64.52	64.52	60.00	100								
C22	70.59	56.25	74.29	58.06	60.61	70.27	62.50	68.75	58.06	86.49	100							
C23	34.78	57.14	41.67	60.00	45.45	46.15	66.67	47.62	60.00	30.77	37.04	100						
P31	57.14	66.67	55.56	50.00	52.94	68.42	66.67	60.61	56.25	63.16	66.67	50.00	100					
P32	60.00	63.16	58.54	59.46	61.54	74.42	57.89	68.42	64.86	69.77	72.73	42.42	80.00	100				
P33	55.00	57.89	63.41	64.86	61.54	83.72	57.89	63.16	64.86	74.42	72.73	36.36	75.56	92.00	100			
C31	64.52	48.28	43.75	50.00	66.67	64.71	62.07	62.07	50.00	70.59	68.57	33.33	66.67	68.29	73.17	100		
C32	50.00	53.85	48.28	72.00	74.07	64.52	53.85	61.54	64.00	58.06	56.25	57.14	54.55	63.16	68.42	75.86	100	
C33	60.00	64.29	58.06	59.26	62.07	72.73	71.43	64.29	66.67	72.73	70.59	43.48	68.57	75.00	75.00	77.42	71.43	100

Note: C = control, P = probiotic 1-3 refers to replicate number in each case, (n=18)

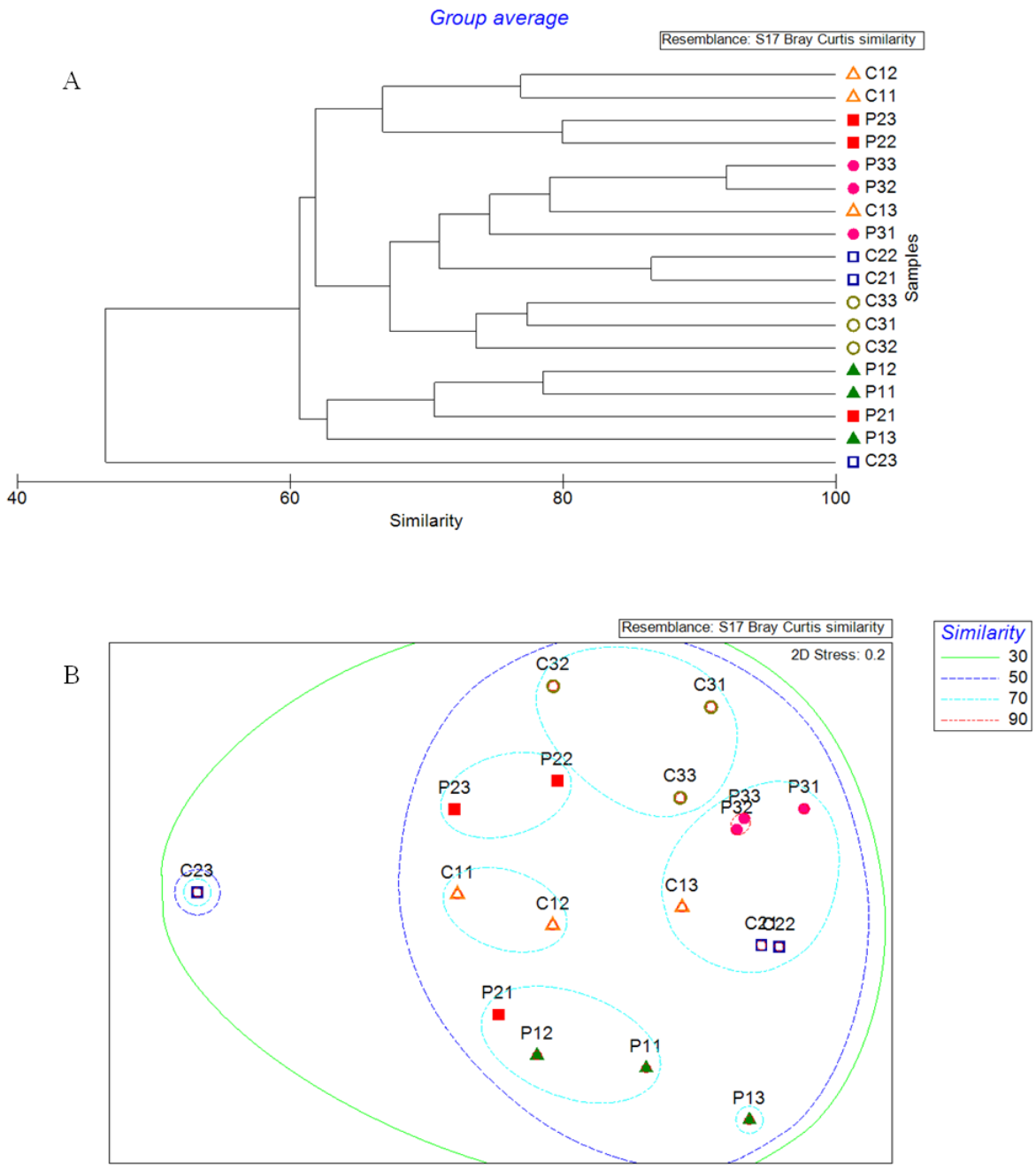


Figure 4.5: (A) Cluster analysis (B) non-metric multidimensional scaling (MDS) analysis based on the PCR-DGGE DNA fingerprints showing percentage and relative similarity of bacterial communities between control and treatment groups in zebrafish gut.

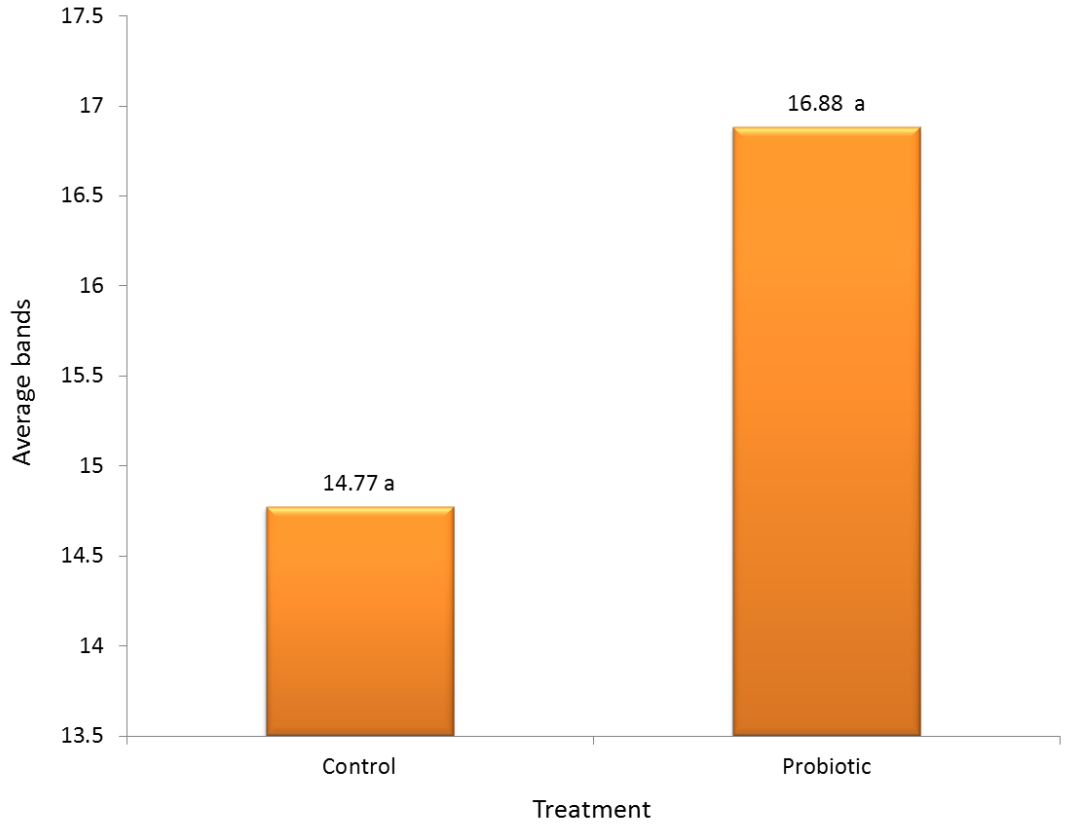


Fig. 4.6 DNA bands average

Table 4.3 Band numbers of bacterial community based on the PCR-DGGE DNA fingerprinting and similarity within treatments.

Treatment	Band number	Similarity
P1	14.66±1.52 ^{b}	70.54±8.26 ^{a}
C1	14.66±3.05 ^{b}	68.70±7.42 ^{a}
P2	12.66±0.57 ^{b}	65.95±13.18 ^{a}
C2	15.00±6.08 ^{ab}	51.43±30.52 ^{a}
P3	23.33±2.88 ^{a}	82.52±8.50 ^{a}
C3	14.66±1.52 ^{b}	74.90±3.11 ^{a}
P value	0.018	0.268

^{a,b} Means with the different superscript in the same column are significantly different ($P<0.05$). Results are mean values from three replications ± standard deviations.

Diversity analysis of gut microflora showed in table 4.4. The Shannon index and Margalef index indicate respectively the diversity, and richness of gut microflora of animals. (Hill et al., 2003) These indexes were used to display the microbial population diversity and richness in the gut, data showed in Table 4.4. The diversity index of bacterial community based on the PCR-DGGE DNA fingerprinting indicated that: at probiotic group had greater Shannon index and Margalef index than control group. However, no significant differences in Shannon index, but good significant in the Margalef index was observed in zebrafish fed with probiotics (*L. rhamnosus*).

Table 4.4: Diversity index of bacterial community in gut based on the PCR-DGGE DNA fingerprinting.

Treatment	Shannon index[1]	Margalef index[2]
P1	2.68±0.10 [a]	5.08±0.37 [ab]
C1	2.67±0.20 [a]	5.07±0.73 [ab]
P2	2.53±0.04 [a]	4.59±0.14 [b]
C2	2.63±0.48 [a]	5.12±1.52 [ab]
P3	3.14±0.12 [a]	7.08±0.64 [a]
C3	2.68±0.10 [a]	5.08±0.37 [ab]
P value	0.085	0.026

Results are mean values from three replications ± standard deviations.
[a,b] Means with the same superscript in the same column are not significantly different (P<0.05).
[1] Shannon diversity index: H‟ = -SUM(pi*Log(pi)).
[2] Margalef species richness: d = (S -1) / log (N). (S: Total species, N: Total individuals)

A number of 25 bands were excised from the PCR-DGGE gel and were subjected to sequence and BLAST analysis (Tab 4.5), after purification as recommended by GATC company around (20-80 ng/µl).

Table 4.5: Summary of the 16S rRNA gene sequence analysis results generated from bands excised from DGGE gel of zebrafish gut samples.

