# Exploring new frontiers in host-microbiome interactions using zebrafish

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Year 2018

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*"Dans le champ de l'observation,*

*le hasard ne favorise que les esprits préparés"*

*L. Pasteur*

A Julio y Karmele

# Acknowledgements

A En primer lugar, tengo que agradecer al Gobierno Vasco por concederme la beca la beca sin la que esta tesis doctoral se habría llevado a cabo. En segundo lugar, me gustaría agradecer también a los proyectos ENVIPHAGE y MAROMEGA por permitirme participar en ellos. Gracias a Alfonso Rodríguez Herrera, del Instituto Hispalense de Pediatría por financiar una pequeña parte de esta investigación e intentar ayudar siempre en todo lo posible.

Me gustaría mostrar mi agradecimiento a mis directores de tesis, Miguel sin el que no podría haber llegado hasta el día de hoy, gracias por ayudarme, apoyarme y aconsejarme siempre; gracias por dejarme volar libre porque he aprendido muchísimo estos años y gracias a tu idea de proyecto he podido comenzar en el mundo de la microbiota, campo que he descubierto que me encanta. Gracias a Andoni, por aceptar la cotutela de mi tesis y a mí al final como alumna de nuevo después de realizar el trabajo de fin de máster en el que comenzó mi interés por la microbiología. También me gustaría agradecer a Lucila por ser mi tutora y vínculo con la universidad, además de al Departamento de Inmunología, microbiología y parasitología por aceptar la tesis dentro del departamento.

Quiero agradecer a la gente de azti que he conocido durante estos cuatro años. A la gente que está y ha pasado por el laboratorio de Biología molecular; Amaia, Nagore, Xabi, Mari, gracias por ayudarme a cuidar mis pececitos, con las extracciones, PCRs y demás tareas. Gracias también por el apoyo moral tanto de la vida laboral como personal durante estos años; la terapia no se paga con dinero. En especial gracias a Xabi por haber compartido conmigo los últimos experimentos de la tesis y haberme servido de tan gran ayuda; sin tus conocimientos y saber hacer no podría haber hecho muchas cosas. Gracias a Iñaki, juntos hemos compartido 3 años de laboratorio, muchas risas, complots, crisis, diseños de experimentos y días de la marmota. Gracias a ti he aprendido casi todo lo que sé del pez cebra. Gracias a Sandra, por ayudarme corregir los papers y a solucionar cualquier duda durante estos años. A Igor primero por ayudarme siempre en el laboratorio con dudas y preguntas, y finalmente por dejarme participar en sus proyectos. A Nadia, sin ti los 3 primeros años no habrían sido lo mismo, ni en el labo ni en lo personal. Lo hemos pasado tan bien y nos hemos ayudado tanto que ahora mismo no me imaginaría estos 4 años sin ti. A los analistas de micro, Iker, Natalia, Cristina, Miguel, que siempre nos han echado un cable cuando lo hemos necesitado. A Itziar Fernandez, que al principio nos tenía por enemigos en la biblioteca pero enseguida nos cogió cariño. A Itziar Tueros y Sara, por compartir sus conocimientos con nosotros y así poder darle un enfoque diferente al tema de las dietas.

A Naiara y Laura, por ayudarme a analizar los datos, dejarme formar parte de sus investigaciones y formar ellas parte de las mías. Por haberme introducido en un mundo de datos, estadística y análisis que pese a mi primera reticencia me ha resultado muy interesante y espero que esté muy presente en mi futuro.

A the becaris. Primero a Nadia y Ekaitz con los que empecé mi andadura doctoral un caluroso 7 de enero. Oihane, que después de coincidir en la carrera nos volvimos a encontrar aquí, sobre todo los últimos meses. A Jani y Mertxe, que ellas ya estaban aquí de antes y nos acogieron con los brazos abiertos. A Jone, Lucía, Leire y Javi que aparecieron en la biblioteca un año después y al resto the becaris con los que desde luego este tiempo no podría haber sido más agradable y feliz. A Ane, con la que comencé en esta locura llamada metabarcoding; juntas hemos echado horas a secuencias y comandos. Al Poron, al que podríamos considerar después de todo parte del Azteam.

A veneno, karma y herpes, a jujaneo y a las 3 maris. Nos ha dado la vida durante este tiempo, y lo que le queda. Gracias por estar ahí siempre, en lo bueno que ha sido muy bueno y en lo malo que lo ha convertido en no tan malo.

Tengo que agradecer también a Leticia del CIC-biogune, a la que conocí por casualidad en Clermont Ferrand pero que enseguida se interesó por mi trabajo y trayectoria; de tal forma que gracias a esto tuve la idea ganadora del concurso de “un reto para el parque” y que finalmente me ha ayudado mucho y ha participado en mi investigación. Sin tu ayuda no podría haber sacado tanto partido a una parte importante para mí de esta tesis y con ello terminado. También a JL que nos ha ayudado mucho y también le hemos mareado mucho. A gabi, de la universidad de Sevilla, que enseguida se quiso involucrar en esta investigación y se puso la día.

Agradecer a mi familia, por su apoyo durante estos años y durante toda la vida. A mis tías, por acompañarme a algún congreso y hacerme sentirme como en casa en momentos de tensión; por llevarme de vacaciones y hacerme desconectar en momentos de estrés.

A mis padres, Julio y Karmele, por supuesto a quienes dedico esta tesis. Sin ellos no podría haber llegado hasta aquí, y a ningún sitio en realidad. Gracias por darme un hogar, el cariño de unos padres, una educación tanto en casa como fuera de ella, por dejarme siempre libertad para conseguir lo que quiero, por venir conmigo a todas partes y sobre todo por apoyarme en todas las decisiones que tomo. Os quiero, aunque sé que no os lo digo lo suficiente. ¡Gracias!

Gracias a mis amigas, por apoyarme siempre, desde pequeñas. Juntas hemos vivido muchos momentos de alegrías y tristezas, pero todos juntas. Y así seguiremos, cantando canciones del cancionero. ¡Carmelitas power! Gracias también a Andere y Lorea; dos personas

que he conocido gracias a la ciencia y se han acabado convirtiendo en una parte muy importante de mi entorno. Si no las hubiera conocido no habríamos acabado en Tokyo siendo big in japan, o secando el malecón en La Habana.

Por último, gracias a Alberto. Gracias por apoyarme en todo, ayudarme sin preguntar y quererme siempre.

…Al karma; esa energía trascendente, invisible e inmensurable que se genera a partir de los actos personales.

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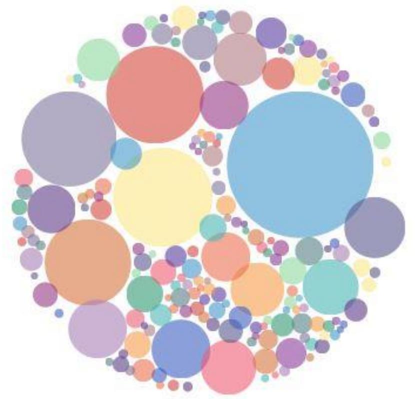
## Abbreviation list

| | |
|---|---|
| ACT | B-actin |
| AMPs | Antimicrobial peptides |
| ARISA | Automated approach for ribosomal intergenic spacer analysis |
| ASC | Caspase recruitment domain |
| ASF | Altered Schaedler flora |
| BCAA | Branched-chain amino acids |
| bp | Base pair |
| CASPA | Caspase 1a |
| DEF1 | Defensin-1 |
| DGGE | Denaturing gradient gel electrophoresis |
| DHA | Docosahexaenoic acid |
| dpf | Days post-fertilization |
| EF1 | Elongation factor |
| ENA | European Nucleotide Archive |
| ER | Endoplasmic reticulum |
| FAMES | Fatty acid methil esthers |
| FDR | False discovery rate |
| GF | Germ-free |
| HCD | High-cholesterol diet |
| HFA | Human flora-associated |
| HFD | High-fat diet |
| HFO | High-fat-DHA enriched diet |
| HPD | High-protein diet |
| hpf | Hours post-fertilization |
| hpi | Hours post-infection |

| | |
|---|---|
| IAP | Intestinal alkaline phosphatase |
| IBD | Intestinal bowel disease |
| IgA | Immunoglobulin A |
| IKK/ NFKB | IκBα kinase/nuclear factor-KB |
| IL10 | Interleukin-10 |
| IL1B | Interleukin-1β |
| IL22 | Interleukin-22 |
| ITS | Internal transcribed spacers |
| LPS | Lipopolysaccharide |
| MALT | Mucosal-associated lymphatic tissue |
| MAMPs | Microbe-associated molecular patterns |
| MMP9 | Matrix metallopeptidase 9 |
| MMPs | Matrix metalloproteinases |
| MPO | Myeloid-specific Peroxidase |
| MUFA | Monounsaturated fatty acid |
| MYD88 | Myeloid differentiation primary response 88 |
| NFKB | Nuclear factor-kB |
| NGS | Next-generation sequencing |
| NLR | NOD-like receptors |
| NLRP3 | NOD-like receptor P3 |
| NMDS | Non-multimetric dimensional scalling |
| NOD1 | Nucleotide-binding oligomerization domain 1 |
| OTU | Operational taxonomic units |
| PCoA | Principal component analysis |
| PRR | Pattern recognition receptors |
| PUFA | Polyunsaturated fatty acids |

| | |
|---|---|
| RIN | RNA integrity number |
| ROS | Reactive oxygen species |
| rRNA | Ribosomal RNA |
| SCFA | Short-chain fatty acids |
| S | Seconds |
| SEM | Standard error of the mean |
| SFA | Saturated fatty acids |
| SIHUMI | Simplified human microbiota |
| TGGE | Temperature gradient gel electrophoresis |
| TLR2 | Toll like receptor 2 |
| TLR4 | Toll like receptor 4 |
| TLR5 | Toll like receptor 5 |
| TLRs | Toll-like receptors |
| TNFA | Tumor necrosis factor |
| T-RFLP | Terminal-restriction fragment length polymorphism |
| UPR | unfolded protein response |
| wpf | Weeks post-fertilization |

# Introduction



# Microbiota

## 1.1. What is it and how it is formed?

All alive organisms in the earth, including animals and plants, have coevolved with the microorganisms in their environment, as microbes colonise the host greatly influencing its physiology and immunity[1]. The term "microbiota" refers to a community of living organisms cohabiting in a determined niche. Animal intestines represent a favorable ecologic niche, the human colon is one of the most densely populated communities, exceeding the soil or the ocean[2]. The mammalian large intestine, is colonised by a number of microorganisms reaching levels of $10^{12}$ - $10^{14}$, more than the number of human cells[3]. The microbial ecosystem of the intestine, called the gut microbiota, includes many species that permanently inhabit the gastrointestinal tract and a variable series of microorganisms that only do so transitorily. The microorganisms, their genes, and their secreted metabolites are commonly known as the "microbiome". The human microbiome comprises the total population of microbes colonising the human body, including the oral cavity, nasopharynx, respiratory tract, gastrointestinal tract, genitourinary tract, and skin[4] (Figure 1). However, recently, the term "microbiome" has been frequently used interchangeably with microbiota[5].

**Figure 1: Mosaic of the microbiomes of the human body** (Illustration by Charis Tsevis).

The axenic epithelial surfaces of all vertebrates are colonised at birth by large communities of microorganisms from the environment which form commensal or mutualistic relationship with their host[6]. Many microorganisms fail to colonise a host because their requirements are not compatible with the conditions and resources in the host. Conversely, others are able to grow and elevate their population. Microbe-microbe interactions are complex and diverse relationships, which are able to drive shifts in microbiome composition that are

generally decisive. They might be competitive, parasitic or predatory, while others are mutualistic. Furthermore, different types of interactions can occur simultaneously[7] and be influenced by different processes and environmental factors which strongly contribute to model the microbiota composition[8] such as age, or diet[9]. However, the initial environmental microbial diversity exerts significant variations in the final microbial community of the host even more to these environmental factors, leading to interindividual variations [10].

## 1.2. The importance for the host

The intestinal microbiota is widely considered a real organ with well-defined functions, composed of different microbes that communicate with each other and with the host. This microbiome exerts an enormous impact on the nutritional and health status of the host, developing a number of immune and metabolic functions[11] (Figure 2), including:

- The maintenance of barrier homeostasis
  The intestinal epithelium constitutes a physical barrier that regulates the transport of substances. The intestinal microbiota contribute to the maintenance of the integrity of the intestinal barrier through the maintenance of cell junctions, and the promotion of epithelial repair after damage[12].
- Immune system development and activation
  The intestinal microbiota is essential for the development and maturation of the immune system, playing an important role in the prevention of pathogenic infection[13], and contributing to the secretion of antimicrobial peptides. It influences the content of lamina propia T cells, immunoglobulin A-producing B cells, intraepithelial T cells and serum immunoglobulin levels[14].
- Structural and metabolic functions
  Microbes provide enzymes and regulate the expression of genes involved in the utilisation of carbohydrates and lipids, maximising the caloric availability of ingested nutrients[15]. They contribute to cholesterol reduction, the biosynthesis of vitamins and isoprenoids, and the metabolism of amino acids. One of the most important metabolic functions of the intestinal microbiota is the utilisation of non-digestible carbohydrates from the diet that the human intestine cannot digest or absorb, into short-chain fatty acids (SCFAs)[16], principally acetic, propionic, and butyric acid. In addition, it appears that the microbiota is capable of modulating the genes that regulate the energy absorption of dietary

carbohydrates and complex lipids by the host, which increases fat storage and leads to body weight gain[17].

- Furthermore, the intestinal microbiota have an impact on the development and function of organs outside the intestinal tract[18]. As a result, it is associated with brain development and subsequent behaviour, linking microbial activity to neurodevelopmental diseases[19].