Band Number	NCBI Accession number	Max. Identity	NCBI BLAST matches
1	NR_025533.1	97%	*Cetobacterium somerae* strain WAL 14325
2	NR_114236.1	96%	*Shewanella algae* strain NBRC 103173
3	NR_117686.1	98%	*Klebsiella pneumoniae* strain DSM 30104
4	NR_025533.1	93%	*Cetobacterium somerae* strain WAL 14325
9	KC010472.1	94%	Uncultured bacterium
10	GU430248.1	83%	*Streptococcus* sp.
11	KJ804042.1	85%	Streptococcus sp. K-72-13-7
12	LK392937.1	94%	Uncultured bacterium
13	NR_024951.1	97%	Pseudomonas thivervalensis strain SBK26
18	JN866573.1	97%	Uncultured bacterium
19	KF256027.1	99%	Uncultured bacterium
21	JQ815676.1	95%	Uncultured bacterium
22	KF256014.1	100%	Uncultured bacterium
23	NR_029216.1	97%	*Propionigenium modestum* strain Gra Succ 2
25	NR_026243.1	97%	*Propionigenium maris* strain 10succ1

Twenty-five bands or OTUs were excised from the PCR-DGGE gel and all bands were sequenced but unfortunately only 15 samples were returned and subjected to BLAST analysis and the others were below the required standard and sequencing data was zero. The results of the trial sequence analysis shown in Table 4.4. The most family BLAST results in zebrafish gut were related to *Cetobacterium* spp., *Shewanella* spp., *Klebsiella* spp., *Streptococcus* spp., *Pseudomonas* spp., *Propionigenium* spp. strains. Otherwise, the sequences of the band numbers 9, 12, 18, 19, 21 and 22 were related to uncultured bacteria. In particular were isolated

Cetobacterium somerae, (band n. 1 and 4) 66,6% of PROBIO samples and 88,8% of CTRL samples; *Shewanella algae* (band n. 2) was isolated in all PROBIO and CTRL samples; *Klebsiella pneumonia* (band n. 3) in 55,5% of PROBIO group and 66,6 % of related CTRL; *Streptococcus sp*. (band n. 11) 66,6% of PROBIO group and 22,2% of CTRL one.

4.5.2 NGS analysis of gut bacterial community

At NGS analysis alpha diversity metrics, which describe the richness and/or evenness of taxa in a single sample, (QIIME,) was at first evaluated. By default, QIIME calculates three metrics: Chao1 (chao1), Observed OTUs (previously known as Observed Species), and Phylogenetic Diversity (Phylogenetic tree). In addition, in the alpha parameters we added the Shannon Index (shannon) to the list of alpha diversity measures that we calculated here. The comparison of OTUs derived from zebrafish intestines revealed that the Chao1 richness and Shannon–Weaver diversity estimates of the intestinal microbiotas from PROBIO and CTRL zebrafish were not statistically different, indicating that Probiotic treatment with *Lb. Rhamnosus* have not particular influence on the overall richness or diversity of the zebrafish gut bacterial community.

In addition to alpha (or within-sample) diversity, community ecologists are often interested in computing beta (or the between-sample) diversity between all pairs of samples in their study. Beta diversity represents the explicit comparison of microbial (or other) communities based on their composition. Beta diversity metrics thus assess the differences between microbial communities. The fundamental output of these comparisons is a square, hollow matrix where a

"distance" or dissimilarity is calculated between every pair of community samples, reflecting the dissimilarity between those samples. (QIIME,). The data in this distance matrix can be visualized with Principal Coordinates Analysis (PCoA). Here, we have calculated beta diversity between intestinal bacterial communities sampled and we analyzed our 16S rRNA gene sequence data sets using the default beta diversity metrics of weighted and unweighted UniFrac, which are phylogenetic measures used extensively in recent microbial community sequencing projects (Caporaso et al., 2011).

To further understand and to compare the composition of the gut microbiotas in zebrafish PROBIO and CTRL one, we subjected these 16S rRNA gene sequences to Principal Coordinates Analysis (PCoA). PCoA plots derived from both unweighted (an assessment of community composition) and weighted (an assessment of community structure which measures the distance between communities based on their phylogenetic lineages) algorithms (Lozupone et al., 2005). PCoA is a technique that helps to extract and visualize a few highly-informative components of variation from complex, multidimensional data. This is a transformation that maps the samples present in the distance matrix to a new set of orthogonal axes such that a maximum amount of variation is explained by the first principal coordinate, the second largest amount of variation is explained by the second principal coordinate, etc. The principal coordinates can be plotted in two or three dimensions to provide an intuitive visualization of differences between samples (http://qiime.org/tutorials) (Fig 4.7 and 4.8).

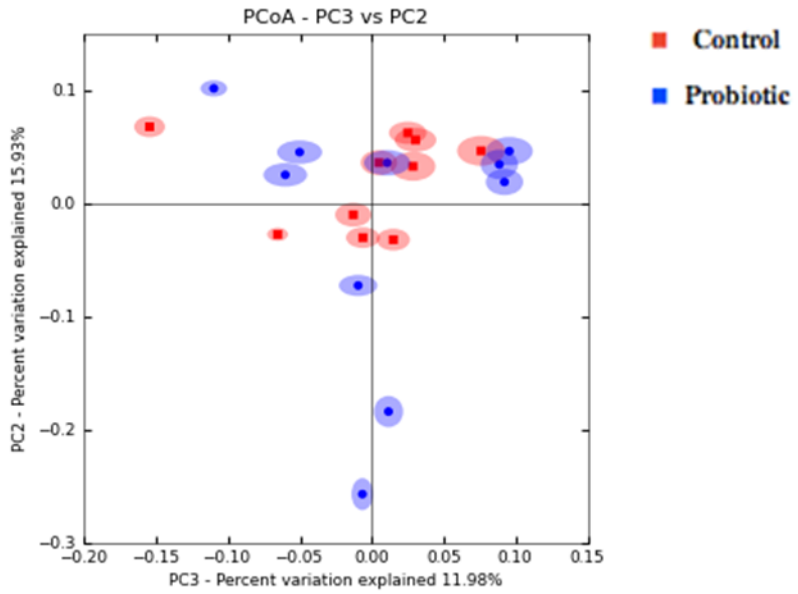


Fig. 4.7 The unweighted Unifrac distances, is an assessment of community composition (OTUs presence/absence). PCoA plot shows the spatial distribution of samples of the groups (control samples are red and the probiotic treated samples are colored blue).

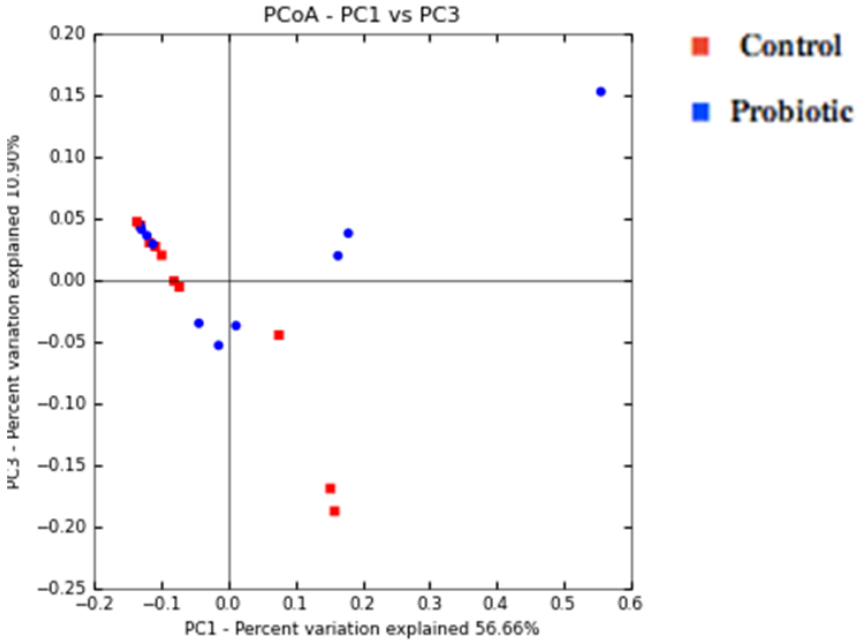


Fig. 4.8 Weighted Unifrac distances (OTUs abundance and weight branches) shows the similarity between phylogenetic lineages of bacteria communities between PROBIO (blue colored) and CTRL group (red colored); this measure also shows the degree of similarity inter-PROBIO group, clustering closer togheter.

All distances were computed according to the Bray-Curtis similarity index. Bray-Curtis dissimilarity demonstrated that 16S rRNA profiles from the the majority of CTRL groups clustered together. Four of the PROBIO samples clustered closer to CTRL whereas the other remaining part of probio samples formed independent groups. Also, five of the PROBIO samples clustered far from all each other formed independent groups. CTRL groups were closer than those PROBIO.

The alpha and beta diversity analysis indicated that these samples were not significantly different from others in their respective group. The unweighted an weighted two-dimensional PCoA showed that PROBIO zebrafish samples clustered together with CTRL zebrafish group establishing a similarity in composition and structure of these gut bacterial communities.

4.5.3 OTUs Comparison of probiotic treated group (PROBIO) and control (CTRL)

Here to provide perspective on the observed relationships between gut microbiota from two different zebrafish group investigated, we compared the taxonomy of intestinal bacterial communities in PROBIO and CTRL zebrafish.

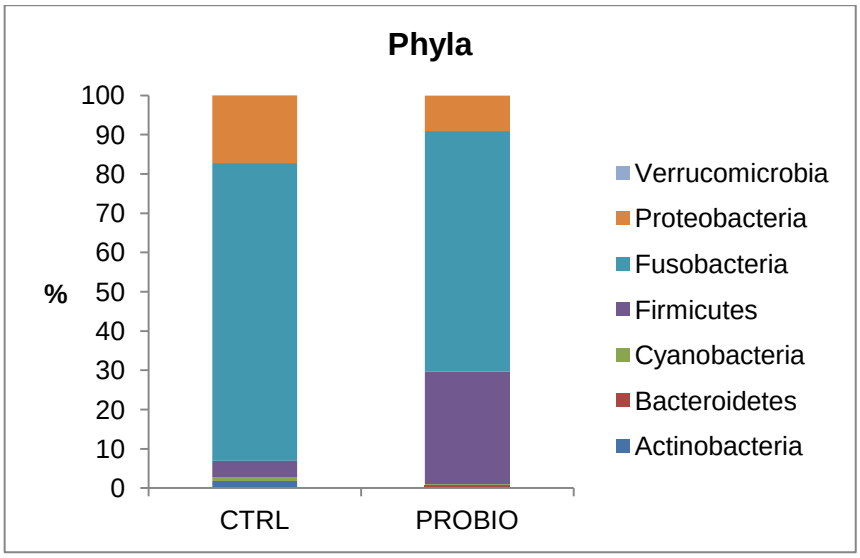


Fig. 4.9 Percentage of OTUs observed (Bar stack plots). Main differences in Phyla of bacteria within the guts of CTRL and PROBIO zebrafish group treated with *Lb rhamnosus* per 28 days.