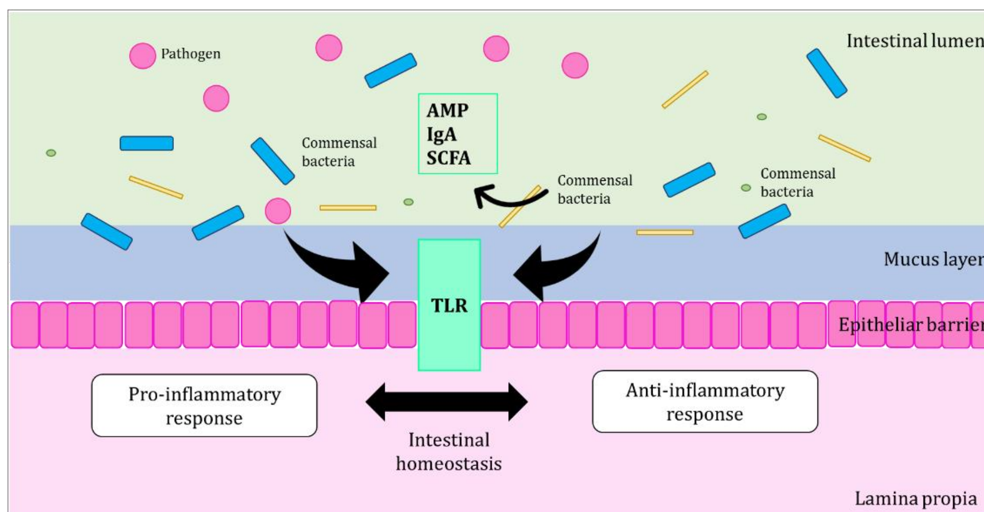



**Figure 2: Molecular interactions between the host, intestinal commensal bacteria and pathogens inside the intestine.**

## 1.3. Eubiosis and dysbiosis status

The microbiome seems to be almost stable during healthy adulthood, called "eubiotic status". This status is characterised by the maintenance of homeostasis where a preponderance of potentially beneficial species versus potentially pathogenic species is essential. In homeostasis, immunoglobulin A (IgA) and antimicrobial peptides (AMPs) are released into the mucus layer, preventing the bacteria from reaching the epithelial surface. Microbe-associated molecular patterns (MAMPs) are bound to the epithelial Toll-like receptors (TLRs). Thus, microbial signalling to epithelial cells can be relayed to the underlying immune and nervous systems to alter gut functions. Moreover, the lamina propia is surveyed by many lymphocytes, phagocytic cells and other immune effector cells which maintain inflammatory cytokines in low concentrations.

The opposite state is called "dysbiosis", where "good bacteria" take over the "bad bacteria" meaning that they no longer have control[20]. Thus, dysbiosis is defined by quantitative

and qualitative microbial alterations[21]. Bacterial diversity is usually considered an indicator of health, because a healthy microbiome composition is required for a number of physiological functions[12]. In contrast, a reduced bacterial diversity has been related to a number of diseases such as obesity and inflammatory diseases in humans[21, 22].

Factors that can disturb the eubiosis state include: lifestyle, antibiotic treatments and pathogens[8]. Consequently, they transform the eubiosis into dysbiosis, characterised by alterations of the intercellular tight junctions that are responsible for maintaining the integrity of the intestinal mucosa[23] . This alteration leads to changes in mucosal barrier permeability, which is crucial to prevent the access to pathogens[24]. Bacteria have evolved multiple methods of subverting epithelial defences and translocating to the lamina propia. Disrupted tight junctions, inflammatory signalling and epithelial cell death create gaps in the barrier, allowing the entry of opportunistic bacteria, antigens and toxins from the lumen, which activate the mucosal associated lymphatic tissue (MALT), and consequently the inflammatory cascade (leukocytes and cytokines)[23]. High levels of inflammatory cytokines, reduced mucus production and impaired antimicrobial production, which allow additional bacteria to reach and traverse the epithelial barrier, lead to intestinal and potentially systemic infections[25] (Figure 3).

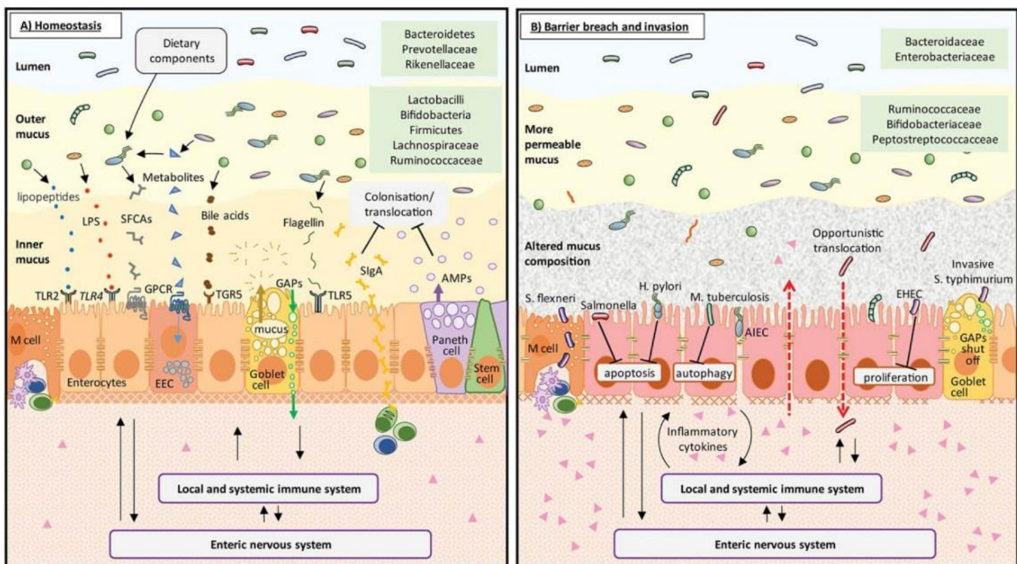



**Figure 3: The intestinal epithelial barrier. A Eubiosis. B Dysbiosis** (Obtained from Parker *et al.*, 2017)[25].

Dysbiosis is also associated with the development of a number of diseases, including type 2 diabetes, allergies, fatty liver disease, or intestinal bowel disease (IBD)[26]. A number of studies consider diet to be the major environmental factor that strongly modulates the intestinal ecosystem, providing selective growth advantages to certain microbial species[27]. Furthermore, the dietary composition also regulates several physiological factors which influence the microbial community but are currently poorly characterised[28]. Thus, different diets are associated with different microbial compositions[29] and, in some cases, with diseases[30]. In addition, the microbiome response to dietary interventions is considered rapid, as it can occur within 24 h [31]. For that reason, gut microbiota can be modulated by dietary interventions, stimulating the growth of potentially beneficial bacteria to improve health status[32].

## 2. Teniques for studying the microbiome

### 2.1. Culture-dependent methods

The study of human microbiome diversity started with Antonie van Leewenhoek. He compared his oral and faecal microbiota in the 1680s, appreciating the differences in terms of microbes between these two habitats and also between healthy and ill individuals[33]. Accordingly, studies of the differences in microbes at different sites of the body, and between healthy and diseased individuals, are as old as microbiology itself[5].

The first gut microbiome studies involved the cultivation of individual bacteria and the co-culture of microbial consortia[34]. The gut microbiome, as mentioned before, is densely populated but, unfortunately, contains only a small percentage of culturable microbes. Some components of the microbiota require special conditions for growth in culture media; therefore, they went undetected. For example, the colonic microbiota have approximately 800 to 1,000 species per individual, but 62% of them remain unknown and 80% of the bacteria identified by metagenomics are regarded as unculturable[35]. For that reason, as culture-dependent techniques do not detect uncultivable microbes, little information about community dynamics is provided. However, cultivation-based approaches are still important today, as they allow the investigation of basic microbial interactions. For example, they are useful to explore the mechanisms by which individual microorganisms modulate the microbiota and host physiology. For that purpose they must be first isolated, identified, and then studied in pure cultures and microbial consortia[36].

One of the latest techniques used to culture isolates of the gut microbiota is “culturomics”. This approach, developed by Lagier *et al.*, combines culture methods with mass

spectrometry (MALDI-TOF), to identify microorganisms, fungi and viruses in a complex ecosystem[37] (Figure 4). Thus, culturomics represents a powerful technique that should revolutionise microbial culture[38].

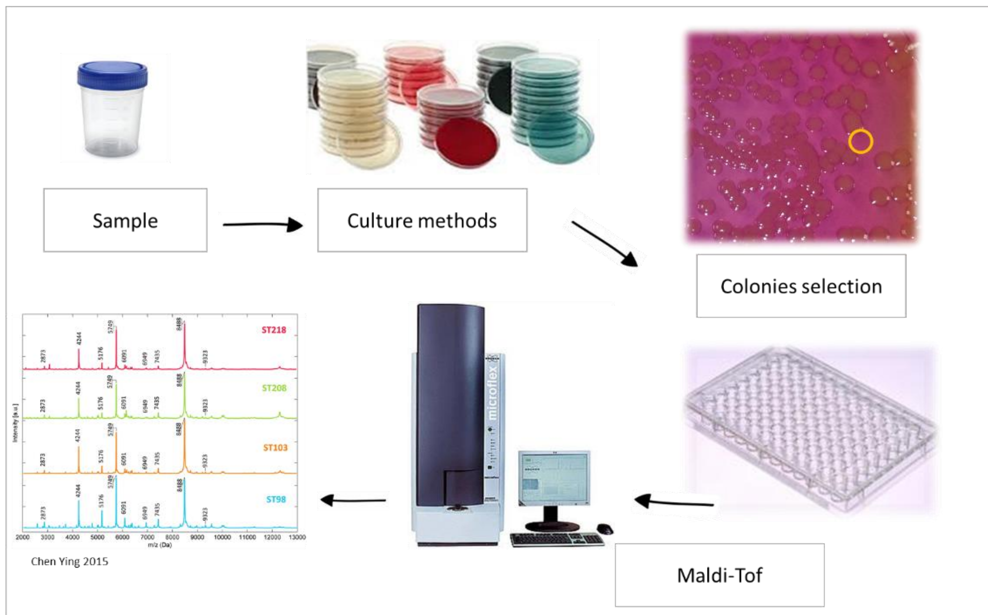



**Fig. 4 Schema of the culturomics technique.**

## 2.2. Molecular techniques

The first report on the sequencing of long DNA sequences, namely by Sanger and Maxam-Gilbert, was published in 1977[39]. From that date, for the next 30 years, large-scale sequencing projects relied on this technology[40]. With the development of molecular biology, different genomic approaches have arisen in the last 10 years for the study of microbial communities. These approaches include terminal-restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), or the automated approach for ribosomal intergenic spacer analysis (ARISA). Unfortunately, these methods provide little taxonomic information[36]. Recent PCR-based sequencing technologies have drastically increased the throughput of microbiome analyses[41]. PCR-based sequencing allows the identification of individuals at a very low concentration within a complex matrix. Consequently, this technique is widely use for the detection of specific bacteria, whereas it is not suitable for the study of the entire microbiome[42].

However, this approach, together with bioinformatics advancements, has opened the door to modern next-generation sequencing (NGS) technology[36].

### 2.3. Next-generation sequencing

The progress in sequence technologies has revolutionised the knowledge of microbiological communities, specifically of the microorganisms living inside and outside the human body. The majority of human microbiome-based studies mainly use one of four types of next-generation sequencing strategies: genome sequencing, shotgun sequencing, transcriptome sequencing and metabolomics (Figure 5).

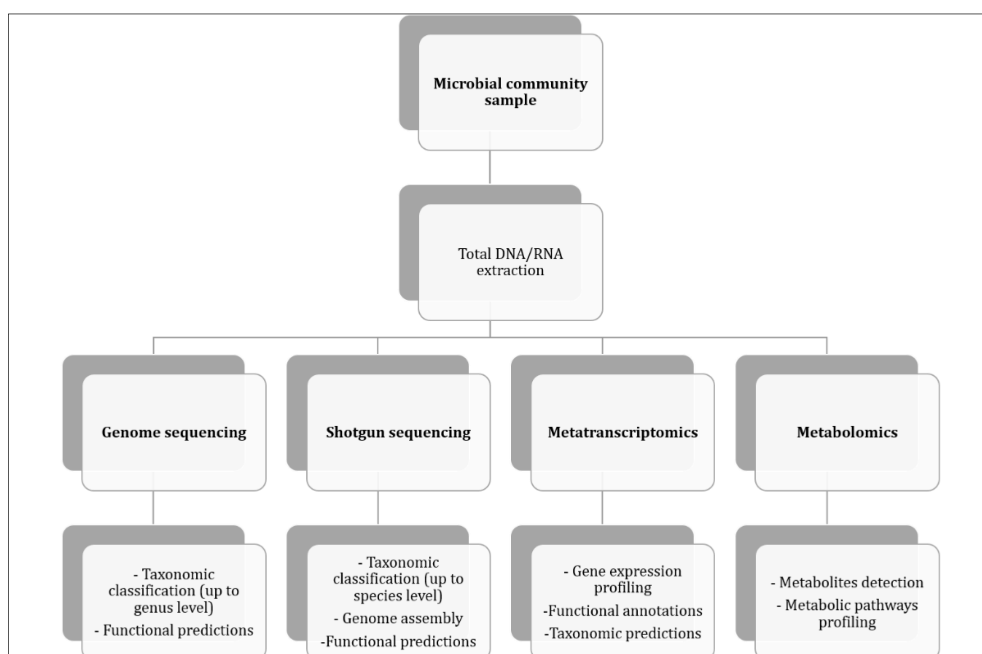



**Figure 5: Schema of NGS techniques.**

- Genome sequencing

This technique is commonly used to study complex microbial communities[43]. It is focused on universal taxonomic markers such as 16S ribosomal RNA (rRNA) for bacteria, 18S rRNA for microeukaryotes and unicellular eukaryotes, and internal transcribed spacers (ITS) for fungal communities[44]. The most extensively used macromolecule in bacterial phylogenetic and taxonomic studies is the 16S ribosomal RNA (16S sRNA)[45], as it is highly conserved between species belonging to the bacteria kingdom and some archaea. These conserved zones are

alternated by hypervariable species-specific regions making bacterial identification possible[46]. Universal primers are usually chosen as complementary to the conserved regions, with the variable region sequence in between[47]. Many primers are available for the amplification of different 16S variable regions, which should be selected based on factors such as the class of bacteria under investigation and the required level of taxonomic resolution (order, family, genus, species, etc.)[48] (Figure 6).