Tab 4.6 Main representative absolute composition of OTUs analyzed per Phyla of bacteria within the guts of CTRL and PROBIO zebrafish group treated with *Lb rhamnosus* per 28 days. Absolute values are calculated as Mean ± SD (standard deviation)

PHYLA	CTRL			PROBIO		
	Mean	±	**SD**	**Mean**	±	**SD**
Fusobacteria	3743	±	1976	3363	±	2367
Proteobacteria	850,7	±	1187,8	495,5	±	666,8
Firmicutes	20,2	±	30,4	158,0	±	193
Bacteroidetes	0,00	±	0,00	4,3	±	9,1
Actinobacteria	91,7	±	249,2	0,18	±	0,4
Cyanobacteria	48,1	±	75,4	10,6	±	4,6

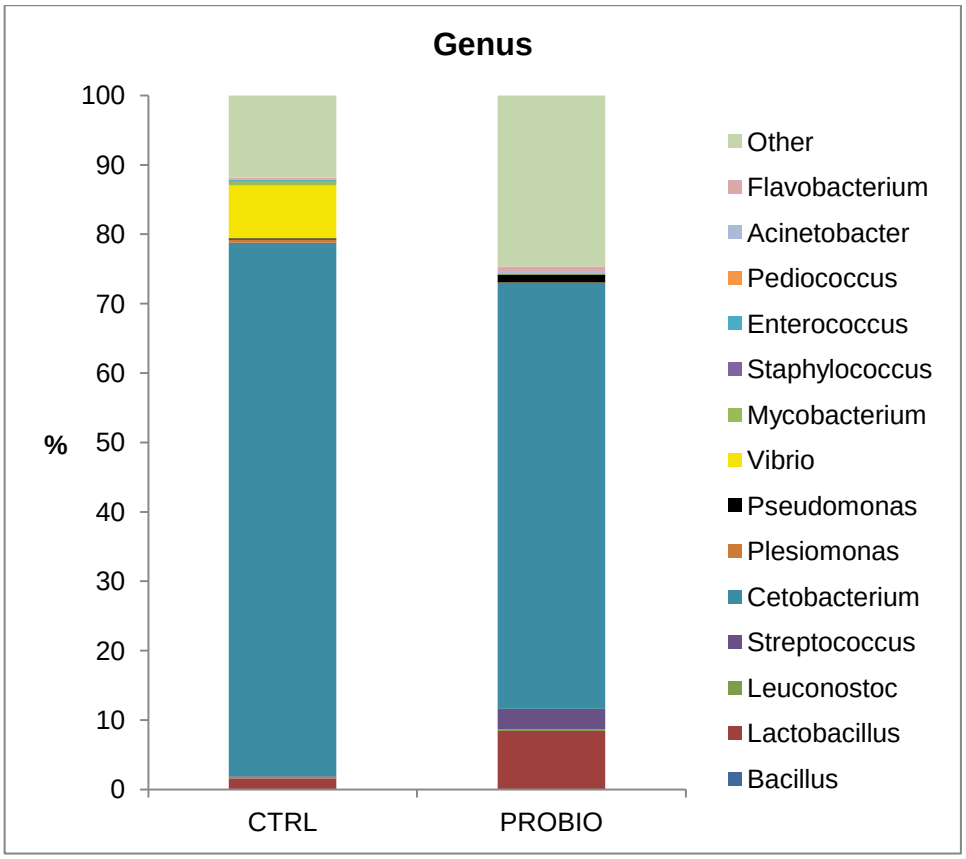


Fig. 4.10 Percentage of OTUs observed (Bar stack plots). Main differences in Genera of bacteria within the guts of CTRL and PROBIO zebrafish group treated with *Lb rhamnosus* per 28 days.

Tab 4.7 Main representative absolute composition of OTUs analyzed per Genera of bacteria within the guts of CTRL and PROBIO zebrafish group treated with *Lb rhamnosus* per 28 days. Absolute values are calculated as Mean ± SD (standard deviation)

GENERA	CTRL		PROBIO	
	Mean ± SD		**Mean ± SD**	
Bacillus	3,0	4,8	3,1	4,6
Lactobacillus	74,8	150,2	458,8	806,8
Leuconostoc	11,9	5,5	12,4	8,2
Streptococcus	4,7	9,9	161,5	202,3
Cetobacterium	3742	1975,7	3363,5	2367,2
Plesiomonas	22,5	40,7	3,6	3,2
Pseudomonas	7	5,9	62,9	139,2
Vibrio	372,6	1172,7	0,9	1,3
Mycobacterium	28,2	89,3	0,04	0,09
Staphylococcus	0,01	0,03	2,7	8,5
Enterococcus	8,2	26	0,01	0,03
Pediococcus	0,01	0,03	2,95	9,2
Acinetobacter	3,1	9,6	12,8	40,6
Flavobacterium	12,2	33,8	43,1	91,1
Others	579	847,1	1350	2022,6

Total OTUs retrieved from PROBIO and CTRL *zebrafish* were binned into six main phyla respectively (Figure 4.9 and Tab 4.6). Fusobacteria ribotypes dominated both groups, accounting for 75,83% (CTRL) and 61,18% (PROBIO) of the OTUs retrieved. Ribotypes representing Proteobacteria, Firmicutes, and Cyanobacteria were present in both groups with relative abundances (Figure 4.9). In order of abundance the CTRL group showed: Fusobacteria (75,83%), Proteobacteria (17,24%), Firmicutes (4,10%), Actinobacteria (1,86%) , and Bacteroidetes (0,00%). In PROBIO group, fed with *Lb rhamnosus* IMC 501 administration: Fusobacteria (61,18%), Firmicutes (28,75 %), Proteobacteria (9,01%), Bacteroidetes (0,78%) and Actinobacteria (0,00%).

Three bacterial class, Proteobacteria and Fusobacteria and Firmicutes appeared consistently in the gut microbiotas of zebrafish analyzed in this study. Proteobacteria and Fusobacteria classes as common members of the gut microbiota in adult zebrafish. Members of these bacterial classes are especially well adapted to conditions in the fish intestine or their surrounding aquatic environment (Rawls et al., 2011).

The group treated with probiotic showed at genus level, the significant abundance of Firmicutes genera (28,75 %), (*Cetobacterium*, *Streptococcus*, *Lactobacillus*) compared to control group (4,10%) (Tab 4.6 and 4.7); in PROBIO group although not significant the aboundance of *Lactobacillus* (8,3%) is representative of the dietary administration of *Lb rhamnosus*. All Proteobacteria, instead, (*Vibrio, Aeromonas, Pseudomonas, Plesiomonas*) decreased. In agreement with Roeselers et

al. (2011), Proteobacteria were detected in the intestines of each fish in our analysis. Although not significant, in the CTRL we found 17.84% of Proteobacteria clones compared with PROBIO group that showed lower percentage of 3.31%.

Our phylogenetic analysis revealed a diverse set of Fusobacteria sequences isolated from the intestines of zebrafish, most of which were related to *Cetobacterium somerae*. The difference among two group was not significant (Figure 4.10 and Tab 4.7).

4.6 Discussion

The zebrafish gut microbiota is numerically dominated at all stages of the zebrafish life cycle by members of the bacterial phylum Proteobacteria, Firmicutes and Fusobacteria also prevalent during larval and adult stages respectively. (Roeselers et al., 2011) In this study DGGE analyses, revealed that dietary *Lb. Rhamnosus* administration increased the number of OTUs. At DGGE, the average DNA bands of OTUs detectable in the fed probiotic group were increased compared with control group, being 16.88 and 14.77 respectively. In addition to the abundance of taxa, it is also recognised that the microbial community ecology contributes to the function of the GIT, potentially supporting positive adaptation to changing conditions, and therefore measures of microbial community ecology are also useful indicators of microbial community modulations (Nayak, 2010).

We use the PCR-DGGE as a first step of detection of bacterial community. These method, although reliable for the analysis of the microbiota, is limited by the resolution of band detection with complex bacterial communities and microbes of low abundance may easily be missed (Sevellac et al., 2014). In contrast NGS showed although limits in the detection of species, showed greater sensitivity.

The DGGE, at 16S rRNA gene sequence analysis results generated from bands excised from DGGE gel of zebrafish gut samples we found *Cetobacterium somerae*, in PROBIO and in CTRL samples. This data is completely in line with Roeselers et al. (2013), where they found it constantly in wild and domesticated zebrafish. *Cetobacterium somerae* has been shown to be indigenous to the digestive tract of multiple freshwater fish species. *C. somerae* (ex Bacteroides type A) is a non-spore-

forming, rod-shaped, microaerotolerant, vitamin B12 (cobalamin) producing Fusobacterium that has been shown to be indigenous to the digestive tract of multiple freshwater fish species that do not require dietary supplements of vitamin B12 (Sugita et al., 1991). *C. somerae* was not detected in the digestive tract of two freshwater fish species which show deficiency symptoms when fed vitamin B12-depleted diets suggesting that *C. somerae* may be involved in determining the vitamin B12 requirements of freshwater fish (Roeselers et al., 2011). The isolation of *Streptococcus sp.* in 66,6% of PROBIO OTUs group and 22,2% of CTRL one is in line with a study reported by Gioacchini et al.,(2014) where the abundance of *Streptococcus* spp. in zebrafish fed *Lb. Rhamnosus* was clearly stimulated by probiotic administration. It is interesting the isolation of *Klebsiella pneumonia* in of PROBIO and in related CTRL DNA samples, To our knowledge, this report comprises the first study on isolation of this bacterium in zebrafish. Microbial studies revealed that *Klebsiella pneumoniae* isolated from *N. japonicus* is the most common pathogen causing ulcers and fin erosions, it cause fin and tail disease in Rainbow trout. *Klebsiella* species were also isolated from gills and intestine of *Tilapia zilli* from creeks around Port Harcourt, Nigeria, from African catfish, from skin mucus of eel fish, *Anguilla Anguilla* (Diana et al., 2012).

One notable exception was the isolation of *Shewanella algae* in all samples (PROBIO and CTRL). The genus *Shewanella* is currently composed of more than 50 species that inhabit a range of marine environs and ecosystems. (Janda et al., 2014). *Shewanella* spp. is Gram-negative bacteria, saprophytes and widely distributed worldwide. It belongs to the microflora of the marine environment but it habits all forms of water and soil, but it has also been isolated from diverse

sources including dairy products, oil, and carcasses. (Vignier et al., 2013). At present, it is unclear exactly how many *Shewanella* species are truly occasional human pathogens. Recent advanced study in the taxonomy and phylogenetic relatedness of members of this genus, show that most human infections are caused by a single species, *S. algae*. (Janda et al., 2014). The pathogenicity of these species remains unclear, partly because they are found in polymicrobial infections, but there is now enough evidence to conclude that some *Shewanella* spp. are pathogenic for humans. *Shewanella* infections are sometimes acquired after exposure to seawater. The most common clinical manifestations seem to be otitis, soft tissue infection, bacteremia, and hepatobiliary infection. Some argue than *S. algae* could be more virulent species. (Janda et al., 2014; Vignier et al., 2013). Here we report the isolation of *Shewanella algae* in all healthy zebrafish. To our knoledge this is the first report of isolation of this strain specie in freshwater fish considering that isolates such as *Shewanella putrefaciens* or *Shewanella* spp. and other pathogen bacteria are commonly seen in ornamental diseased fish (Rose et al., 2013) well as in lake whitefish (*Coregonus clupeaformis*) (Sevellec et al., 2014) and them are also common in caught and domesticated zebrafish. Most of the clinical isolates found in literature are *Shewanella putrefaciens*, but recent data suggest that many of these isolates should be classified as the genetically distinct species *Shewanella algae*, a more virulent species. This misclassification is largely caused by the use of conventional systems that are unable to identify *S. algae.* As has been suggested elsewhere, some of the *S. putrefaciens* infections reported during recent years were probably caused by *S. algae* (Vignier et al., 2013). The presence of *Shewanella algae* in all our samples might suggest zebrafish as a possible reservoir of this pathogen. This finding has a double meaning for both

public health considering the serious bacterial infections in human, both for further study of this infection in an animal model as *Danio rerio*, for human and animal health. This finding is important to pay more attention to animal handling by researchers, technicians and all people in contact with fishes in the facility. A routine environmental and microbiological monitoring program should be applied in the major fish or aquatic animals facility to well understand the epidemiology of *Shawanella algae.*

The NGS analysis supports the major presence of phyla, Fusobacteria Proteobacteria Firmicutes and Cyanobacteria with predominance of Fusobacteria in both PROBIO and CTRL in line with DGGE analysis. In order of abundance in CTRL group: Fusobacteria (75,83%), Proteobacteria (17,24%), Firmicutes (4,10%), Actinobacteria 1,86% , and Bacteroidetes (0,00%). In PROBIO group, treated with *Lb rhamnosus*: Fusobacteria (61,18%), Firmicutes (28,75 %), Proteobacteria (9,01%), Bacteroidetes (0,78%) and Actinobacteria (0,00%).

The isolation of *Streptococcus* in 2,94% of PROBIO group and in 0,09 % of CTRL is statistically significant (*p value*=0.02). This result confirms the DGGE analysis and with the study reported by Gioacchini et al.,(2014) where the abundance of a strain of *Streptococcus* in zebrafish fed *Lb. rhamnosus* was clearly stimulated by probiotic administration. It belongs to lactic acid bacteria (LAB) group including *Lactobacillus.*

The zebrafish gut microbiota, like that of humans and other mammals, are dominated by the bacterial phyla Proteobacteria, Firmicutes, and Bacteroidetes (Rawls et al., 2004; Rawls et al., 2006; Roeselers et al., 2011; Semova et al., 2012;

Cantas et al., 2011). However, the impact of diet on the zebrafish gut microbiota, and their relationship to the microbiota in the surrounding aqueous environment, is unknown. In line with these researches, our study identified members of the Proteobacteria and Fusobacteria and Bacteroidetes phyla as common prevalent members of the gut microbiota in adult zebrafish.

Gastro intestinal tract (GIT) and feces can serve as an enrichment site for pathogenic bacteria such as *Aeromonas*, *Pseudomonas* and *Vibrio* species, belonging to Proteobacteria phylum. The use of probiotics with antagonistic activity may be used to reduce or inhibit pathogens activities (Balcázar et al., 2008). In this study, feeding zebrafish probiotic *Lb rhamnosus* showed a reduction of this genera supporting the antagonistic activity role of this probiotic strain. The intestinal microbiota dysbiosis in zebrafish with inflammatory bowel disease (IBD)-like colitis was characterized by an increased proportion of Proteobacteria and a decreased of Firmicutes (*Lactobacillus, Streptococcus group*), which were significantly correlated with enterocolitis severity. At the same time other researchers have documented changes in the human gut microbiota associated with IBD, especially a dramatically reduced diversity in the phylum Firmicutes and concomitant increase in Proteobacteria (He et al., 2013). There is also now increasing evidence that dysbiosis modulates peripheral and central nervous system function, leading to alterations in brain signalling and behaviour (Bercik et al. 2011; Collins et al. 2013;). The composition of the zebrafish gut microbiota can have direct impacts on disease pathogenesis. Inflammatory bowel disease can be modeled in zebrafish also using a chemical called oxazolone, which induces intestinal inflammation (Brugman et al. 2009). In zebrafish treated with the

antibiotic vancomycin, Fusobacteria became the dominant phyla in the gut microbiota, and the inflammatory response observed in response to oxazolone was markedly decreased. Treatment with colistin sulfate increased γ-Proteobacteria in the gut microbiota, and these zebrafish developed intestinal inflammation in response to oxazolone treatment. These results demonstrate that certain members of the microbiota, such as the γ-Proteobacteria, may help drive intestinal inflammation in an experimental model of colitis. Proteobacteria have been observed in human chronic inflammatory diseases. This study in zebrafish suggests that members of the Proteobacteria may increase propensity for inflammatory responses in the gastrointestinal tract. Different studies have also shown an increase of some opportunistic pathogenic Proteobacteria and a decreased proportion of Firmicutes phylum (He et al., 2013). In our study indeed we assisted to a significant increase of Firmicutes (p=0,038), The zebrafish digestive tract is similar to that of mammals in its development, organization and function. Zebrafish are well suited for studying host-bacterial interactions as they have innate and adaptive immune systems similar to higher vertebrates (Trade et al., 2004)

The relationship between (gut microbiota) and host physiology is an interesting translational area of zebrafish digestive system research. A study tested the ability of anaerobic bacteria derived from the human intestine to colonize the zebrafish gut, suggesting that the zebrafish could be used as an experimentally malleable system for modeling host–microbiota interactions in humans.

Although not significant differences exist between PROBIO and CTRL group, in this study, feeding zebrafish probiotic *Lb rhamnosus* showed a reduction of

Proteobacteria genera the greather microbiota present in dysbiosis, supporting the antagonistic activity role of this probiotic strain. Here we demonstrated a significant increase of Firmicutes Phyla, These results underscore the need to identify the selective pressures governing microbial community within the intestinal habitat and the benefit of dietary supplementation of probiotics such as *Lb rhamnosus* or other strains. This information could semplify the development of safe and effective methods for manipulating gut microbiota composition to promote the health of humans and other animals.

CHAPTER 5

EXPRESSION OF BDNF MRNA IN ZEBRAFISH TREATED WITH A PROBIOTIC STRAIN, *LACTOBACILLUS RHAMNOSUS*

5.1 Abstract

Several researches on human and animal models have been evaluated to assess the role of microbiota on brain function, providing how this microcosmic word can influence brain chemistry and behaviour. Neurotrophic factors are included in the big brain chemistry. Alterations of gut microbiota has been associated with stress and decreased brain neurotrophic factor expression in the CNS. The BDNF gene expression in zebrafish has been documented in many tissues and organs.

The purpose of this study was to determine whether probiotic strain *Lactocabillus rhamnosus* influences brain neurochemistry in zebrafish. Brain-derived neurotrophic factor (BDNF) mRNA expression was evaluated in zebrafish, for the first time, with dietary administration for 28 days of this probiotic strain. The probiotic treated group showed a statistically significant near two-fold increase in BDNF expression compared to the control group. Furthermore, we assessed also the BDNF localization by immunohistochemistry. BDNF seems distributed in all regions of the brain, without any remarkable differeces compared to control group.

5.2 Introduction

Progresses has already been made in understanding the bi-directional crosstalk governing the gut-brain axis. Accordingly, not only the brain can affects gut functions, but the gut can also induces changes in the central nervous system (CNS) (O'Sullivan et al., 2011). Although there is now compelling evidence for a link between the enteric microbiota and brain function, we are only just beginning to realize the physiological impact of the microbiota on this process (Al-Asmakh et al., 2012).

CNS can influence gut microbiome through neural and endocrine pathways in both direct and indirect manners. The autonomic nervous system (ANS) and hypothalamus–pituitary–adrenal (HPA) axis that liaise the CNS and viscera can modulate gut physiology such as motility, secretion and epithelial permeability as well as systemic hormones, which in turn affect the niche environment for microbiota and also host-microbiome interaction at the mucosae (Cryan and Dinan, 2012). Microbiota is also implicated in the alteration of neurotrophic factors, which constitute of an extensive and heterogeneous class of proteins which play roles in controlling neuronal function and maintaining cellular integrity (O'Sullivan et al., 2011). Particularly, neurotrophins constitute a family of structurally related proteins required for the development and function of the vertebrate nervous system where they regulate survival, differentiation and synaptic plasticity of specific neuronal populations. members of the family are brain derived neurotrophic fact (BDNF), Nerve Growth Factor (NGF), Neurotrophin-3 (NT-3), Neurotrophin 4 (NT-4) and the fish specific Neurotrophin 6 (NT-6).

Brain-derived neurotrophic factor (BDNF) is a neurotrophin of particular interest to neuroscientists, due to its clearly defined impact on several aspects of brain function and potential relevance to brain diseases. Furthermore, evolutionary studies have demonstrated that among all neurotorphins, BDNF is the most well conserved throughout vertebrates evolution (Tettamanti et al., 2009; Lanave et al., 2007). The BDNF protein contains a domain shared by all neurotrophins characterized by six strictly conserved cysteine residues, fundamental for the correct folding of the molecule. This feature, together with the high percentage of amino acid identity shown by vertebrate BDNF, indicates that this factor reached an optimally functioning structure very early in vertebrate evolution, thus hindering further variations (Götz et al., 1992), due to an increased selective pressure on the coding region. While all neurotrophins bind to the common p75 receptor, the binding to three distinct Trk tyrosine kinase receptors mediates the specificity in the activity of these neurotrophic polypeptides (Chao, 2003; Reichardt, 2006). BDNF acts on neurons of the central and peripheral nervous system (Levi-Montalcini and Calissano, 1979; Leibrock et al., 1989). In mammals, BDNF has been extensively studied. BDNF is highly expressed in the mammalian hippocampus and plays a major role in synaptic transmission (Berninger et al., 1999) and plasticity (Thoenen, 1995). BDNF activating its intracellular signalling pathways through binding to the tropomyosin-related kinase (Trk) receptor TrkB has a relevant function in memory, learning and in the development of the nervous system (for review, see Chao et al., 2003).

Changes in the expression and/or function of BDNF may be relevant to a range of human psychiatric disorder conditions, such as drug addiction, (Bolanos and

Nestler, 2004), depression (Duman, 2002), schizophrenia (Weickert et al., 2003) and bipolar disorder (Thome et al., 1998). Alteration in BDNF also including Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (Adachi N, 2014; Frade and Lopez-Sanchez, 2010; Ventriglia M, 2013). Several studies have reported reduced brain-derived neurotrophic factor (BDNF) levels in the brain and in the serum and plasma of patients with psychosocially stressed. Stress and the biological systems involved in the stress response have been suggested to play a role in BDNF changes. High levels of both glucocorticoid hormones and pro-inflammatory cytokines, two key players in the response to stress, have been associated with decreased BDNF levels in first-episode psychosis patients (Mondelli et al., 2011). In teleostean models, data already available in literature report the expression of BDNF in the developing brain of zebrafish (De Felice et al., 2014) and in the adult brain of different teleostean species (D'Angelo et al., 2014; Vissio et al., 2008). BDNF has also been observed in other organs and tissues of adult and developing zebrafish (Germanà et al., 2010).

Several researches on human and animal models have been evaluated to assess the role of microbiota on brain function, providing how this microcosmic world can influence brain chemistry and behaviour. Neurotrophic factors such as BDNF are included in the big brain chemistry. Alterations of gut microbiota has been associated with stress and decreased brain neurotrophic factor expression in the CNS.

Bercick et al., (2011) demonstrated that administration of oral antimicrobials to SPF mice transiently altered the composition of the microbiota and increased

exploratory behaviour and hippocampal expression of BDNF. The proliferation of the *Bifidobacteria* and *Lactobacilli* strains in the large intestine have anxiolytic and mnemonic effects in rodents (Bravo et al., 2011) and humans (Cryan and Dinan, 2012). O'Sullivan et al.,(2011) have shown that treatment with B. breve 6330 increased levels of BDNF total mRNA in rats Bravo et al., (2011) demonstrated antidepressant and anxiolytic-like properties of a probiotic *Lactobacillus rhamnosus* in mice. Evidence for an involvement of the vagus nerve and the central gamma-aminobutyric acid (GABA) system in the modulation of emotional behavior by these bacteria was also provided. Strains of *Lactobacillus* and *Bifidobacterium* secrete gamma-aminobutyric acid (GABA). This is the main inhibitory neurotransmitter in the brain regulating many physiological and psychological processes, with dysfunction in the system implicated in anxiety and depression. The changes in behaviour and GABA receptor expression following *Lb. rhamnousus* treatment were also in keeping with studies of GABA B1b-deficient animals, indicating an important role for this subunit in the development of cognitive processes, including those relevant to fear (Forsythe and Kunze, 2012).

To our knowledge no study on zebrafish has so far been conducted to describe the impact of probiotics on neurobiological consequences. In the present investigation, we administrated the probiotic strain *Lactobacillus rhamnosus* to zebrafish to assess whether also in zebrafish *L. rhamnosus* has effects on the levels of mRNA BDNF in the CNS.