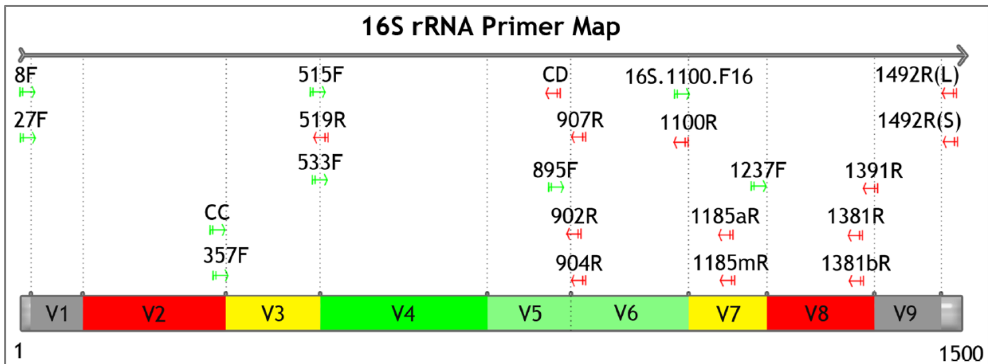



**Figure 6: Different 16S rRNA variable regions** (http://lutzonilab.org/16s-ribosomal-dna/).

Once the primer pairs are selected, they are used to perform PCR after the community DNA is extracted. This process is followed by high-throughput sequencing of PCR amplicons[36]. When the sequencing has been completed, computational methods and techniques complementing experimental approaches are necessary to analyse the data obtained from the sequencer. The most frequently utilised bioinformatic software platforms are Mothur[49] and QIIME[50], which focus on exploratory analysis and visualisation of taxonomic composition.

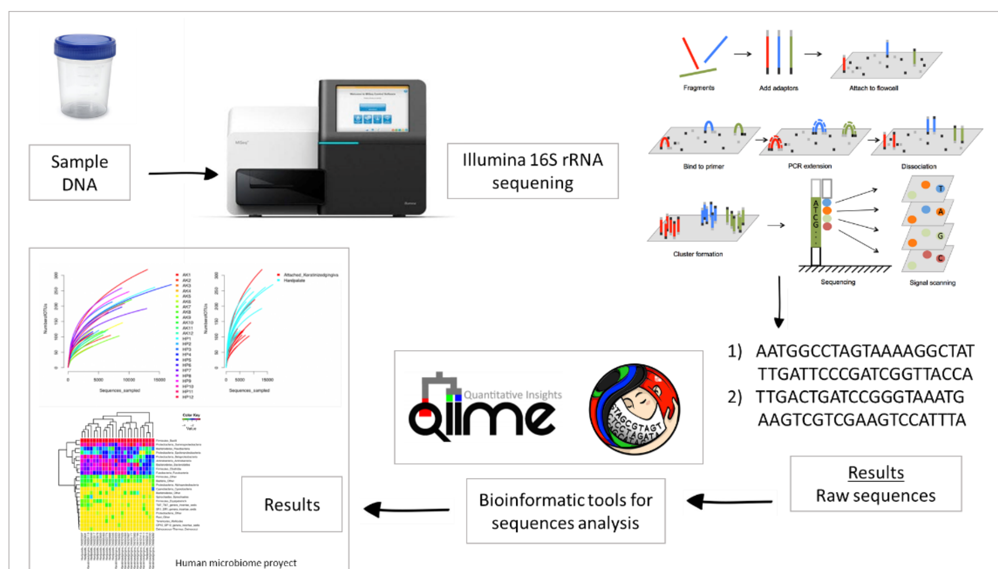



**Figure 7: Illumina sequencing method and bioinformatic analysis.**

- <u>Shotgun sequencing</u>

Whole-genome shotgun sequencing extends the 16S/18S rRNA amplicon by sequencing random fragments of the genome, allowing the reconstruction of the entire microbial genome and providing information about gene content and bacterial metabolic pathways[51]. Thus, it allows taxonomy to be accurately defined at the species level, whereas taxonomy increases to the family or genera level in 16S rRNA sequencing[52]. However, WGS is more expensive and requires more extensive data analysis[53]. In addition, the generated gene functional information represents only potential functions since it is impossible to determine whether the gene is differentially expressed under the studied conditions[36].

- <u>Transcriptome sequencing</u>

Metatranscriptomics analyses the RNA transcript pool expressed by a community. As the mere presence of a gene in a metagenome does not guarantee its expression, metatranscriptome sequencing provides information concerning the microbial processes that are active in a community. This technique allows the regulation and changes in microbial gene expression profiles to be monitored, which is particularly interesting in studies based on changes in the microbiota in response to perturbations. To date, metatranscriptomics have mainly been applied in environmental studies, whereas its application in the human microbiota is still rather limited[54].

- Metabolomics

Metabolomics is the systematic study of all small molecules within a biological system. This approach plays a key role in connecting host phenotype and microbiome function and has the advantage that metabolites and metabolic pathways are relatively conserved across species. Coupling metabolomics with metagenomics has great potential to understand host-microbial interactions and the specific functions of microbes in the community[55]. However, the expansion of metabolite databases and the development of advanced statistical algorithms is necessary to connect these heterogeneous data matrices[56].

### 2.4. Human Microbiome Project

The Human Microbiome project, an international collaborative project, was launched in 2007 with the aim of collecting and integrating genomic information from many diverse human microbiomes[57]. It was focused on a better understanding of the roles of human microbes on human biology including their relationship with health and disease[58]. Before these projects were started, several studies reported human microbiome data; however, this was the first multiple scaling project involving individuals from over the world. The project included metagenomics and 16S analysis of the microbiota inhabiting various body sites at different time points, and in several hundreds of healthy and disease-afflicted subjects. At the end, they concluded that microbes play an important role in the health of the host. In addition, the diversity and abundance of each host's microbes varied widely among healthy subjects, depending on the unique species of bacteria accumulated over a lifetime[59].

## 3. Microbiome study models

As previously described, microbial communities are complex ecosystems. The establishment of interactions between microbes, with the host and other environmental factors, such as diet, are key steps in microbial studies. To address this aim, several technical innovations have been developed to allow the study of whole gut microbial communities under physiologically relevant conditions[60]. For that reason, experimental model systems are necessary. *In vitro* models are simplified model systems that allow mechanistic studies to be performed from the absence of a complete physiological

environment, and animal models, which permit the study of the microbes in an alive ecosystem.

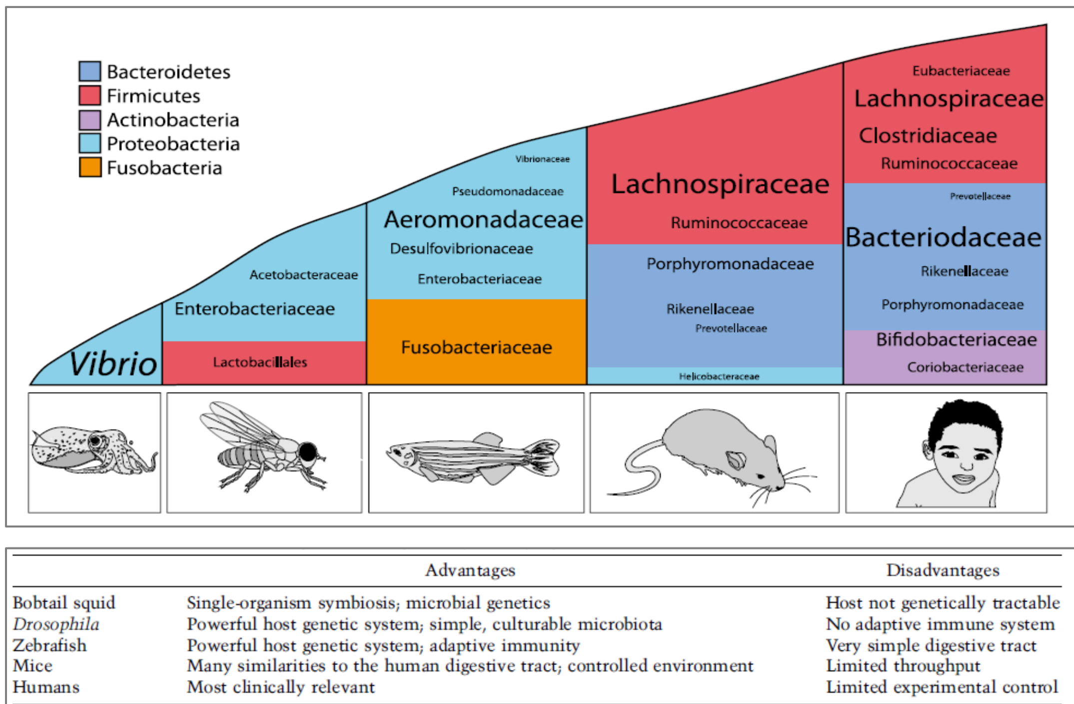
### 3.1. *In vitro*

Different *in vitro* models have been developed from the first one described by Gibson and Macfarlane in 1988[61]. They closely mimic the microbial composition and activity in different regions of the human gut. As an advantage, they are easy, cheap and highly reproducible. Moreover, it is possible to take samples during different steps and there are no ethical constrains[60]. However, they lack the ability to factor in host-specific modulators of the gut microbiota[36].

*In vitro* models can be divided into two types: luminal models and mucosal models. The first type ranges from the simplest batch fermentations to complex compartmental continuous systems. In a small reactor vessel, the gut can be approximated with a variety of cultures where desired conditions can be developed. Nutrients are constantly added as food intake and waste products are periodically removed[62]. In contrast, mucosal models are developed to study the relationship between microbes and the mucosa layer. They are an alternative for human mucosal studies regarding the difficulty and invasiveness of sampling[63].

### 3.2. *In vivo*

Preferably, human microbiome studies should be performed in human clinical trials. Unfortunately, important ethical and economic considerations certainly restrain this approach. Alternatively, model organisms provide opportunities to perturb and study the host–microbiome interplay with a level of experimental control that is not achievable in human studies[64]. As a result, several *in vivo* models are currently available depending on the pursuit, which range from simple multicellular organism, such as the cnidarian *Hydra*[65], to non-mammalian and mammalian models (Figure 8).




| | Advantages | Disadvantages |
|---|---|---|
| Bobtail squid | Single-organism symbiosis; microbial genetics | Host not genetically tractable |
| *Drosophila* | Powerful host genetic system; simple, culturable microbiota | No adaptive immune system |
| Zebrafish | Powerful host genetic system; adaptive immunity | Very simple digestive tract |
| Mice | Many similarities to the human digestive tract; controlled environment | Limited throughput |
| Humans | Most clinically relevant | Limited experimental control |

**Figure 8: The structure of the microbiota across species and a table with considerations when choosing a model organism for studying the microbiome** (Obtained from kostic *et al.*, 2013**)** [64] .

The use of animal models to study the human microbiome is possible due to gnotobiotic animals. This term refers to individuals who harbour a previously known “gnoto” microbial composition “biotic”. Gnotobiotic experiments are based on the ability to raise animals in the absence of any microorganisms (germ-free GF) and then colonise them with single specific microbial species (monoassociation) or complex consortia[66]. The concept of gnotobiotics was developed in 1896 when the first GF animals, guinea pigs, were raised by aseptic caesarean section and maintained for up to 13 days[67]. This initial report was soon followed by the successful production of GF chickens, goats, birds, amphibians and fish[68, 69]. Live gnotobiotic animals provide novel methods for the cultivation of difficult-to-grow microbes[70], offering the possibility to study host responses to gut microbiota and microbial products[71], biological processes[72], or host-microbe interactions[73].

- <u>Mammalian models</u>

Mammalian models offer the advantage of the community structure being conserved between them, almost at the phylum level. Mammals, including humans, are dominated by *Bacteroidetes* and *Firmicutes*[74]. Indeed, 99% of mouse genes are shared with humans at the host genetic level. The availability of both inbred and outbred strains and collections of knockout, knock-in, and transgenic mutants, make the murine model useful to study the host genetics. These advantages make murine models the most utilised in human microbiome studies[64]. However, within mammalian models, rats and pigs may provide a superior model of the human gut microbiome, as they are able to retain more of the original input community[75]. In addition, pig is considered the best non-human-primate model for many aspects of human gastrointestinal biology[76]. However, mammalian models are expensive to maintain in controlled environments.

There are a number of different murine models with which to study the human microbiota. Gnotobiotic mice colonised with a pure bacterial culture (monoassociation) represent the most reductionist approach for obtaining information about host-microbe specificity, the ecological niche of that particular microbe, and mechanisms of pathogenicity without competition from other species. However, it enables the study of specific factors involved during bacterial colonisation[77]. A step up in complexity, the association of GF animals with few (5-15) species, provides a more complex yet simple enough model with which to investigate host-microbe and microbe-microbe interactions. One of the first defined complex bacterial communities described was the so called altered Schaedler flora (ASF), which was primarily developed to obtain specific pathogen-free mice in breeding facilities[78]. However, the members of the ASF did not represent dominant human intestinal bacteria. For that reason, several models called SIHUMI (simplified human microbiota) were developed first in rats[79] and later in mice[80, 81], which also fulfilled some critical metabolic features. Finally, the most complex model is the human flora-associated (HFA) rodent model. This model is developed by colonising GF mice with faecal suspensions originating from human donors. Investigators had hoped that HFA rodents would mimic the microbiota of the human intestinal tract, therefore being more relevant for predicting the situation in humans. However, they encountered difficulties in achieving an exact match in microbial profiles between donor and recipient mice microbiota[31].