5.3 Materials and methods

5.3.1 Animals and husbandry

Adult 4–6-month-old male and female zebrafish (*Danio rerio*) (~ 30:70%) of heterozygous "wild type" strain were obtained from local commercial distributors (Carmar sas, Napoli). All fishes were given at least 14 days to acclimate to the laboratory environment and housed in groups of 12 fishes per 30-L tank. All tanks were filled with deionized water before introducing the fishes. Fishes were fed two times daily with commercial food (SERA Vipagran®, Germany). The fishes were fed the diets at 1.5%–2% of bodyweight per day automatically using Rondomatic 400 (Grässlin, Germany). The room and water temperatures were maintained at 25–27 °C. Illumination (1010 ± 88 lx) was provided by ceiling-mounted fluorescent light tubes on a 14-h cycle (D:N=14h:10h) consistent with the standards of zebrafish care (Westerfield M., 2000). All fishes used in this study were experimentally naïve. Two experimental groups were evaluated: a control group (CTRL) (n. 16 animals) and a probiotic-treated group (PROBIO) (n. 16 animals).

5.3.2 Probiotic administration

The control group (CTRL), was fed twice per day with a commercial diet only and a probiotic-treated group (PROBIO), was fed twice per day the commercial diet and twice with the lyophilized probiotic strain *L. rhamnosus* IMC 501, provided by Synbiotec s.r.l. (Camerino, Italy) at a final concentration of 10^6 colony-forming units/g (0,01 g/l) for 28 days (as reported in general chapter at 2.3). Both groups are constituted by animals randomly chosen from two different biological replicates.

5.3.3 RT-qPCR analysis of BDNF

Fishes were euthanized by immersion in overdose 500 mg/ L-1 of 3-aminobenzoic acid ethyl ester [MS-222] buffered to pH 7.4 (Sigma–Aldrich, USA). After death, fishes were dissected under a light source and with sterilized micro surgical blade and forceps, brain was entirely excised and replaced into sterile 1.5 mL micro centrifuge tubes (MCT) with 1 ml of RNAlater® sterile solution (Life Technologies, USA) and stored at -80°C until use for analysis. The total RNA was extracted using the Ambion Pure Link® RNA Mini Kit (Life Technologies, Carlsbad CA, USA). Briefly, brain samples were thawed on ice, weighed, and macerated in lysis buffer containing β-mercaptoethanol. The RNA was applied to a silica filter, washed three times and eluted. DNase treatment was performed using Ambion Pure Link® DNase Set (Life Technologies, Carlsbad CA, USA). Isolated RNA was quantified on Nanodrop® ND-2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, NC, USA) and analysed for quality using the Agilent 2100 Electrophoresis Bioanalyzer Nano- Chip (Agilent, Stockport, UK) according to the associated protocol. All of the RNA samples had an RNA integrity number (RIN) greater than 8. Total RNA was reverse transcribed to cDNA, using the SuperScript® VILO™ cDNA Synthesis Kit and an oligo dT primer. BDNF and β-actin primers (Tab. 5.1) were used to amplify 30 ng of the first strand cDNA. The reactions were conducted in technical triplicates and biological duplicates with Power SYBR® Green Master mix (Applied Biosystems) following manufacturing instructions. The reactions were run in the 7500 ABI Thermal Cycler (Applied Biosystems) using the following thermal cycle: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C, 15 sec and 60°C, 1 min. The melting curve stage was: 95°C, 15 sec; 60°C, 1 min; 95°C, 30 sec; 60°C, 15 sec.

The Real-Time PCR Miner online tool (Zhao and Fernald, 2005) was used to calculate the PCR efficiency (E) and optimal threshold cycle (CT) for each well. The mean relative expression ratio (Rn) and standard error of the BDNF transcript was calculated using the actin gene as the endogenous control applying the formula

$Rn = R_0$ BDNF $/R_0$ actin = $(1+E \text{ target})^{-CT \text{ BDNF}} / (1+E \text{ control})^{-CT \text{ actin.}}$

Tab.5.1 β-actin and BDNF primers used for RT-qPCR

***Danio rerio* gene**	**Primer Sequences**
β-actin forward	CACAGATCATGTTCGAGACC
β-actin reverse	GGTCAGGATCTTCATCAGGT
BDNF forward	ATAGTAACGAACAGGATGG
BDNF reverse	GCTCAGTCATGGGAGTCC

5.3.4 Statistical Analysis

Data are presented as means ± standard error of the mean. Statistical analysis was performed using the two-tailed t test. A *p* value of less than 0.05 was considered significant.

5.3.5 Immunohistochemistry

4 adult zebrafish (male and female) heads were fixed by immersion in Bouin's fluid for 24 h at room temperature (RT), dehydrated in ethanol series and embedded in paraffin wax. Transverse 5–7 μm thick sections were cut. Microtomical sections were serially stained by luxol fast blue, cresyl violet, and immunocytochemistry. After dewaxing in xylene and rehydration, slides were washed in PBS and then

placed in target retrieval solution (Citric buffer pH 7.4) brought to boil using microwave, and then gently boiled for 10 min at 10% power, then left in the solution to cool for 30 min. Sections were washed with PBS and treated with 3% H_2O_2 (20 min), washed with phosphate buffered saline solution (PBS) pH 7.4 and incubated in a humid chamber for 24 h at 4 °C with anti-NGF (sc-549, Santa Cruz Biotechnology, Santa Cruz, CA) diluted at 1:300 with PBS containing 0.2% TritonX-100, 0.1% bovine serum albumin, and 4% normal goat serum (NGS) (cod. S1000, VECTOR Lab, Burlingame, CA). After incubation, the sections were washed in PBS and incubated with EnVision for 30 min at RT. The sections were washed and the immunoreactive sites obtained were visualized using a fresh solution of 10 mg of 3,3′-diaminobenzidine tetrahydrochloride (DAB) (cod. D5905, Sigma-Aldrich) in 15 ml of a 0.5 M Tris buffer, pH 7.6, containing 1.5 ml of 0.03% H2O2. Slides were observed and analyzed by Nikon Eclipse 90i. The digital raw images were optimized for image resolution, contrast, evenness of illumination, and background by using Adobe Photoshop CS5 (Adobe Systems, San Jose, CA).

5.4 Results

5.4.1 RT-qPCR

The results for BDNF expression level measured in PROBIO and CTRL group are showed in Fig. 5.1. The PROBIO group had a statistically significant near two-fold increase in BDNF expression compared to the CTRL group.

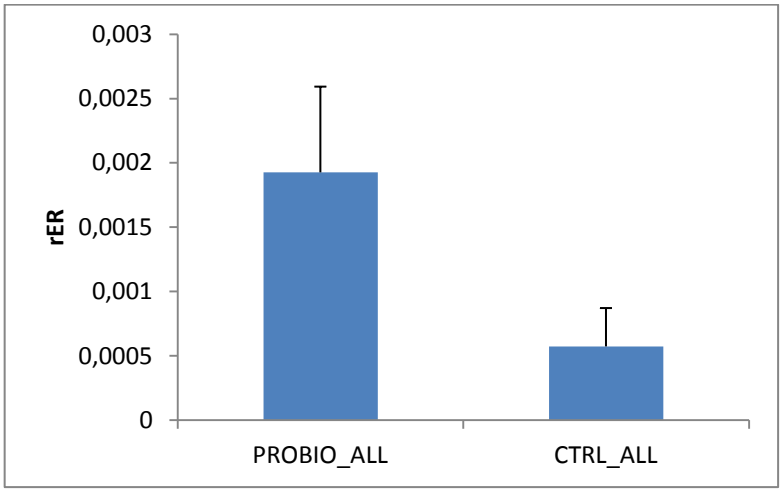


Fig. 5.1. BDNF brain expression in *L. rhamnosus* treated group (PROBIO) and controls. Bars represent standard error. rER, relative expression ratio.

5.4.2 Immunohistochemistry

The anatomical nomenclature used for the description of BDNF immunoreactivity follows that from "neuroanatomy of the zebrafish brain" atlas by Wullimann (1996). Cells displaying immunoreactivity to BDNF were observed in all encephalic regions, although with different pattern of distribution. Immunoreactivity has been observed either in fibers and in cytoplasm of neurons. Grouped BDNF positive small neurons were seen in the dorsal and ventral zones of telencephalon, and some positive cells were also recognized along the ventricle. Sparse positive neurons were seen in the preoptic area of diencephalon, and in some thalamic nuclei, in the dorsal hypothalamus (Fig a) and hypothalamic inferior lobe mainly in neurons localized in the caudal zone of periventricular hypothalamus. In the

mesencephalon, positive small neurons were observed thorughout the layers of in the optic tect and in numerous fibers of the longitudinal tori (Fig. b). Strong positivity to BDNF was detected in the cerebellum, in recognizable Purkinje cells of either the valvula and body (Fig c,d). Finally, immunoreactivity was seen in the medulla oblongata, in large neurons of anterior and intermediate reticular formation.

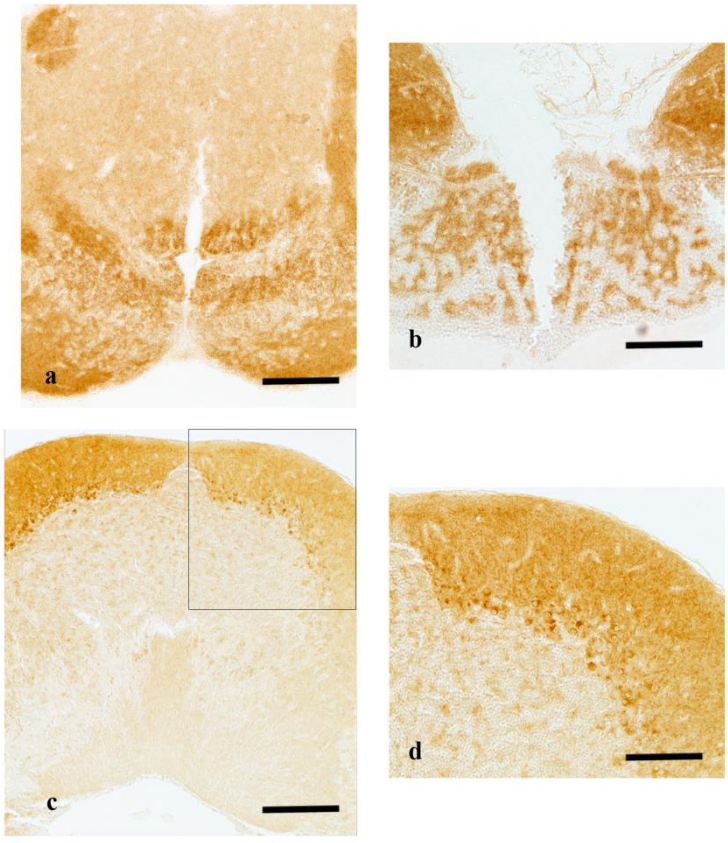


Fig 5.2 IHC: a. Immunoreacitivity in numerous fibers and some small neurons of dorsal hypothalamus. b. Positivity to BDNF in fibers of the longitudinal tori. c. Positivity in the citoplasm of neurons of Purkinje and in the molecular layer of cerebellum body. d. High magnification of rectagle shown in c. Scale bars: a-c= 100 µm; bd= 50 µm.

5.5 Discussion and Conclusion

It is known that gut microbiota impacts various aspects of host physiology including modulation of gut–brain communication. Here we provide evidence, using qPCR, that *Lactobacillus rhamnosus* dietary administration in zebrafish increased the transcription of BDNF mRNA in brain, without distinguishing between the pro - and mature BDNF forms. The results are consistent with those reported in mammals (O'Sullivan et al., 2011). Furthermore, the neuroanatomical localization of BDNF protein seems to confirm observations carried out in previous studies (Gatta et al., manuscript in preparation). The specificity of the antibody as already been tested in Western blot experiments (Gatta et al., manuscript in preparation), demonstrating that BDNF antiserum identifies three bands of ~14 kD ~26kD and ~39 kD, suggesting that the antibody recognizes pro and mature BDNF forms. The wide distribution of BDNF in the brain of adult zebrafish underlye the key role of this molecule in the maintenance of the nervous system. Given the increased expression of BDNF at mRNA levels, it is arguable that probiotics do have positive effects on brain, by regulating the synthesis of BDNF.