- Non-mammalian models

Simple animals, such as *Drosophila melanogaster*, the fruit fly, have contributed greatly to the understanding of basic cellular and developmental biology. *Drosophila* are recognised as a powerful model to study innate immunity and microbial pathogenesis. As they are simple microbiota, they form a tractable system with which to explore molecular host-microbiome interactions that may be applicable to higher-order microorganisms[82].

Zebrafish is the simplest vertebrate model with a number of features that make it an attractive experimental system. Despite the simple model complexity being distant from mammalian physiology, they are cost-effective and ethically acceptable solutions.

# 4. Zebrafish

## 4.1. General characteristics

The zebrafish (*Danio rerio*) is a small subtropical freshwater fish, belonging to the Cyprinidae family, originally described by Hamilton from the Kosi River, Bengal in 1822[83] (Figure 9). Its distribution in the wild includes rivers, small streams, channels, water ponds and paddy fields from Myanmar, Pakistan, India, Bangladesh, and Nepal with slightly alkaline (pH 8) and clear water[84]. Zebrafish presents a high adaptability to fluctuations in water physicochemical conditions, due to the climate fluctuation because of the monsoon season[85]. Zebrafish can tolerate a temperature range of 15–35°C, although the optimal temperature is around 28°C. Similarly, it can survive in water with pH values between 5.5 and 9 and tolerate salinity values between 10 to 1000 μS[86].

The lifespan of zebrafish in captivity is around two to three years, although this may be extended to over five years in ideal conditions. They are omnivorous, primarily eating zooplankton, phytoplankton, insects and insect larvae, although they can eat a variety of other foods, such as worms and small crustaceans. In zebrafish-husbandry facilities, current feeding practices involve a diet based on rotifers and dried-food pellets used for other freshwater aquarium species. The fulfilment of nutritional requirements is essential for the fish's survival during the larval stage[87]. Laboratory-reared zebrafish, unlike those in natural conditions, do not feed on the bottom of the tanks, they seek food in the water column and on the surface. For that reason, excessive food accumulation at the bottom can lead to water contamination[88].

**Figure 9: Picture of the appearance of a zebrafish adult (Michigan Science Art).**

Zebrafish are photoperiodic in their breeding, and produce embryos every morning, shortly after sunrise, by external fecundation. Under favourable conditions, females are able to spawn every two days, although the egg number produced may be variable due to stress factors[89]. Zebrafish embryonic development is very rapid: at 120 hours post-fertilisation (hpf), the fish's major organs are already functional, and hatching occurs at 2.5–3 days post-fertilisation (dpf). The embryonic stage covers from 0 to 3 dpf. This period is characterised by a rapid morphological transformation, and finishes at approximately 72 h with mouth protrusion[90]. In the juvenile state, the larva triples its length and progresses through a series of morphological changes that transform the overall body morphology into the juvenile configuration[91]. Then, larvae grow and acquire most adult characteristics, except sexual maturity. Finally, when they reach sexual maturity, they are considered adults, which normally happens at 3 months post-fertilisation[92].

### 4.2. Gastrointestinal tract

Numerous studies have shown that intestinal architecture and anatomy in cyprinid teleost fish is closely related to that in mammals[93]. Zebrafish gut tube morphogenesis finishes at 34 dpf; however, the size and appearance of the epithelial cells continue to increase until the onset of exogenous feeding (120 dpf). Three intestinal segments may be defined based upon the histological appearance of the epithelial folds and the distribution of differentiated epithelial cell types[94]. The anterior intestine, often referred as to as the intestinal bulb, has a bigger lumen than the posterior part and may therefore function as a reservoir comparable to the stomach. However, like other cyprinids, zebrafish are stomachless[95] (Figure 10).

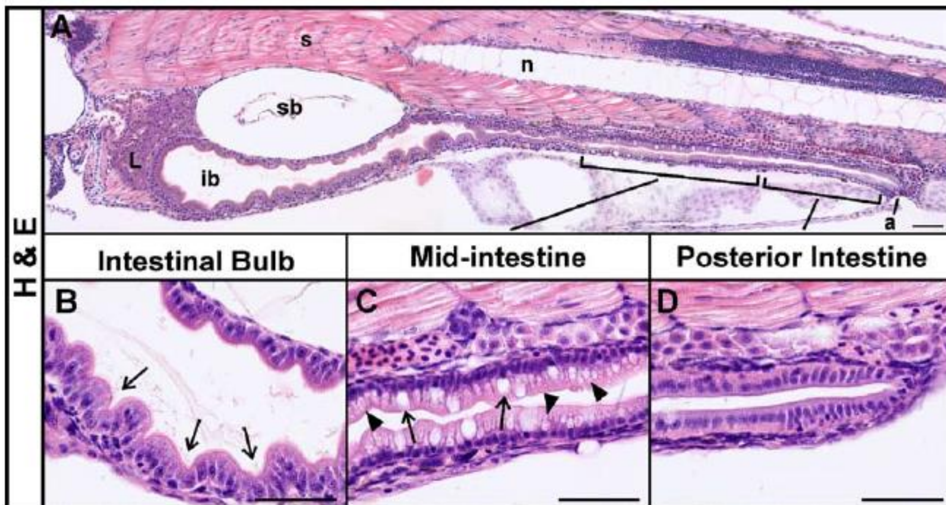



**Figure 10: Histology of zebrafish larvae (obtained from Ng *et al.*, 2005)**[96].

Illumination of the intestinal wall, which is transparent, reveals the presence of large randomly-shaped epithelial folds, resembling the finger-like intestinal villi of mammals and other amniotes. Fold height is smaller in the mid than in the anterior intestine. Many folds are oriented circumferentially, whereas a significant percentage of them are randomly organised. Histological sections through the anterior, mid and posterior intestinal segments reveal, as shown in Figure 11, that the folds are comprised of a single layer of epithelium that rests upon a connective tissue core similar to the lamina propia of the mammalian small intestine[97].

Epithelial cells within the intestine are joined apically through a complex set of junctional complexes (tight junctions, adherent junctions and desmosomes)[96]. These complexes act as a barrier, restricting the movement of membrane components between the apical and basolateral cellular domains. Three of the four principal cell types within the mammalian small intestinal epithelium, columnar-shaped absorptive enterocytes, the most numerous, goblet cells and enteroendocrine cells, are present within the anterior zebrafish intestine[98] (Figure 11). The terminal region of the mild intestine is comprised of specialised enterocytes that appear to play a role in mucosal immunity. This region may be analogous to the mammalian ileum, where antigen presenting cells (M cells) and submucosal lymphoid aggregates, known as Peyer's patches, are located[99, 100]. The short posterior segment architecture and absence of absorptive enterocytes, suggest that this region may be analogous to the colon of higher vertebrates[97].

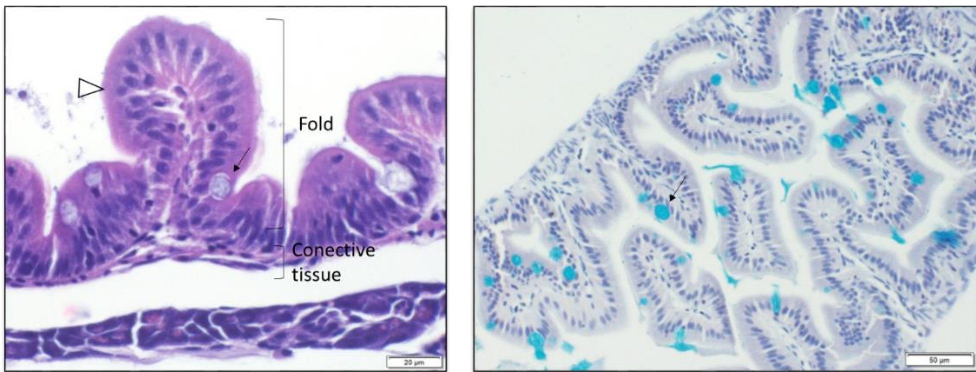



**Figure 11: Histological cross section of zebrafish mid intestine.** Intestinal folds are represented by enterocytes (triangle) and goblet cells (arrows). (Picture from Azti, non-published).

Apart from the morphological resemblance to mammals, the zebrafish intestine seems to also share metabolic functions. The second part of the zebrafish intestine closely resembles the caecum and rectum of the human large intestine in glycolysis, oxidoreductase activity, and the metabolism of amino acids, amine derivatives, organic acids, carboxylic acids and alcohol, whereas the third part of the zebrafish intestine resembles the human rectum, where only the metabolism of membrane lipids is enriched. Moreover, aquaporin genes are highly expressed in both sections[101].

### 4.3. Immune system

Adult zebrafish have both innate and adaptive branches of the immune system, similar to that seen in mammals or other jawed vertebrates. It was shown that the zebrafish thymus, as a primary immune organ, remains rudimentary throughout the early larval stage and only acquires a complex shape from 4 weeks post-fertilisation (wpf)[102]. Thus, zebrafish appear to be incapable of mounting an antibody response until early adulthood, 4 wpf, before which they rely exclusively on innate defence mechanisms[103]. The late activation of the adaptive immune system allows the study of the innate immune system in zebrafish embryo or larvae without any interference from adaptive immunity. Zebrafish haematopoiesis produces most if not all immune mammalian differentiated cell types, including, leucocytes, macrophages, neutrophils and granulocytes[104]. As in mammals, the development of immune cells during embryogenesis occurs in distinct waves of primitive and definitive haematopoiesis[105]. The innate immune functions provide a robust defence, beginning early in development. Macrophages and

neutrophils can be recruited to the site of infection and phagocytose invading bacteria as early as 30 hpf and 52 hpf, respectively[106, 107].

The first line of defence in the larval zebrafish gut is formed by the anti-microbial peptides (AMPs) produced by the intestinal epithelial cells. AMPs are potent, broad spectrum antimicrobials that can kill Gram-negative and Gram-positive bacteria, enveloped viruses, fungi and even transformed or cancerous cells[108]. Multiple defensin-like genes encoding for an important AMP family have been discovered in zebrafish, which resemble beta-defensin family members of birds and mammals[109]. The elevated expression of beta-like defensin is found in the mid-intestine. Most immune cells and vacuolated (M-like) cells reside in this region, indicating its role in immune function[110]. Additional potent mechanisms of the innate immune system in zebrafish are reactive oxygen species (ROS), which mediate the killing of pathogens, and intestinal alkaline phosphatases (IAP), enzymes which are abundant in the brush border of the intestinal epithelial cells which dephosphorylate and detoxify the endotoxin component of bacterial lipopolysaccharide (LPS)[111]. In addition, several homologues of pattern recognition receptors (PRR) have been found in zebrafish[112], such as TLRs. These receptors are located on the cell surface, on endosomal compartments and in the cytosol, and are able to sense the presence of pathogens or commensal microbiota by recognising MAMP[113]. The triggering of the innate immune response in zebrafish results in the transcriptional induction of well-conserved transcription factors, such as members of the adaptor protein MyD88[114] intracellular pattern recognition receptor NOD2, or several cytokines and chemokines, whose function is conserved between zebrafish and mammals[110]. Some of these cytokines are NF-κB[115], TNF[116] or IL-1β[117] (Figure 12). This species also has an active complement system with three activation routes: the classical, alternative, and lectin pathways[118].

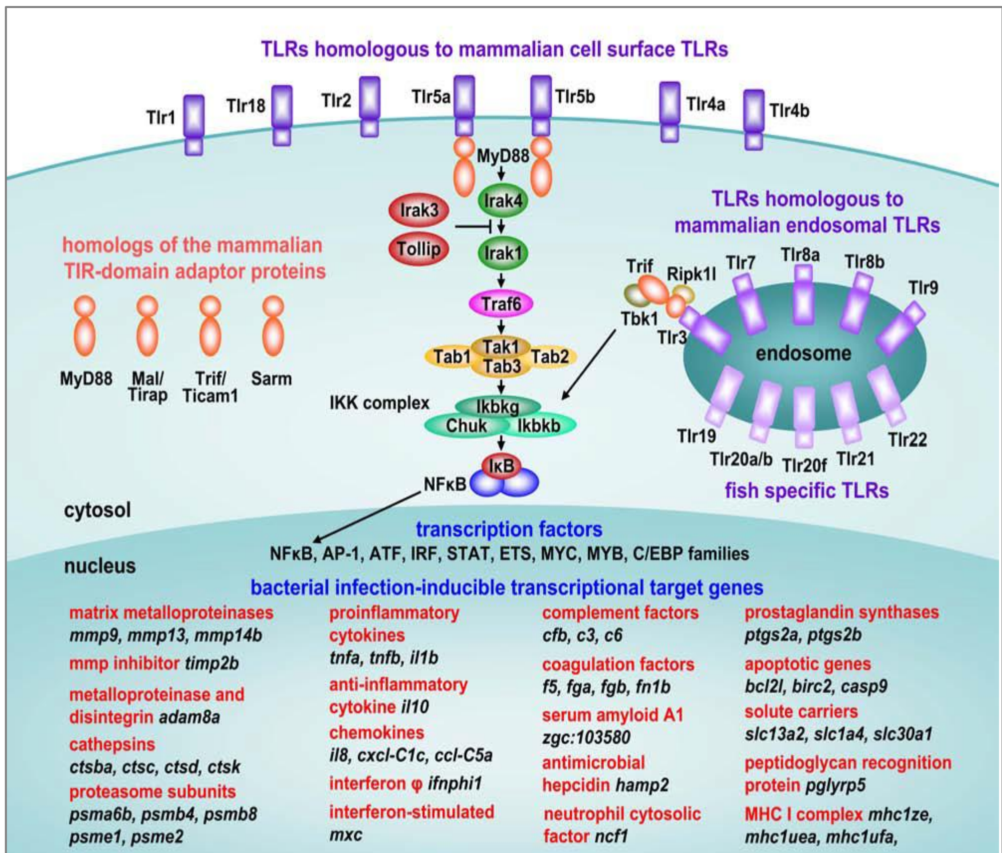



**Figure 12: Components of the TLR pathway and genes commonly induced during the innate immune response of zebrafish to bacterial infection** (Obtained from Meijer, 2011) [112].