A number of studies has demonstrated that gut bacteria influence BDNF levels. How this alterations in brain chemistry are related to specific behavioral changes is unclear (Forsythe and Kunze, 2012). So, what is the relationship between gut bacteria and the brain for human and animal health? Explication in understanding of the microbiome–gut– brain axis comes from studies of how distinct microbial stimuli activate the vagus and the nature of the signals transmitted to the brain that lead to differential changes in the neurochemistry of the brain (Forsythe and Kunze, 2014). Heart, lungs, pancreas, liver, stomach, and intestines send

information to the brain, via sensory fibers, by the vagus nerve (Browning et al., 2003). This communication of visceral signals from the vagus to brain, and particularly to neural circuitry associated with mood and anxiety, suggests the role of this nerve in the direct communication between gut bacteria and the CNS. Gut bacteria can influence memory, mood, and cognition and are clinically and therapeutically relevant to a range of disorders (Galland, 2014). Evidences from animal model studies show that gut microorganisms can activate the vagus nerve and that such activation plays a critical role in mediating effects on the brain chemistry and, subsequently, may have effects on behavior (Forsythe and Kunze, 2014).

The study reports, for the first time, the effects of oral administration of the probiotic *Lactobacillus rhamnosus* 501 IMC on BDNF brain expression in adult zebrafish. The results showed that the intestinal microbiota can influence CNS in terms of neurotrophins expression.

Clarification of the precise pathway of communication underlying the microbiota–gut–brain axis in zebrafish will require vast interdisciplinary efforts because our results suggest that the pathway may involve production of neuroactive substances modulating the microbiota. Sheding light on the mechanisms underlying the central effects of gut bacteria, overall the neurotrophic effects of probiotics strain, is important because it may lead to the discovery of alternative pathways and substrates to treat brain disorders that do not always respond to prescribed drugs, or when used adjunctively with conventional medications. Finally, understanding this microbiota-gut-brain axis may have important implications for the development of microbial or nutrition based therapeutic

strategies for mood disorders. This first investigations on zebrafish lays the foundation for advancements in knowledge of the microbiota–gut–brain axis, compelling evidence for the link between gut bacteria and brain function in this popular “striped” animal model.

CHAPTER 6

FINAL DISCUSSION

The results of this study may provide the evidence for a microbiota–gut–brain axis which influences brain biochemistry and modulates behavior in adult zebrafish. This is supported by several lines of evidence. First, the "perturbation" of the microbiota, feeding zebrafish with the probiotic strain *L. rhamnosus* IMC 501, apported change in shoaling behavior, increased significantly brain BDNF expression and, although not highly significant it leads to shifts in gut microbial populations. Modulation of central neurotrophin expression, thus, may play a role in the induction of behavior changes in animal models (Bercik et al., 2011).

This study highlights that using probiotic strain, intestinal commensals may play a critical role in behavior and central neurotrophin expression. These bacteria may be capable of producing and delivering neuroactive substances which act on the brain-gut axis.

Analysis of recent studies reveals that gut bacteria influence BDNF levels, particularly in the hippocampus. How this alterations in brain chemistry are related to specific behavioral changes is unclear (Forsythe and Kunze, 2012), but it will likely be a focus of future research efforts.

In the hippocampus, BDNF is associated with memory and learning, but recent evidence indicates that increases in hippocampal BDNF are associated with anxiolytic and antidepressant behavior. The increase in hippocampal BDNF seen in mice is therefore consistent with their gregarious behavior. The amygdala is also

associated with memory and mood disorders and this studies have shown that BDNF expression is increased in the amygdala during fear learning. Amygdala hyperactivity has also been implicated in depression and anxiety and lower levels of BDNF in the amygdala mice are therefore consistent with the observed increase in exploratory behavior. (Bercik et al., 2011). Von Trotha et al., (2014) observed that homologous structures to the mammalian amygdala and hippocampus are respectively contained in the medial (Dm) and the lateral (Dl) domains of the adult zebrafish pallium. This finding showed Dm as a territory that shares developmental origin, gene expression and neuronal connections with the mammalian basolateral amygdale (BLA). Like the mammalian BLA, Dm is activated both upon acute administration of the rewarding drug amphetamine and following conditioning during drug-seeking behavior. These results suggest an evolutionary conserved function of the amygdala in the processing of positive emotions and induction of motivated behavior in zebrafish (Von Trotha et al., 2014), and provides that further studies on behavior and neurochemical this cyprinidae might be required to understand the microbiota-gut-brain communication axis better.

Elucidating the mechanisms underlying the central effects of gut bacteria is important because it may lead to the discovery of alternative pathways and substrates to treat brain disorders that do not always respond to prescribed drugs.

In this study, Firmicutes phyla increased significantly at the expense of representatives of the the Proteobacteria fractions in PROBIO group. Feeding zebrafish probiotic *Lb rhamnosus,* althought not significan showed a reduction of Proteobacteria genera abundance, supporting the antagonistic activity role of this probiotic strain. A higher proportion of Proteobacteria and a lower amount of

Firmicutes (*Lactobacillus* group), which were significantly correlated with enterocolitis severity, characterized the intestinal microbiota dysbiosis in zebrafish with inflammatory bowel disease (IBD) -like colitis. At the same time different researchers showed evidence of changes in the human gut microbiota associated with IBD, particularly a dramatic decrease of diversity in the phylum Firmicutes and a related increase in Proteobacteria (He et al., 2013). The zebrafish digestive tract is similar to that of mammals in its development, organization and function. Zebrafish are well suited for studying host-bacterial interactions as they have innate and adaptive immune systems similar to higher vertebrates (Trade et al., 2004) Hi level of proteobacteria is also associated, in zebrafish, with inflammatory bowel disease (IBD)-like colitis. At the same time other researchers have documented changes in the human gut microbiota associated with IBD, especially a dramatically reduced diversity in the phylum Firmicutes and concomitant increase in Proteobacteria (He et al., 2013). Alterations in diet can lead to marked shifts in gut microbial populations. In this study zebrafish fed a diet containing *Lb rhamnosus* (PROBIO group) were found to have a greater diversity of gut bacteria than those receiving commercial food only (CTRL group). In this study we may speculate that a significantly increased of Firmicutes phyla at the expense of Proteobacteria, in PROBIO group, should be the reader key of abundance of BDNF in zebrafish. Furthermore, zebrafish receiving *Lb rhamnosus* exhibited different shoaling behavior in response to the video tracker test. While no causal relationship was established, this study provides early support for the suggestion that, in addition to any direct effects of dietary components, diet-induced changes in bacterial diversity and neurotrophin factors, may influence behavior.

In this research, a correlation between microbiota modulation and behavioral effects is supported by the demonstration that two groups of zebrafis showed different behavioral patterns in terms of distance variance (DV), nearest distance (ND), the shoaling size area occupied (OA) and water column position (CP). Here we speculate the hypothesis that whether the behavioral changes might be attributed to specific alterations in the microbiota after administration of *Lb rhamnosus.*

More recently it was demonstrated that long-term (28- day) oral administration of a *Lb. rhamnosus* strain (JB1) could alter the normal behavior of adult balb/c mice. Chronic treatment with the bacteria reduced anxiety-like behavior as assessed in an elevated plus maze and decreased the time spent immobile in a forced swim test. Overall, changes induced with this strain of *L. rhamnosus* were indicative of reduced anxiety and decreased depression-like behavior (Forsythe and Kunze, 2012). This study is in line with our own work proposed here.

Thus, in answer to the question" what the relationship between gut bacteria and the brain means for human and animal health", we could show that a lot of interesting studies support that the composition of the gut microbiota may be associated with psychiatric conditions, as it has been proposed in case of schizophrenia (Dinan et al 2014) anxiety and depression and obesity (Forsythe and Kunze, 2012) .

This study on zebrafish is at the very early stages of understanding the complex communication systems of the microbiota-gut-brain axis. The interest in this mechanisms will provide us with new understanding of the symbiotic relationship

between the gut microbiota and their host, in humans animals or fish. In conclusion using different techniques such as video-tracking software, q-RT PCR and NGS we are understanding the ways in which the microbiota influences the brain. It is becoming increasingly apparent that behaviour, neurophysiology and neurochemistry can be affected in many ways through modulation of the gut microbiota. This communication mechanisms will be crucially important for the development of any microbiota-based and microbiota specific therapeutic strategies for CNS diseases. We may assert that this form of interkingdom signaling, based on bidirectional neurochemical interactions between the host's neurophysiological system and the microbiota should be also robust in zebrafish, consolidating it an interesting animal model, one of main character in the theatre of this translational momentum.

Future studies will also help us to identify the potential for microbial-based therapeutic strategies that may aid in the treatment of mood disorders, stress, for human and animal health.

REFERENCES

Adachi N, Numakawa T, Richards M, Nakajima S, Kunugi H, 2014 New insight in expression, transport, and secretion of brain-derived neurotrophic factor: Implications in brain-related diseases. World J Biol Chem. 2014 Nov 26;5(4):409-28. doi: 10.4331/wjbc.v5.i4.409.

Al-Asmakh, M, Anuar, F, Zadjali F, Rafter J, Pettersson, S, 2012. Gut microbial communities modulating brain development and function. Gut Microbes 3, 366–373.

Avella MA, Place A, Du SJ, Williams E, Silvi S, Zohar Y, Carnevali O, 2012. Lactobacillus rhamnosus accelerates zebrafish backbone calcification and gonadal differentiation through effects on the GnRH and IGF systems. PLoS One.;7(9):e45572. doi: 10.1371/journal.pone.0045572

Bolaños CA, Nestler EJ. 2004. Neurotrophic mechanisms in drug addiction. Neuromol Med; 5(1):69-83.

Balcazar JL, Vendrell Dl, de Blas I, Ruiz-Zarzuela I, Muzquiz JL, 2009 Effect of Lactococcus lactis CLFP 100 and Leuconostoc mesenteroides CLFP 196 on Aeromonas salmonicida infection in brown trout (Salmo trutta). J Mol Microbiol Biotechnol, 17 , pp. 153–157

Bates J. M., Mittge E., Kuhlman J., Baden K. N., Cheesman S. E., Guillemin K. (2006). Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. Dev. Biol. 297 374–386 10.1016/j.ydbio.2006.05.006

Bercik P, Collins SM, 2014. The effects of inflammation, infection and antibiotics on the microbiota-gut-brain axis. Adv Exp Med Biol. 2014;817:279-89. doi: 10.1007/978-1-4939-0897-4_13.

Bercik P, Denou E. Collins, J Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy K D, Verdu EF and Collins SM, 2011. The intestinal microbiota affect central levels of brain -derived neurotropic factor and behavior in mice. Gastroenterology 141: 599-609 e593.

Berninger B, Poo M, 1999. Exciting neurotrophins. Nature. 28;401(6756):862-3.

Bested AC, Logan AC, and Selhub EM, 2013. Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: Part I – autointoxication revisited. Gut Pathog 2013, 5:5 doi:10.1186/1757-4749-5-5

Blaser RE and Goldsteinholm K, 2012. Depth preference in zebrafish, Danio rerio: control by surface and substrate cues. An Behav; 83:953-959. doi: 10.1016/j.anbehav.2012.01.014

Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, Bienenstock J, Cryan JF, 2011. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proc Natl Acad Sci USA 108:16050–16055.