## 4.4. Microbiota development

The epithelial surfaces of zebrafish and all other vertebrates are colonised at birth by large communities of microorganisms that form commensal or mutualistic relationship with their host[6]. Host responses to microbial encounters frequently involve communications between multiple host cell lineages and tissues. Teleost possess physiological and immunological features common to all vertebrates as well as a complex gut microbiota[97].

Zebrafish larvae hatch from the axenic environment within their protective chorion around 3dpf, when the intestine and other surfaces are colonised by microbes within 12-24 h [119, 120]. In much the same way as mammalian newborns are first colonised by microbes at birth, fish initially acquire their gut microbes from the environment upon opening of their digestive tract, which typically occurs a couple of days after hatching. Domesticated lab-reared zebrafish develop a gut microbiota similar to that of zebrafish born in their natural habitat[121]. The

community composition of larval zebrafish is more similar to communities associated with the surrounding environment than is adult zebrafish, indicating a greater role of environmental exposure early in development[122]. This is consistent with observed correlations in humans between birth delivery mode and the composition of the intestinal microbiota[123].

Zebrafish gut microbiota is primarily represented by Proteobacteria, Fusobacteria and Bacteroidetes bacterial groups at all stages of the zebrafish life cycle, representing up to 90% of the zebrafish intestinal microbiota, as reported in other fish species[124]. This core gut microbiota includes the genera *Aeromonas*, *Shewanella*, *Pseudomonas*, Stenotrophomonas, *Vibrio*, *Burkholderia*, *DIAPhorobacter*, *Cetobacterium*, *Streptococcus*, *Bacillus*, *Cloacibacterium* and *Propionibacterium*[125]. Among the mentioned bacterial genera taxa, Cantas *et al.* isolated and identified 13 cultivable species from the zebrafish adult intestine, such as *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *Photobacterium damselae* or *Pseudomonas fluorescens*[126]. This is in marked contrast to the mammalian gut microbiota, which is dominated by members of the Fimicutes and Bacteroidetes phyla[74]. Nonetheless, the zebrafish microbiota, although differing from the mammalian microbiota in terms of dominant phyla, induces a very conserved host response during colonisation and development[71], modulates host metabolism, contributes to the normal intestinal development and function[41], [30] and is also influenced by diet[127]. It has also been shown that the composition of zebrafish microbiota, like in mice, can convey disease susceptibility in a model of intestinal inflammation, emphasising the usefulness of zebrafish as a model to study host microbe interactions in health and disease[128].

### 4.5. The use of the zebrafish as an animal model

The establishment of zebrafish as a prominent model organism was largely driven by the desire of developmental biologists for a genetically tractable system for understanding early vertebrate embryogenesis. The pioneering studies using zebrafish embryos were largely focused on the genetic analyses of early development and organogenesis during the first 3 dpf[129]. Now, the zebrafish species have been fully characterised morphologically and physiologically, and the genome is fully sequenced and available[130]. Zebrafish share high genetic homology with humans, and doffers a cost-effective and ethical solution for many biomedical studies. According to the EU directive 2010/63/EU, zebrafish embryos are considered to be an alternative animal model, so their use is not restricted by regulation for animal welfare[131].

Zebrafish has a number of features that makes it an attractive research tool. It shares a considerable amount of homologies with humans, at the cell, tissue, organ and system levels[132].

A single cross produces a large number of offspring, which develop rapidly. The facilities are simple and highly controlled, where the environment can be easily sampled and manipulated[9], permitting statistically robust studies[133]. Additionally, zebrafish is relatively inexpensive to maintain, reducing the time and cost of carrying out *in vivo* investigations[132]. The transparency of embryos and larvae allowed the first successful examination of the colonisation dynamics of bacteria within live, developing hosts[119]. The ability to genetically manipulate both host cells and microbes to express fluorescent proteins allows for real-time non-destructive observations of spatial and temporal variation in host-microbe interactions in developing zebrafish[134]. Its powerful lifespan (1-2 years) allows the evolution of the relationship between the host and its microbiota to be studied[135].

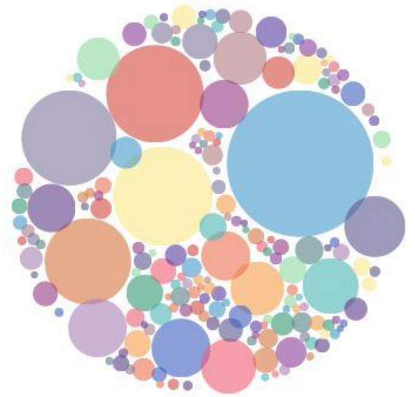
Gnotobiotic techniques are straightforward in fishes since they develop *ex utero* and eggs can be surface-sterilised shortly after fertilisation[136]. Gnotobiotic studies in teleost, as in mammals, allow for the documentation of a broad array of host responses to gut microbiota; however, zebrafish have revealed advantages over mice in identifying microbial signalling pathways influencing development[9]. Nevertheless, while multi-generational gnotobiotic lines are able to be maintained in mice, this is not yet possible in fishes.

The zebrafish gnotobiotic model has been widely use in mono- or simple associations (2 bacteria) to study the mechanisms of pathogen infections[134] or the interactions between pathogens and probiotics[137]. In contrast, even though many attempts to colonise zebrafish with human complex associations have been performed, no clear result has yet been reported. Rawls *et al*. tested the effect of habitat in assembling a community in 2006. They reciprocally colonised axenic zebrafish and mice, and reported that the zebrafish colonised with mice microbiota changed in the proportions of divisions from the mouse input, calling this phenomena "teleostification"[9]. It was predicted that different habitats might influence the microbial assembly, preventing the growth of anaerobic bacteria inside the zebrafish digestive tract. In another attempt, Toh *et al*. colonised zebrafish larvae with a defined community of human gut-derived bacterial strains in 2013. This community was based on 5 obligate and 25 facultative anaerobes. At 3 different time points, they only recovered 2 strains out of 30, all of which were facultative anaerobes. This result might be explained by the inability for selected microbes to adapt to oxygen exposure and the simplicity of the introduced community.

## 4.6. Zebrafish to study the impact of dietary fat on microbiome

Research in zebrafish as a model to study the impact of dietary nutrients has been widely extended. There are several studies which have investigated the effect of proteins, gluten, or immunostimulants[138, 139], on the intestinal microbiome[140, 141] and immune system in zebrafish. Soybean meal-based diets have been deeply studied in aquaculture species and zebrafish, as it is widely use as protein source in aquaculture nutrition. Many studies have demonstrated that soybean meal induces intestinal inflammation in zebrafish[144, 145] and also changes intestinal microbiota in aquaculture species[142]. In addition, zebrafish has recently gained popularity in lipid research because its lipid absorption and metabolism share remarkable similarities with mammals[143] and the microbiota is also involved in both actions[144]. There have been many studies focused on the beneficial effect of bioactive compound such as Yuzu peel or green tea on lipid accumulation and metabolism, as a screening for human nutrition[145, 146]. In contrast, few studies have used zebrafish to study the impact of dietary fat on the intestinal microbiome. As a result, it is now clear that high-fat diets (HFD) led to variations in fish environment and on the intestinal microbiome of zebrafish at larvae and adult states[121, 147] . In contrast, a high-cholesterol diet (HCD) did not changed the microbial community composition of zebrafish larvae despite it induced inflammation[148]. Though there are few studies focused on the study of the effect of dietary fat on the zebrafish microbiome, immunity and body fat accumulation the relationship is not clear yet.

# Hypothesis and objectives



In the last 10 years, the technological revolution and advances in sequencing techniques have allowed the development of new technologies for the study of complex microbial communities. Therefore, not only has the study of the composition of the human flora advanced, but many diseases have also been related to changes in the microbiota, such as Alzheimer's disease or intestinal bowel disease. In addition, the characterisation of the impact of diet on the microbiota and host's health has been established.

Studies performed in humans are very expensive, ethically reprehensible and difficult to achieve. Thanks to humanised rodent models, many advances have been accomplished, but they are expensive to maintain. In this context, simple animal models such as zebrafish, where all of these disadvantages become advantages, are desirable. Nevertheless, to date, a humanised zebrafish model has not been described.

With this concern, the hypothesis of this study is that zebrafish might be a viable model to study the human intestinal microbiota and its relationship with diet.

To demonstrate this hypothesis, the following specific objectives are proposed:

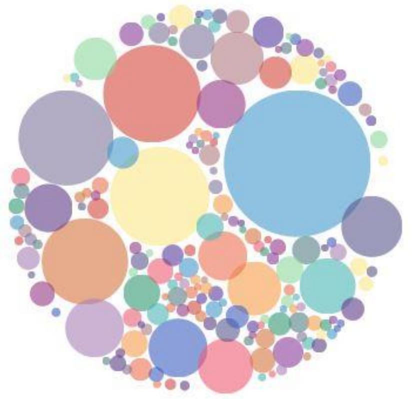
1. To establish a colonisation protocol using obligate anaerobe bacterial strains in order to test their ability to colonise axenic zebrafish larvae intestine tract.

2. To develop a humanised zebrafish model with human-derived microbiota previously extracted from a healthy donor to colonise axenic larvae.

3. To study the effect of a high-fat diet administration in zebrafish larvae, during the period of 3 to 30 dpf, on the intestinal microbiome and the immune system.

4. To study the effect of a high-fat diet and a high-fat diet supplemented with a commercial fish oil on the zebrafish adult intestinal microbiome, the immune system, lipid profile and predictive metabolic functions.

# Results



# Zebrafish gut as a house for human intestinal bacteria

**PUBLICATION** | **Arias-Jayo, N., Ramirez-Garcia, A.; Pardo M.A. Zebrafish gut as a house for human intestinal bacteria. JISM, 2017,3.**

**CONFERENCE**

*Gut microbiology*
Poster and oral communication. "Zebrafish gut as a house for human intestinal bacteria" Arias, N., Ramirez-Garcia, A.; Pardo M.A. (France 2016)

*World congress on targeting microbiota*
Oral communication. "Zebrafish gut as a house for human intestinal bacteria" Arias, N., Ramirez-Garcia, A.; Pardo M.A. (France 2016)

## 1. Abstract

In this chapter, germ-free (GF) zebrafish larvae intestine was colonised with five bacterial species, members of the human gut microbiome, including obligate and facultative anaerobes (*Escherichia coli, Enterococcus faecalis, Bifidobacterium breve, Lactobacillus casei* and *Eubacterium limosum*). Larvae were infected with each bacterium (monoassociation) and with all bacteria (consortia), and the colonisation was monitored by culturing on different media, by q-PCR analysis and metabarcoding. After the infection, all the strains remain inside the zebrafish gut almost during 48 hours post infection (hpi). This suggests that the developing zebrafish could be a suitable model for studying certain species of the human gut microbiota.

## 2. Introduction

The human gut houses a vast microbial community that is vital for maintaining host health[149]. Considering the human body as an environment and the human microbiota the entire collection of microorganisms living on the surface and inside our body[42] humans are called as "superorganisms"[30]. Consequently, we have two genomes, one inherited from our parents and the other acquired, i.e., the microbiome.

The intestinal microbiome thrives in a nutrient rich and thermostable environment and provides the host with metabolic nutrition, the facilitation of energy extraction, the competitive exclusion of pathogenic microorganisms and many other beneficial functions[150]. The gut resident microbes are crucial for normal immune development and homeostasis, as well as regulatory effects on epithelial growth, differentiation and cytoprotection, thus exemplifying a balanced symbiotic relationship between the host and its resident bacterial flora. Compositional perturbations of the microbiota (dysbiosis) have been associated with diseases, as described in the introduction section, and allergies. Hence, maintaining compositional and functional stability within the gut microbiome is essential to host health, as demonstrated by dysbiosis detected at the onset of nonpathogenic chronic diseases[36].

The complexity and the high inter-individual variability of the human gut microbiota are inherent problems in the study of host-microbe interactions. GF animals offer the opportunity to circumvent some problems. For that reason, it would be desirable to have a simple animal model to study the interactions between the gut microbiota and the host[9].

Zebrafish has become a popular new model organism for biomedical research, due to their physiological and genetic homology to mammals, high sensitivity to environmental modifications, and ease of experimental (behavioral, genetic and pharmacological) manipulation. In addition, its genome is fully sequenced and available, they have quick reproduction, potential for high-throughput screening, low cost, and at larvae state they are optically transparent[132, 151].

# 3. Materials and methods

## 3.1. Zebrafish husbandry

Zebrafish embryos were obtained from wild-type adult zebrafish (*D. rerio*, Hamilton 1822) bred in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain) following standard conditions. fishes were maintained at 27°C in 60 l tanks, with aerated freshwater; according to standard protocols[152]. Fish were fed with a pellet-formulated diet (Gemma Micro 300; Skretting) and reared on a 12 h light/12 h dark cycle.

Convencionalized zebrafish larvae (monoassociation and consortia) were maintained under gnotobiotic conditions with autoclaved diet (ZF biolabs) three times per day[136] and one medium change, for up to 10 (dpf).

All experimental procedures were approved by the regional animal welfare body (NEIKER-OEBA-2015-005).

## 3.2. Procedure for obtaining germ-free larvae

Zebrafish embryos were collected directly from the breeding tanks immediately after fertilization and germ-free larvae were obtained following a well-established protocol[153]. Briefly, embryos were washed with a sterilized embryo wash buffer (EWB) solution (embryo water (EW): CaCl2 294 mg/ml, MgSO4 7H2O at 123.3 mg/ml, NaHCO3 at 63 mg/ml, KCl at 5.5 mg/ml and supplemented with methylene blue 0.01% (w/v)), antibiotic solution (AB) (kanamycin 15 µg/ml, ampicillin 300 µg/ml and amphotericin B 1.25 µg/ml), 0.02% (w/v) polyvinylpyrrolidone (PVP) solution for 2 min, 0.003% (v/v) bleach solution for 1 h and finally with EWB solution. Afterwards, the embryos were incubated overnight in AB solution. The following day 50 embryos were collected and transferred to a Petri plate (5.5 cm diameter×1.0 cm) to be immersed in 5 ml EWB solution and treated with two UV light pulses of 1.6 kV (Pulsed UV System XeMatica 1:2L-SA, SteriBeam Systems, GmbH) to inactivate bacteria present in the

sample. The entire procedure was carried out inside a laminar flow cabinet to maintain sterile conditions; sterile solutions and materials were also used.