Brierley AS, Cox MJ, 2010. Shapes of krill swarms and fish schools emerge as aggregation members avoid predators and access oxygen. Curr Biol;20:1758–62.

Browning KN, Mendelowitz D (2003) Musings on the wanderer: what's new in our understanding of vago-vagal reflexes? II. Integration of afferent signaling from the viscera by the nodose ganglia. Am J Physiol Gastrointest Liver Physiol 284:G8–G14

Brugman S, Liu KY, Lindenbergh Kortleve D, Samsom JN, Furuta GT, Renshaw SA, Willemsen R, Nieuwenhuis EES, 2009. Oxazolone-induced enterocolitis in zebrafish depends on the composition of the intestinal microbiota. Gastroenterology 137: 1757–1767

Cachat J, Stewart A, Utterback E, Hart P, Gaikwad S, et al. (2011) Three-Dimensional Neurophenotyping of Adult Zebrafish Behavior. PLoS ONE 6(3): e17597. doi:10.1371/journal.pone.0017597

Cantas L., Sorby J. R., Alestrom P., Sorum H. (2012). Culturable gut microbiota diversity in zebrafish. Zebrafish 9, 26–37 10.1089/zeb.2011.0712

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, et al., 2011. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7: 335–336.

Carnevali et al., 2014. Probiotic Applications in Temperate and Warm Water Fish Species. In: Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics. 10: 255-289. Ed. D.Merrifield and E Ringo, Wiley & Sons, Ltd UK.

Carnevali O, Avella MA, Gioacchini G, 2013. Effects of probiotic administration on zebrafish development and reproduction. Gen Comp Endocr, 188:297-302.

Castillo DV, Escobar ML, 2011. A role for MAPK and PI-3K signaling pathways in brain-derived neurotrophic factor modification of conditioned taste aversion retention. Behav Brain Res 217, 248–252.

Cerf-Bensussan N, and Gaboriau-Routhiau V, 2010. The immune system and the gut microbiota: friends or foes? Nature Reviews Immunoly, 10, 735-744.

Chao CC, Ma YL, Chu KY, Lee EH, 2003.Integrin alphav and NCAM mediate the effects of GDNF on DA neuron survival, outgrowth, DA turnover and motor activity in rats. Neurobiol Aging; 24(1):105-16.

Choi DC, Maguschak KA, Ye K, Jang SW, Myers KM, Ressler KJ.2010) Prelimbic cortical BDNF is required for memory of learned fear but not extinction or innate fear. Proc Natl Acad Sci USA 107, 2675–2680.

Clarke KR and Gorley RN, 2006. PRIMER v6: User Manual/Tutorial. Plymouth, UK, Primer-E.

Clements KD, Angert ER, Montgomery WL and Choat JH, 2014. Intestinal microbiota in fishes: what's known and what's not. Molecular Ecology, 23: 1891–1898. doi: 10.1111/mec.12699

Collins SM, Kassam Z & Bercik P, 2013. The adoptive transfer of behavioral phenotype via the intestinal microbiota: experimental evidence and clinical implications. Curr Opin Microbiol 16, 240–245.

Cryan, J.F., Dinan, T.G., 2012. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat Rev Neurosci 13, 701–712.

D'Angelo L, De Girolamo P, Lucini C, Terzibasi ET, Baumgart M, Castaldo L, Cellerino A, 2014. Brain-derived neurotrophic factor: mRNA expression and protein distribution in the brain of the teleost Nothobranchius furzeri. J Comp Neurol ;522(5):1004-30. doi: 10.1002/cne.23457.

T.C. Diana and C. Manjulatha, 2012. Incidence and Identification of Klebsiella pneumoniae in Mucosal Buccal Polyp of Nemipterus japonicus of Visakhapatnam Coast, India. Journal of Fisheries and Aquatic Science, 7: 454-460. doi: 10.3923/jfas.2012.454.460

Dimitroglou A, Merrifield DL, Carnevali O, Picchietti S, Avella M, Daniels, CL, Güroy D and Davies SJ, 2011. Microbial manipulations to improve fish health and production: a Mediterranean perspective. Fish and Shellf Immunol 30, 1–16.

Dinan TG, Borre YE, and Cryan JF, 2014. Genomics of schizophrenia: time to consider the gut microbiome? Mol Psych; 19, 1252–1257; doi:10.1038/mp.2014.93

Dinan TG, Crayan JF, 2013. Melancholic microbes: a link between gut microbiota and depression? Neurogastroenterol Motil; 25(9):713-9.

Dinan TG, Stanton C, and Cryan JF, 2013. Psychobiotics: A Novel Class of Psychotropic Biol Psych 74:720–726. http://dx.doi.org/10.1016/j.biopsych.2013.05.001

Duman RS, 2002. Pathophysiology of depression: the concept of synaptic plasticity. Eur Psychiatry ;17 Suppl 3:306-10

Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav Brain Res; 205:38-44.

Engeszer RE, Patterson LB, Rao AA, Parichy DM, 2007. Zebrafish in the wild: a review of natural history and new notes from the field. Zebrafish. Spring;4(1):21-40.

Fijan S, 2014. Microorganisms with claimed probiotic properties: an overview of recent literature. Int J Environ Res Public Health. 5;11(5):4745-67. doi: 10.3390/ijerph110504745.

Forsythe P and Kunze WA, 2012. Voices from within: gut microbes and the CNS. Cell. Mol. Life Sci. doi 10.1007/s00018-012-1028-z

Forsythe P, Bienenstock J, Kunze WA, 2014 Vagal pathways for microbiome-brain-gut axis communication. Adv Exp Med Biol.;817:115-33. doi: 10.1007/978-1-4939-0897-4_5.

Frade JM, Lopez-Sanchez N. 2010. A novel hypothesis for Alzheimer disease based on neuronal tetraploidy induced by p75 (NTR). Cell Cycle 9, 1934–1941.

Germanà A, Laurà R, Montalbano G, Guerrera MC, Amato V, Zichichi R, Campo S, Ciriaco E, Vega JA, 2010. Expression of brain-derived neurotrophic factor and TrkB in the lateral line system of zebrafish during development. Cell Mol Neurobiol; 30(5):787-93. doi: 10.1007/s10571-010-9506-z.

Gerlai R, 2014. Fish in behavior research: Unique tools with a great promise!, J Neurosci Met; 234: 54-58, http://dx.doi.org/10.1016/j.jneumeth.2014.04.015

Gerlai R, 2014. Social behavior of zebrafish: From synthetic images to biological mechanisms of shoaling. J Neurosci Methods; doi:10.1016/j.jneumeth.2014.04.028

Gerlai R, Chatterjee D, Pereira T, Sawashima T, Krishnannair R, 2009. Acute and chronic alcohol dose: population differences in behavior and neurochemistry of zebrafish. Genes Brain Behav;8:586–99.

Gioacchini G, Giorgini E, Merrifield DL, Hardiman G, Borini A, Vaccari L, Carnevali O, 2012

Probiotics can induce follicle maturational competence: the Danio rerio case. Biol Reprod; 86(3):65. doi: 10.1095/biolreprod.111.094243.

Gioacchini G, Giorgini E, Olivotto I, Maradonna F, Merrifield DL, Carnevali O, 2014. The influence of probiotics on zebrafish Danio rerio innate immunity and hepatic stress. Zebrafish. 11(2):98-106. doi: 10.1089/zeb.2013.0932.

Götz R, Raulf F, Schartl M, 1992 Brain-derived neurotrophic factor is more highly conserved in structure and function than nerve growth factor during vertebrate evolution. J Neurochem59(2):432-42

He Q, Wang L, Wang F, Wang C, Tang C, Li, Q, Zhao Q. (2013). Microbial fingerprinting detects intestinal microbiota dysbiosis in Zebrafish models with chemically-induced enterocolitis. BMC Microbiology, 13, 289. doi:10.1186/1471-2180-13-28.

Hill TCJ, Walsh KA, Harris JA, Moffeett BF, 2003. Using ecological diversity measures with bacterial communities FEMS Microbiology Ecology 43:1-11 DOI: http://dx.doi.org/10.1111/j.1574-6941.2003.tb01040.x

Howe K, Clark MD, Torroja CF, et al., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503.

Huber, I., Spanggaard, B., Appel, K.F., Rossen, L., Nielsen, T. and Gram, L. (2004) Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (Oncorhynchus mykiss, Walbaum). Journal of Applied Microbiology 96, 117–132.

Huynh G and Heinrich G, 2001. Brain-derived neurotrophic factor gene organization and transcription in the zebrafish embryo. Int J Dev Neurosci. Nov;19(7):663-73.

Janda JM, Abbott SL, 2014. The genus *Shewanella*: from the briny depths below to human pathogen Crit Rev Microbiol.; 40(4):293-312. doi: 10.3109/1040841X.2012.726209. Epub 2012 Oct 8.

Kalueff AV, Echevarria DJ, Stewart AM, 2014. Gaining translational momentum: more zebrafish models for neuroscience research. Prog. Neuropsychopharmacol Biol Psychiatry http://dx.doi.org/10.1016/j.pnpbp.2014.01.022

Kalueff AV, Stewart AM, Gerlai R, 2014a. Zebrafish as an emerging model for studying complex brain disorders. Trends in pharmacol sci;35(2):63-75. doi:10.1016/j.tips.2013.12.002.

Kalueff et al.,2013. Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond. zebrafish; 10, 1. doi: 10.1089/zeb.2012.0861

Kostic AD, Howitt MR, Garrett WS, 2013. Exploring host-microbiota interactions in animal models and humans. Genes Dev; 27(7):701-18. doi: 10.1101/gad.212522.112.

Lanave C, Colangelo AM, Saccone C, Alberghina L, 2007. Molecular evolution of the neurotrophin family members and their Trk receptors. Gene ;394(1-2):1-12.

Leibrock J, Lottspeich F, Hohn A, Hofer M, Hengerer B, Masiakowski P, Thoenen H, Barde YA, 1989. Molecular cloning and expression of brain-derived neurotrophic factor. Nature; 341(6238):149-52.

Levi-Montalcini R, Calissano P, 1979. The nerve-growth factor. Sci Am. Jun;240(6):68-77.

Levi-Montalcini R, Meyer H, Hamburger V, 1954 In vitro experiments on the effects of mouse sarcomas on the spinal and sympathetic ganglia of the chick embryo. Cancer Res. 14: 49–57

Llewellyn MS, Boutin S, Hoseinifar SH, Derome N, 2014. Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries Front Microbiol.; 5: 207 doi: 10.3389/fmicb.2014.00207

Lozupone C, Knight R 2005 UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 71: 8228–8235.

Lyte M, 2011. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. Bioessays 33:574–581.

Merrifield DL and Carnevali O, 2014. “Probiotic modulation of the gut microbiota of fish” In Aquaculture Nutrition: GutHealth, Probiotics and Prebiotics, Edn1,edsDL Merrifield and E Ringø (Hoboken,NJ:Wiley BlackwellScientificPublication); 8:185-222

Merrifield DL, Dimitroglou A, Foey A, Davies SJ, Baker RTM, Bogwald J, Castex M, Ringø E, 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture, 302, 1-18.

Milani C, Hevia A, Foroni E, Duranti S, Turroni F, et al. (2013) Assessing the Fecal Microbiota: An Optimized Ion Torrent 16S rRNA Gene-Based Analysis Protocol. PLoS ONE 8(7): e68739. doi:10.1371/journal.pone.0068739

Mondelli V, Cattaneo A, Belvederi Murri M, Di Forti M, Handley R, Hepgul N, Miorelli A, Navari S, Papadopoulos AS, Aitchison KJ, Morgan C, Murray RM, Dazzan P, Pariante CM, 2011. Stress and inflammation reduce brain-derived neurotrophic factor expression in first-episode psychosis: a pathway to smaller hippocampal volume. J Clin Psychiatry (2011) 72:1677–8410.4088/JCP.10m06745

Montiel-Castro AJ, González-Cervantes RM, Bravo-Ruiseco G, Pacheco-López G, 2013. The microbiota-gut-brain axis: neurobehavioral correlates, health and sociality. Frontiers in Integrative Neuroscience;7:70. doi:10.3389/fnint.2013.00070.