Sterility was routinely tested after 96 hpf (hours post fertilization), by culturing on general aerobic and yeast and molds culture media (Petrifilm aerobic, and Petrifilm yeast and molds count plates, 3M) and by PCR amplification using primers targeting 16S ribosomal RNA gene (63f: CAGGCCTAACAGATGCAAGTC and 1387r: GGGCGGWGTGTACAAGGC)[154].

### 3.3. Germ-free larvae colonisation

GF zebrafish laevae were colonised at 5dpf with desired bacteria (*E. coli, E. faecalis, B. breve, L. casei and E. limosum*) separately (monoassociation) and in association (consortia), inoculating each pure culture into the surrounding water at a final density of $10^7$ - $10^8$ CFU/ml.

### 3.4. Colonisation confirmation

Colonisation success was tested at different time points (24, 48, 72 hpi, 5 and 7 dpi). Three pools of five larvae were taken at each time point, euthanized with tricaine methanesulfonate (MS-222, Aldrich), washed and homogenized in pre-reduced Buffered Peptone Water, supplemented with cysteine (0.25 g/l) and K1 vitamin (0.001‰) (Oxoid). Serial dilutions were spotted onto Wilkins Chalgren general media; to MacConkey, KF, MRS (Oxoid) and Bifidobacterium agar (BD), as specific culture media in order to count and confirm each colony. Media were incubated at 37°C in anaerobiosis during 48 h. All the procedures, manipulation and incubations carried out in this section were performed inside *in vivo* & microaerophilic workstation (Ruskinn technology) maintaining anaerobic conditions. The identification of each suspected *E. limosum* colony was performed by qPCR (LightCycler 480 Instrument II, Roche), because there was no specific medium for it. ELIM-F (5´-GACTTAGGCGCAGAAAAATTCC) and ELIM-R (5´-CAAACCAGCCATACGGCATT). The PCR conditions consisted of initial activation for 10 min at 95°C, followed by 45 cycles (15 s at 95°C and 30 s at 60°C) and cooling at 40°C for 30 s.

Furthermore, total genomic DNA was extracted from 3 pools of five larvae following the manufacturer's instructions (QIAamp DNA mini kit, Qiagen) at 48 hpi and the microbial composition was characterized by Illumina sequencing of 16S rRNA gene amplicon (Biopolis, Valencia, Spain). The microbial community composition was characterized by sequencing the V3-V4 16S rRNA region using the primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21[155]

in an Illumina Miseq Platform. Paired reads were merged using flash[156] after removing primer sequences with Trimmomatic. The rest of the bioinformatic analysis was performed using the mothur platform[49] (1.37.2). The merged reads were quality trimmed (with a minimum Phred average quality score of 35 over a 50 bp window) and aligned against a reference SILVA alignment, keeping only those positions that start and stop in the same alignment space. The resulting sequences (median length of 414 bp) were denoised by a pre-clustering method allowing 1 mismatch[157] as recommended[158], and chimeras were removed using Uchime. For operational taxonomic unit (OTU) construction, reads were clustered at the genetic distance cut-offs 0.01 and 0.03 substitution per nucleotide, using the average linkage method. The final OTU table was rarefied to a depth of 3036 sequences per sample and results are shown to 97% sequence similarity. Graphics were drawn in excel.

## 4. Results and discussion

Using this protocol, all the strains included in the study were identified inside the zebrafish gut for 48 hpi (Figure 13), whereas previous studies only found two species in very low concentrations[149].

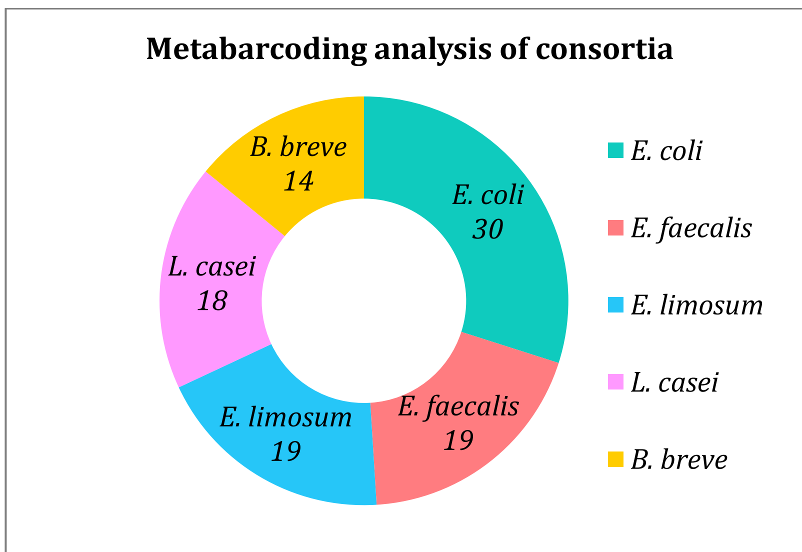



**Figure 13: E. *coli, E. faecalis, L. casei, B. breve* and *E. limosum* percentage contribution in consortium at 48 hpi by 16S sequencing.**

These strains were selected because they are commensal bacteria in the human intestine, belong to different taxonomic filum and classes, and they are facultative and obligate anaerobes. *E. limosum* was included because Toh *et al.* introduced this strain in their experiment, and was

the only specie they found, together with *Lactobacillus paracasei*, inside the zebrafish gut 72 hpi[149].

On the one hand, facultative anaerobes are able to survive up to 10 days, but obligate anaerobes only survive 48 h (Figure 14). This could be due to the oxygen pressure that makes possible facultative anaerobes growth, limiting obligate anaerobes survival[149]. At the beginning, obligate anaerobes might be able to survive in low/restricted oxygen conditions, but when facultative anaerobes colonise and grow obligate anaerobes die out because of the interspecific competition for the niche and resources[9].

Furthermore, these kinds of bacteria are not commonly found in the zebrafish gut, so they could find difficulties to establish themselves and grow.

In monoassociations, in general, higher counts for all species used were monitorized. This could be due to the absence of interspecific competition, as well as the metabolism derived from the bacterial growth.

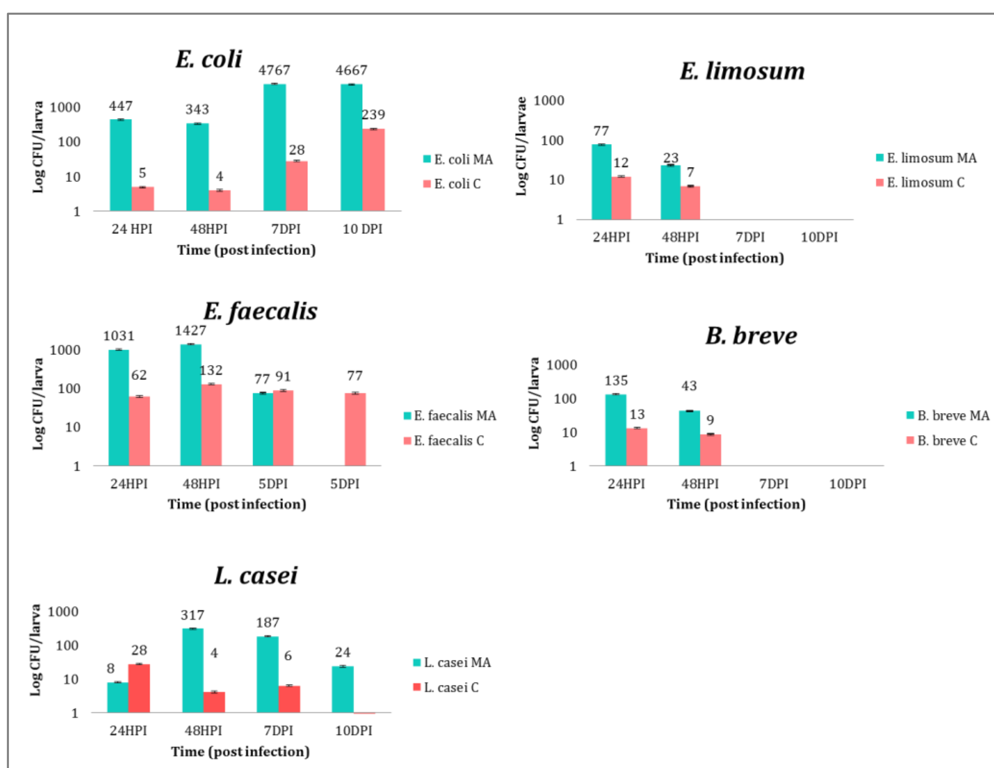



**Figure 14: *E. coli, E. faecalis, L. casei, B. breve* and *E. limosum* concentration inside zebrafish intestine (CFU/larvae), for monoassociation experiment (green) and for consortium (orange).**

Therefore, we conclude that different human microbiota derived bacteria, including some obligate anaerobes, are able to colonise the zebrafish gut. This data suggests that using this method, working in anaerobiosis, to colonise the developing zebrafish could be a suitable model for studying the human gut microbiota, how to model it, the interactions with the host, and related diseases, as a result, diabetes, obesity or intestinal bowel disease.

In the near future, we will use this protocol to, elucidate if zebrafish could be used as a model to study the whole human intestinal microbiota.

# Zebrafish axenic larvae colonisation with human intestinal microbiota

**PUBLICATION**

**Arias-Jayo, N., Alonso-Saez L., Ramirez-Garcia, A.; Pardo M.A.**
**Zebrafish axenic Larvae Colonisation with Human Intestinal Microbiota. Zebrafish, 2018,3.**

**CONFERENCE**

*SEGHNP*
Poster and oral communication. "Modelo para el estudio de la microbiota intestinal humana utilizando el Pez cebra (*Danio rerio*)" Rodriguez, A., Arias, N., Ramirez-Garcia, A., Martin, G., Pardo, M.A. (Donostia 2017)

## 1. Abstract

In this chapter, we took a step further to identify for the first time the bacterial phylotypes from human intestinal microbiota able to colonise the zebrafish larvae digestive tract. Five days post-fertilisation (dpf) germ-free (GF) zebrafish larvae were colonised successfully with the human intestinal microbiota previously extracted from a donor. The composition of the transferred microbial communities was analyzed by high-throughput sequencing. As a result, species with relevant interest because of their linkage to dysbiosis occurred in different human diseases, such as, *Akkermansia muciniphila, Eubacterium rectale, Faecalibacterium prausnitzii, Prevotella spp.* or *Roseburia spp*. were successfully transferred inside the zebrafish gut. This information has a remarkable interest in order to establish a simplified-human microbiota (SIHUMI) model in zebrafish.

## 2. Introduction

The human microbiota lives in a close relationship with their host, forming a complex and dynamic ecosystem, which has a large impact on several aspects of our physiology, promoting host health and fitness. The gut microbiome encodes 10 million microbial genes, 400-fold more than the human gene complement. For that reason, the gut microbiota is widely recognized as an active and integral organ of the human body, which provides necessary functions to our life[159]. Host-associated microbiota is generally very diverse and differs from microbial communities in the external environment, but its composition is driven by poorly understood processes [7].

Some of the forces that shape the composition of the gut microbiota may include stochastic processes such as genetic diversification, ecological drift, or deterministic interactions between species, individuals, and the environment, which create defined niches. The host also determines in diverse ways the gut environment. For example, the immune system can act as an environmental filter to limit or expand the available niches. However, the abundance and types of nutrients acquired from the diet, including secreted molecules into the gut by the host, are thought to largely determine the niche space in the gut[160]. Furthermore, the gut microbiota composition varies with developmental age within an individual, as well as among the individuals of the same species[2].

Currently, there is a strong interest in studying the microbiota composition in the human gut[42,10], the interactions with the host, the dietary effects[28] and the relationship between the

microbiota dysbiosis and diseases or well-being[161,12]. Moreover, over the past decade, novel technological advances have facilitated a better understanding of the host-microbe interactome pathways that affect human health, diseases, and aging. These approaches include high-throughput DNA sequencing[36] or bioinformatic tools and gnotobiotic animal models[162,150]. Among the studies using animal models, Du *et al.*[163] used human microbiota extracted from patients with intestinal bowel disease to colonise germ-free mice and study the changes in microbial composition during the disease. Chung *et al.*[164] also used a gnotobiotic mice model colonised with human gut microbiota to study the gut immune maturation. Furthermore, a few other studies have used SIHUMI to colonise germ-free mice[165] and rats[79] to study the dietary regulation of the microbiota [166] or the effect of pathogens[80].

To date, several models of GF zebrafish have been colonised with human pathogens[167], probiotics, or a mixture of them[168]. However, as mentioned in the introduction section, there are no reports of any SIHUMI model using zebrafish, nor a gnotobiotic model colonised with the total human microbiota. Currently, there is only one study published describing the colonisation of GF zebrafish with human derived bacteria, where only a minor number of species where successfully transferred (2 out of 30 species)[149]. Performing the same type of experiment, we succeeded in transferring five out of five bacteria species, commonly found in human gut, including three obligate anaerobes, being all of them maintained during 48 h inside the zebrafish digestive tract[169].

# 3. Materials and methods

## 3.1. Zebrafish husbandry and germ-free larvae

Zebrafish embryos were obtained from wild-type adult zebrafish bred in the AZTI Zebrafish Facility following the standard conditions and procedures described in the previous chapter. Embryos were collected directly from the breeding tanks immediately after fertilization and GF larvae were obtained following a well-established protocol aforementioned. All experimental procedures were approved by the regional animal welfare body (NEIKER-OEBA-2015-005).

## 3.2. Human sample collection and stock preparation

A human stool sample was aseptically collected from a healthy donor according to a sampling protocol approved by the Regional Health Research Ethics Committee (NEIKER-OEBA-2015-005), and processed in less than 1 h. Ten g of stool were weighted and

homogenized in 100 mL of Brain Heart Infusion (Oxoid) supplemented with 0.5% (w/v) yeast extract (Pronadisa) + 0.0005% (w/v) hemin (Fluka) + 0.0001% (w/v) K1 vitamin (Fluka) + 0.005% (w/v) cysteine (Sigma) 60 s in a Stomacher (Masticator IUL Instrument). The slurry was then passed through 300, 250 and 150 µm stainless steel laboratory sieves to remove big particles[170], aliquoted, mixed with 20% glycerol (Sigma-Aldrich) and kept at -80◦C until use.

All the procedure was carried out inside an anaerobic chamber (invivo & microaerophilic workstation, Ruskinn technology) maintaining anaerobic conditions.

### 3.3. Germ-free larvae colonisation

Five dpf GF larvae were colonised with the human intestinal microbiota by static immersion following the infection protocol previously established in the first chapter with some modifications[169]. Briefly, one aliquot of the human fecal slurry was thawed at 4°C and inoculated into previously oxygen reduced PBS (1X) (Fisher) medium supplemented with 0.01% (w/v) K1 vitamin (sigma), 0.05% cysteine (Sigma) and 0.05% (w/v) yeast extract (Oxoid) (rPBS+) at a final density of $10^5$ CFU/mL, inside an anaerobic chamber. Then, outside in aerobic conditions, it was poured into a petri dish containing zebrafish larvae inside a laminar flow cabinet.

After 24 h incubating at 27◦C shaking at 50 rpm (Heidolph unimax 1010), the whole medium was removed, and larvae were washed with clean rPBS+ medium twice. Finally, the slurry was added again at a final density of $10^4$ CFU/ml in fresh medium. Larvae were further incubated for 24 h. Afterword they were washed with sterile rPBS+, passed into a new petri dish and maintained for 6 h in fresh rPBS+ medium. To pool all samples, larvae were washed twice in a Tween 20 0.1% (Merck) baths and after that twice in fresh sterile PBS (1X) to remove bacteria attached on the skin[137]. Finally, larvae were collected in 6 pools of 20 individuals (Figure 15). GF and media controls were not taken.

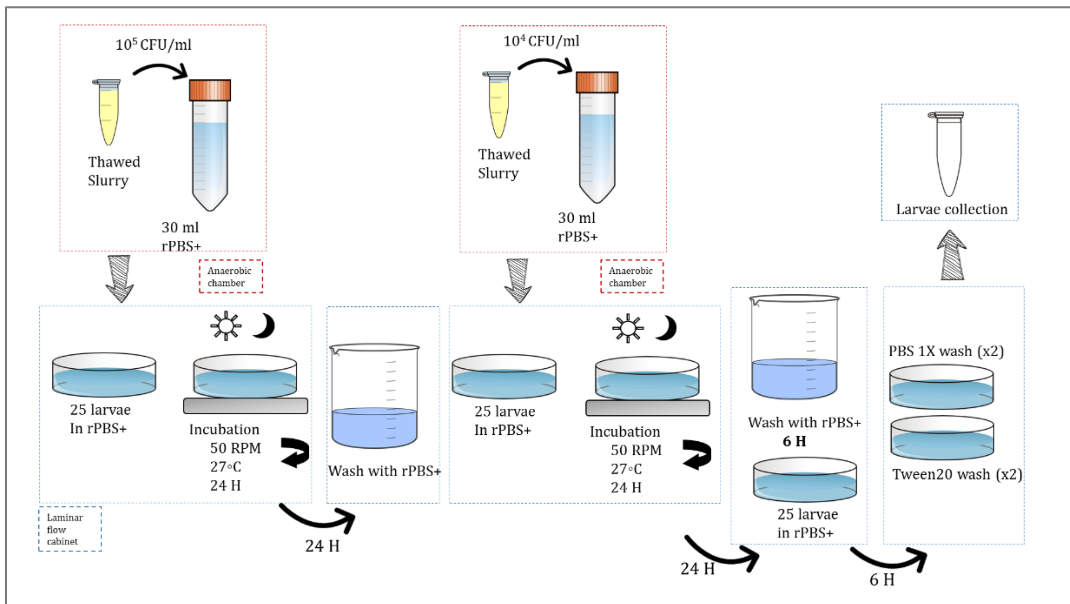



**Figure 15. Schematic overview of colonisation procedure.**

The toxicity of the medium, was tested as well, comparing the larvae mortality and the swimming behavior in the presence of rPBS+ medium with larvae in PBS shaking at 50 rpm for 5 days. To determine the optimal bacterial concentration of the slurry for the colonisation 4 replicates of 30 five dpf germ-free larvae were exposed to different slurry concentration: $10^3$, $10^4$, $10^5$ and $10^6$ CFU/ml following the protocol explained above during 72 h. The larvae were not fed during this experiment.

### 3.4. Analysis of community composition of microbiomes by metabarcoding

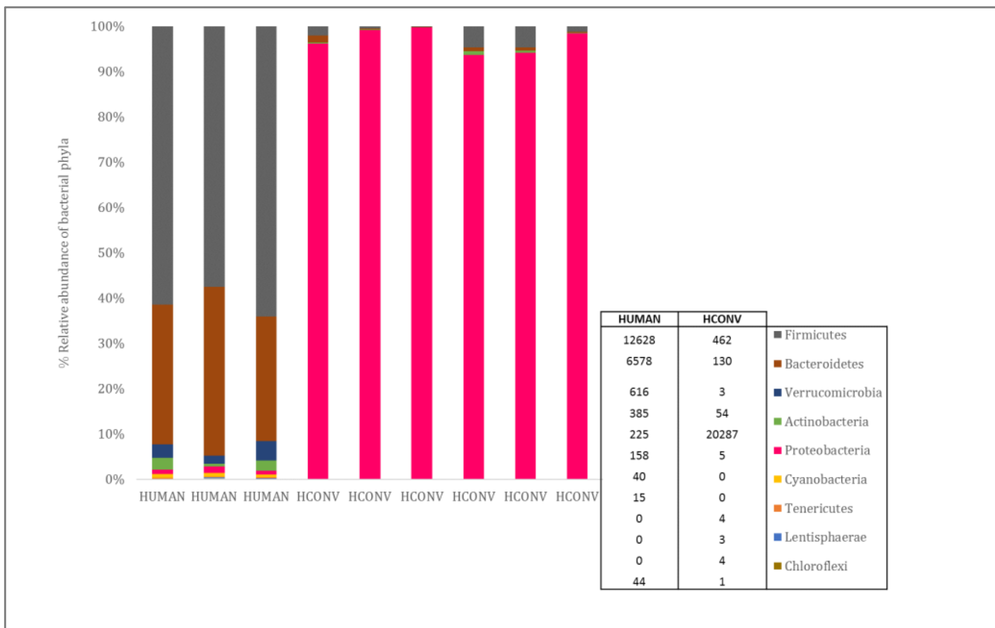
Total gDNA was extracted from 6 pools of 20 human conventionalized larvae (HCONV), from the human feces (HUMAN) in triplicate (QIAamp DNA mini kit, Qiagen) and quantified using a Nanodrop 1000 spectrophotometer (Thermo). The microbial community composition was characterized as in chapter 1. For OTU construction, reads were clustered at the genetic distance cut-offs 0.01 substitution per nucleotide, using the average linkage method. Taxonomic assignment of the OTUs was obtained by classification with SILVA taxonomy (version 128) using the Wang approach[171] and in some cases by BLAST (Basic Local Alignment Tool – NCBI) comparing the most representative sequence from the rep. file from Mothur with the database. Only OTUs identify in at least half of the HCONV samples were taken into account; however, in HUMAN samples all OTUs were considered.

## 4. Results

During the first 24 h at least 90% of zebrafish larvae survived up to $10^5$ CFU/ml slurry concentration; however, at $10^6$ CFU/mL, less than 50% survival was detected. The optimal concentration to ensure at least 90% survival rate was $10^4$ CFU/ml if the exposure time was increased 24h more.

The composition of the microbial community of six pools of 20 GF larvae colonised with the human gut microbiota (HCONV) and the human (HUMAN) input were compared by 16S rRNA sequencing using an Illumina Miseq platform. The total number of sequences per sample was rarefied to 20961 reads and assigned to 3538 OTUs at 0.01 distance cutoff. Among the OTUs detected, bacteria represented a 98.7% and 99.9% of the HUMAN and HCONV samples, respectively, with only a minor contribution of Archaea. All archaeal reads were affiliated with *Methanobacteriaceae* family (Euryarchaeota phylum), and most of them with the genus *Methanobrevibacter*.

Within Bacteria, 9 phyla were found in HUMAN samples, being Firmicutes (61,04%), Bacteroidetes (31,80%) and Verrucomicrobia (2,98%) the most abundant. Seven out of the 9 phyla found in HUMAN samples were also detected in HCONVs, including the three phyla previously mentioned, and Actinobacteria, Proteobacteria and Cyanobacteria. Among them, Proteobacteria was the most abundant in HCONV samples, contributing 95% of the reads, despite it contributed only 1% in HUMAN. Members of Tenericutes, Lentisphaerae and other unclassified Bacteria presenting very low proportion in HUMAN samples were not identified in HCONV. Finally, in some HCONV samples Chloroflexi, Parcubacteria and Saccharibacteria phyla were detected, in contrast to HUMAN samples where they not were identified. (Figure 16).




| HUMAN | HCONV |
|---|---|
| 12628 | 462 |
| 6578 | 130 |
| 616 | 3 |
| 385 | 54 |
| 225 | 20287 |
| 158 | 5 |
| 40 | 0 |
| 15 | 0 |
| 0 | 4 |
| 0 | 3 |
| 0 | 4 |
| 44 | 1 |

**Figure 16. Relative abundance at phylum level in Human (HUMAN) and zebrafish larvae colonised with human intestinal microbiota (HCONV) represented in stacked bar chart.**

At family level, nearly 40% of the families (20) and 30% of the genera (50) detected in HUMAN samples were recovered inside the HCONV. The 20 families are listed in Figure 17. In HUMAN samples *Ruminococcaceae* (Firmicutes) was the predominating family, followed by *Lachnospiraceae* (Firmicutes), *Prevotellaceae* (Bacteroidetes) and *Bacteroidaceae* (Bacteroidetes). While in HCONV the most abundant family was *Enterobacteriaceae* (Proteobacteria), including the genera *Coprococcus, Eubacterium* and *Faecalibacterium*. The majority of Firmicutes families, the most abundant phylum in HUMAN samples, were also detected in HCONV (*Lachnospiraceae*, *Ruminoccocaceae, Lactobacillaceae, Christensenellaceae, Erysipelotrichaceae, Acidaminoccocaceae, Veillonellaceae* or *Streptococcaceae* among others) (Figure 17), incuding the genera *Blautia, Dorea, Coprococcus, Faecalibacterium, Eubacterium, etc. Peptococcaceae, Clostridiaceae, Carnobacteriaceae* and *Defluviitaleaceae* families were not detected in HCONV samples.

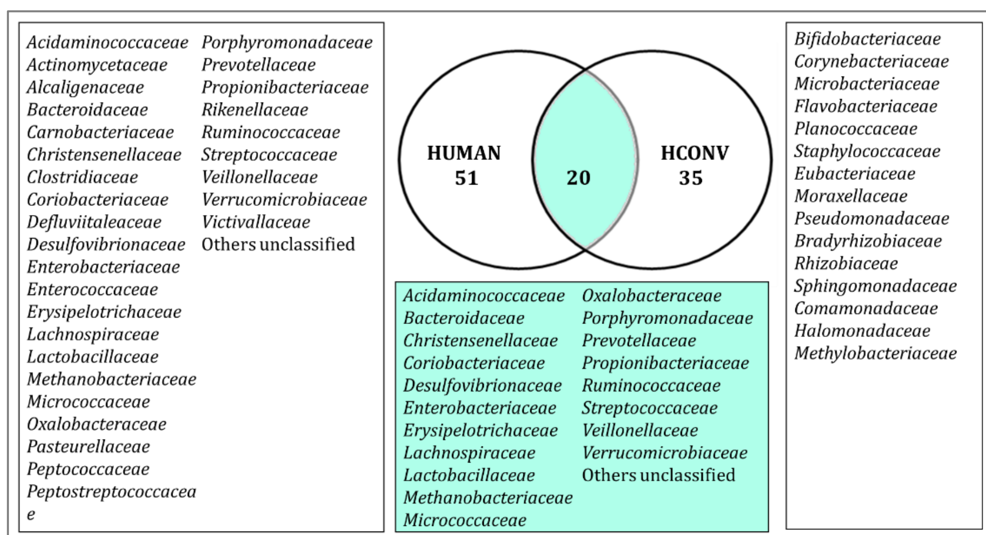
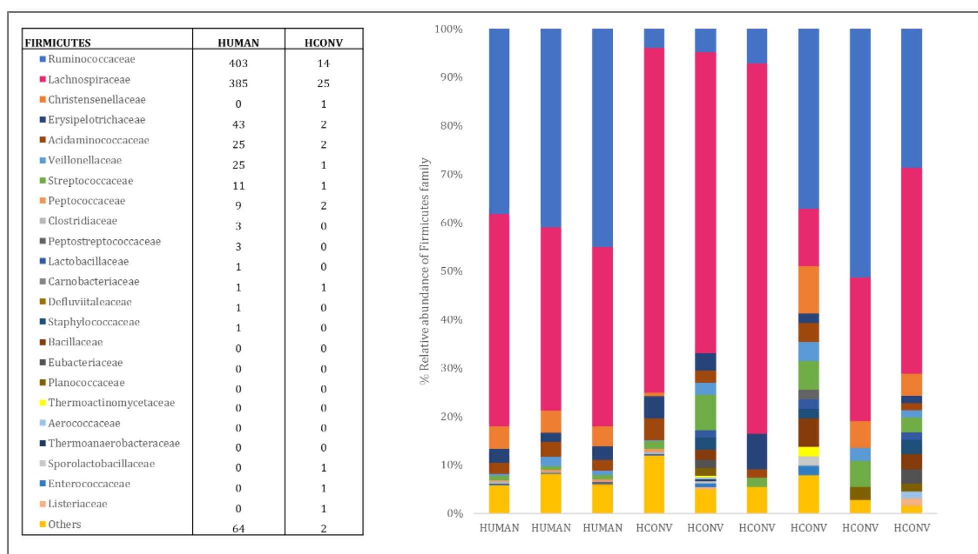