Muyzer, G., deWaal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59, 695–700.

Nayak SK, 2010. Probiotics and immunity: a fish perspective. Fish Shellfish Immunol.;29(1):2-14. doi: 10.1016/j.fsi.2010.02.017.

Nowicki M, Tran S, Muraleetharana A, Markovic S, Gerlai R, 2014. Serotonin antagonists induce anxiolytic and anxiogenic-like behavior in zebrafish in a receptor-subtype dependent manner. Pharmacol Biochem Behav; 126 :170–180 http://dx.doi.org/10.1016/j.pbb.2014.09.022

O'Sullivan, E. Barrett, S. Grenham, P. Fitzgerald, C. Stanton, R.P. Ross1,2, E.M.M. Quigley1,5, Cryan JF and Dinan TG, 2011. BDNF expression in the hippocampus of maternally separated rats: does Bifidobacterium breve 6330 alter BDNF levels? Benef Micr; 2(3): 199-207

O' Mahony SM, Clarke G, Borre YE, Dinan TG, Cryan JF, 2014. Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. Behav Brain Res; 277:32-48. doi: 10.1016/j.bbr.2014.07.027.

Pagnussat N, Piato AL, Schaefer IC, Blank M, Tamborski AR, Guerim LD, Bonan CD, Vianna MRM and Lara DR, 2013. One for All and All for One: The Importance of Shoaling on Behavioral and Stress Responses in Zebrafish. Zebrafish. 10(3):338-42. doi: 10.1089/zeb.2013.0867

Panula P, Sallinen V, Sundvik M, Kolehmainen J, Torkko V, Tiittula A, et al., 2006. Modulatory neurotransmitter systems and behavior: towards zebrafish models of neurodegenerative diseases. Zebrafish;3:235–47.

Rawls JF, Mahowald MA, Ley RE, Gordon JI. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell. 2006;127(2):423–433.

Rawls JF, Samuel BS, Gordon JI, 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc Natl Acad Sci USA.;101(13):4596–4601.

Rhee SH, Pothoulakis C, Mayer EA, 2009 Principles and clinical implications of the brain-gut-enteric microbiota axis. Nat Rev Gastroenterol Hepatol; 6(5):306-14. doi: 10.1038/nrgastro.2009.35.

Ringø E, and Birkbeck TH, 1999. Intestinal microflora of fish larvae and fry. Aquac Res, 30, 73-93.

Ringø E, Dimitroglou A, Hoseinifar SH and Davies SJ, 2014.“Prebiotics in finfish:anupdate,”in Aquaculture Nutrition: GutHealth,Probiotics and Prebiotics, Edn1,edsD.L.Merrifield and E.Ringø (Hoboken, NJ: Wiley - BlackwellScientificPublication).

Ringø E, Olsen RE, Mayhew TM, Myklebust R, 2003. Electron microscopy of the intestinal microflora of fish. Aquaculture, 227, 395-415.

Robinson RC, Radziejewski C, Spraggon G, Greenwald J, Kostura MR, Burtnick LD, Stuart DI, Choe S and Jones EY, 1999. The structures of the neurotrophin 4 homodimer and the brain-derived neurotrophic factor/neurotrophin 4 heterodimer reveal a common Trk-binding site. Protein Science 8: 2589-2597.

Roeselers, G., Mittge, E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K. and Rawls, J.F. (2011) Evidence for a core gut microbiota in the zebrafish. ISME Journal. DOI 10.1038/ismej.2011.38.

Rose JD, 2007. Anthropomorphism and 'mental welfare' of fishes. Dis Aquat Organ;75:139–54.

Rose S, Hill R, Bermudez LE, Miller-Morgan T, 2013. Imported ornamental fish are colonized with antibiotic-resistant bacteria. J Fish Dis; 36(6):533-42. doi: 10.1111/jfd.12044.

Sánchez-Ramos C1, Bonnin-Arias C, Guerrera MC, Calavia MG, Chamorro E, Montalbano G, López-Velasco S, López-Muñiz A, Germanà A, Vega JA, 2013. Light regulates the expression of the BDNF/TrkB system in the adult zebrafish retina. Microsc Res Tech; 76(1):42-9. doi: 10.1002/jemt.22133.

Savignac HM, Corona G, Mills H, Chen L, Spencer JP, Tzortzis G, Burnet PW, 2013. Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-D-aspartate receptor subunits and D-serine. Neurochem Int. 2013 Dec;63(8):756-64. doi: 10.1016/j.neuint.2013.10.006.

Sekirov I, Russell SL, Antunes LCM, Finlay BB, 2010. Gut Microbiota in Health and Disease. Physiological Reviews, 90, 859-904.

Semova I, Carten JD, Stombaugh J, Mackey LC, Knight R, Farber SA, et al. (2012). Microbiota regulate intestinal absorption and metabolism of fatty acids in the zebrafish. Cell Host Microbe 12, 277–288 10.1016/j.chom.2012.08.003

Sevellec M, Pavey SA, Boutin S, Filteau M, Derome N, Bernatchez L. Microbiome investigation in the ecological speciation context of lake whitefish (Coregonus clupeaformis) using next-generation sequencing. J Evol Biol. 2014 Jun;27(6):1029-46. doi: 10.1111/jeb.12374. Epub 2014 Apr 29.

Spanggaard, B., Huber, I., Nielsen, J., Nielsen, T., Appel, K.F. and Gram, L. (2000) The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. Aquaculture 182, 1–15.

Stewart AM, Braubach O, Spitsbergen J, Gerlai R and Kalueff AV, 2014 Zebrafish models for translational neuroscience research: from tank to bedside. Trends in Neurosci Vol. 37, 5. http://dx.doi.org/10.1016/j.tins.2014.02.011

Stewart AM, Ullmann JF, Norton WH, Parker MO, Brennan CH, Gerlai R, Kalueff AV, Molecular psychiatry of zebrafish. Mol Psychiatry. 2015 Feb;20(1):2-17. doi: 10.1038/mp.2014.128.

Sugita H, Miyajima C, Deguchi Y, 1991. The vitamin B12-producing ability of the intestinal microflora of freshwater fish. Aquaculture 92: 267–276.

Tellez G, Higgins S, Donoghue A, and Hargis B, 2006. Digestive physiology and the role of microorganisms. J. Appl Poult Res 15, 136–144.doi:10.1093/japr/15.1.136

Thoenen H, 1995. Neurotrophins and neuronal plasticity. Science 270: 593-598.

Tran S and Gerlai R, 2013. Individual differences in activity levels in zebrafish (Danio rerio). Behav Brain Res. 257: 224–229. doi:10.1016/j.bbr.2013.09.040.

Trede NS, Langenau DM, Traver D, Look AT, Zon LI. The use of zebrafish to understand immunity. Immunity. 2004;13(4):367–379. doi: 10.1016/S1074-7613(04)00084-6

Tyler WJ, Alonso M, Bramham CR and Pozzo-Miller LD, 2002. From acquisition to consolidation: on the role of brain-derived neurotrophic factor signaling in hippocampal-dependent learning. Learning and Memory 9: 224-237

Tsuchiya C, Sakata T, Sugita H, 2008. Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. Lett Appl Microbiol 46: 43–48

Ventriglia M, Zanardini R, Bonomini C, et al. 2013. Serum Brain-Derived Neurotrophic Factor Levels in Different Neurological Diseases. BioMed Research International;2013:901082. doi:10.1155/2013/901082.

Vignier N, Barreau M, Olive C, Baubion E, Théodose R, Hochedez P, Cabié A, 2013. Human infection with *Shewanella putrefaciens* and *S. algae*: report of 16 cases in Martinique and review of the literature. Am J Trop Med Hyg.; 89(1):151-6. doi: 10.4269/ajtmh.13-0055.

Vissio PG, Cánepa MM, Maggese MC, 2008 Brain-derived neurotrophic factor (BDNF)-like immunoreactivity localization in the retina and brain of Cichlasoma dimerus (Teleostei, Perciformes). Tissue Cell; 40(4):261-70. doi: 10.1016/j.tice.2008.01.001

Von Trotha JW, Vernier P, Bally-Cuif L. Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish. The European Journal of Neuroscience 2014;40(9):3302-3315. doi:10.1111/ejn.12692.

Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, Feldstein AE, Britt EB, Fu X, Chung Y-M, et al., 2011. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 472: 57–6

Weickert CS, Hyde TM, Lipska BK, Herman MM, Weinberger DR and Kleinman JE, 2003. Reduced brain-derived neurotrophic factor in prefrontal cortex of patients with schizophrenia. Molecular Psychiatry 8: 592-610

Westerfield M (2000) The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio Rerio). Eugene, OR: University of Oregon.

Wong K, Elegante M, Bartels B, Elkhayat S, Tien D, Roy S, et al. Analyzing habituation responses to novelty in zebrafish (Danio rerio). Behav Brain Res 2010;208:450–7.

Yoshii A, Constantine-Paton M (2010) Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. Dev Neurobiol 70, 304–322.

Zhao S, Fernald RD (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. J Comput Biol 12: 1047–1064.

ACKNOLEDGMENT

I would like to dedicate this PhD thesis to my mum, dad and Emanuela for believing in everything I have ever done. Thanks my dear family for your immeasurable love.

I would like to thank my supervisor Prof. Alessandro Fioretti for him support and guidance throughout my studies and all research time. I am extremely grateful for the help, encouragement, thoughtfulness and understanding he has provided me over theese courious years and for the formative influence on my life.

I would also like to acknowledge the members of my supervisory team, Professors. Luciano Gaudio, Serena Aceto, Claudio Agnisola All have given very willingly of their time and expertise to aid me in my academic pursuits and have provided me with invaluable advice on many occasions.Thanks to Prof. Paolo de Girolamo, my doctoral course coordinator, for all tips.

I would deeply like to thank a lot my thesis referees, Prof. Ted Dinan and Carmine Pariante. I was really glad to recieve their their precious words opinions and suggestions and their works and papers have amplified my curiosity encouraging my efforts.

Many thanks to Dr. Daniel Merrifield who have offered me the opportunity of going to the Plymouth University developing an important biomolecular part of this research project; Thanks to all Plymouth University reaserchers of 310 MicroLab for their support and precious expertise in molecular biology.

I want to acknowledge all my Lab and Departement mates, particularly Ludovico Dipineto for his humour, friendship and support which have helped me through difficult times. I'm also thankful to Francesca Menna from the beginning of my

research course, for believing me, for all lively discussions; thanks to Livia D'Angelo for her nice and smiling support and professional time; thanks to Mariarosaria Calabria for her maternal arms and I am thankful to all guys of Centro Sperimentale di Varcaturo, especially Tamara, Fede, Antonio, il Gargiulo, Gigi, Giovanni and Giovanni, Ilia, Daniela, Sergio, Sofia and all the others guys for their companionship during the best and worst of daily science and lunch time.

I would also like to thank the beautiful people of Plymouth University for their friendship, emotional support and good time spent. I am truly thankful to many people, that have helped and supported me, there is no words to acknowledge them all.

Finally I would thank all my new and old friends, life companions. I truly would never have accomplished this goal without all of your brilliant eyes and all of your seasonal colours.

Thanks to all time spent in beautiful and courious company.