**Figure 17. Summary of families identified only in human samples and detected in both human samples and colonised zebrafish (HCONV) by 16S sequencing.**

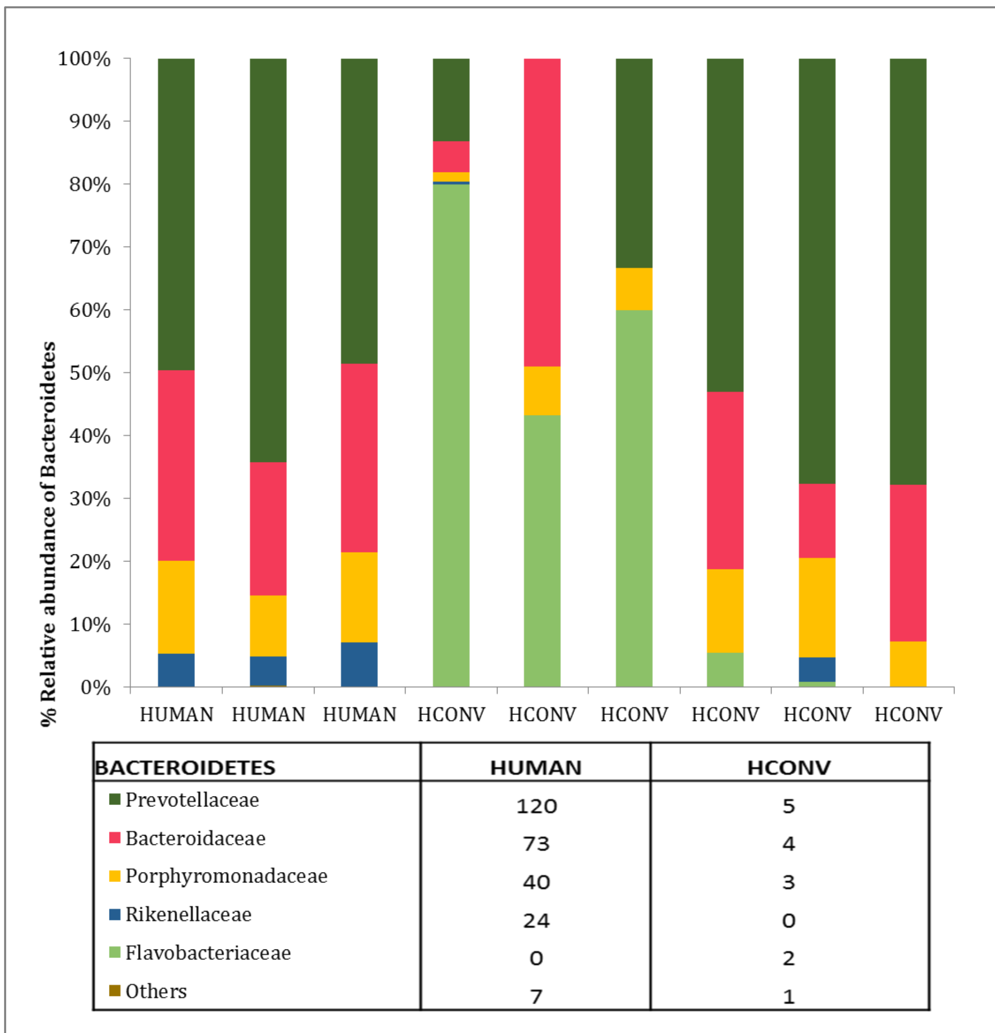


| FIRMICUTES | HUMAN | HCONV |
|---|---|---|
| Ruminococcaceae | 403 | 14 |
| Lachnospiraceae | 385 | 25 |
| Christensenellaceae | 0 | 1 |
| Erysipelotrichaceae | 43 | 2 |
| Acidaminococcaceae | 25 | 2 |
| Veillonellaceae | 25 | 1 |
| Streptococcaceae | 11 | 1 |
| Peptococcaceae | 9 | 2 |
| Clostridiaceae | 3 | 0 |
| Peptostreptococcaceae | 3 | 0 |
| Lactobacillaceae | 1 | 0 |
| Carnobacteriaceae | 1 | 1 |
| Defluviitaleaceae | 1 | 0 |
| Staphylococcaceae | 1 | 0 |
| Bacillaceae | 0 | 0 |
| Eubacteriaceae | 0 | 0 |
| Planococcaceae | 0 | 0 |
| Thermoactinomycetaceae | 0 | 0 |
| Aerococcaceae | 0 | 0 |
| Thermoanaerobacteraceae | 0 | 0 |
| Sporolactobacillaceae | 0 | 1 |
| Enterococcaceae | 0 | 1 |
| Listeriaceae | 0 | 1 |
| Others | 64 | 2 |



**Figure 18. Relative abundance of families belonging to Firmicutes phylum in Human (HUMAN) and zebrafish larvae colonised with human intestinal microbiota (HCONV) represented in stacked bar chart.**

Similarly, HUMAN and HCONV shared half of the families within Bacteroidetes phylum (Figure 18). In this case, *Prevotellaceae*, *Bacteroidaceae* and *Porphyromonadacedae* were the three most abundant in both, including genera such as *Prevotella, Bacteroides* and *Parabacteroides* (Figure 19).




| BACTEROIDETES | HUMAN | HCONV |
|---|---|---|
| Prevotellaceae | 120 | 5 |
| Bacteroidaceae | 73 | 4 |
| Porphyromonadaceae | 40 | 3 |
| Rikenellaceae | 24 | 0 |
| Flavobacteriaceae | 0 | 2 |
| Others | 7 | 1 |

**Figure 19. Relative abundance of different families of Bacteroidetes phylum in Human (HUMAN) and zebrafish larvae colonised with human intestinal microbiota (HCONV) represented in stacked bar chart.**

Verrucomicrobia was the third most abundant phylum in HUMAN samples. It was represented by three families, but only one (*Verrucomicrobiaceae*, and particularly the genus *Akkermansia*) appeared in 2 out of the 6 HCONV pools. Graphs from Verrucomicrobia and other phyla not detailed here are in supplementary material Figure 1.

When samples were compared at the OTU level (0.01 distance cutoff), large quantitative differences were found between HUMAN and HCONV samples. Considering only OTUs appearing in at least one of the three HUMAN samples and half (3/6) of the HCONV samples, only 2.5% of total HUMAN OTUs were found in HCONV; being the HCONV community less rich

and less diverse. *Escherichia* affiliated OTU was the most abundant one inside the HCONV community, colonizing the 95% of the larvae.

Of 3538 OTUs, 3270 are present in HUMAN samples, whereas 410 (12%) are recovered in HCONV. In HUMAN OTUs are distributed in Bacteroidetes (596), Firmicutes (2366), Verrucomicrobia (36), Actinobacteria (72), Proteobacteria (30), Cyanobacteria (23), Methanobacteria (12) and Others (146). Around 2.5% of them (85) are present in at least half of HCONV samples (Bacteroidetes (11), Firmicutes (49), Verrucomicrobia (1), Actinobacteria (5), Proteobacteria (17), Cyanobacteria (1) and Methanobacteria (1) (Figure 20). Furthermore, 23 OTUs were detected in at least half of HCONV samples that were not recovered in in HUMAN, where 11 of them were human related taxons. In order to identify the most representative species of each OTU, we also analyzed the most representative sequence corresponding to each OTU in BLAST (Basic Local Alignment Search Tool- NCBI). The results showed that OTUs closely related to human species with scientific and medical interest, were identified (*Faecalibacterium prausnitzii, Akkermansia municiphila,* or *Eubacterium rectale*). These results are shown in Figure 21.

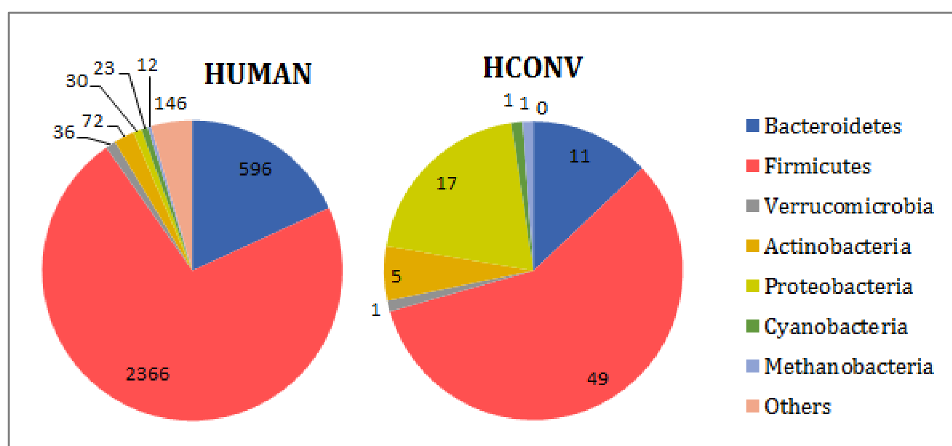



**Figure 20. Taxonomic composition of sequences retrieved from both human (HUMAN) and zebrafish conventionalized with human intestinal microbiota (HCONV) represented in a pie chart graph.** Number of OTUs at 0.01 distance cutoff from each phylum are indicated in numbers.

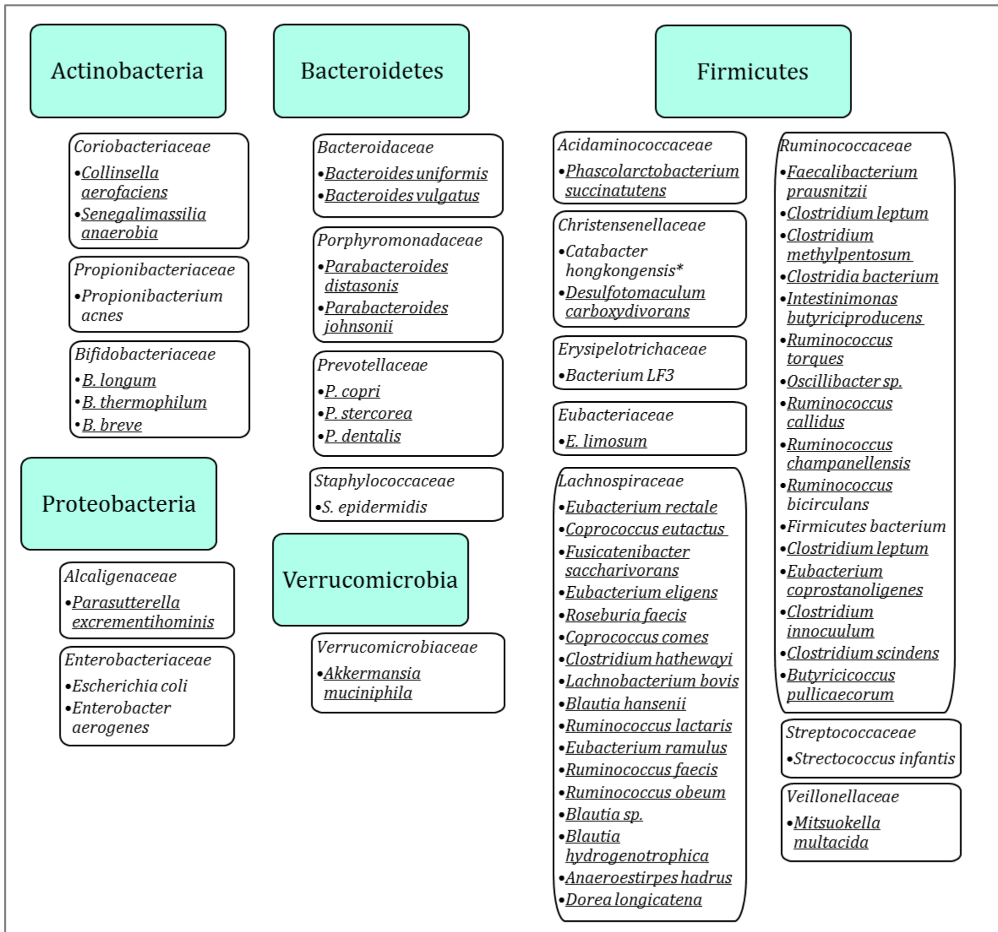



**Figure 21. Species that appeared in both human (HUMAN) and conventionalized zebrafish (HCONV).** The distance cutoff was 0.01 with the corresponding taxonomic information provided by Mothur related to Silva database (v128, Phylum and Family). Species underlined are obligate anaerobes (Bergey's manual of systematic bacteriology), species with * no information was found.

## 5. Discussion

In this study, we demonstrated that up to 60 human intestinal bacterial phylotypes are able to colonise the digestive tract of germ-free zebrafish larvae. In earlier works, mice intestinal microbiota was transplanted into germ-free zebrafish larvae and *vice versa*[9]; and two human gut-derived bacteria (*E. limosum* and *L. paracasei*) were detected as able to successfully colonise the developing gastrointestinal tract of germ-free larval zebrafish[149]. More recently, we were able to colonise axenic zebrafish larvae with five bacteria commonly found in human intestine (*E. coli*, *E. faecalis*, *E. limosum*, *B. breve and L. casei*) recovering all of them, including obligate anaerobes, during the first 48 h[169]. However, these reports did not clarify which human phylotypes in a complex microbiota sample were able to establish inside the zebrafish digestive

tract. In this work, we have successfully colonised the digestive tract of GF zebrafish larvae with the whole human intestinal microbiota, adjusting the appropiate inoculum concentration to ensure larvae survival, and describing for the first time which human bacteria phylotypes are able to establish inside the zebrafish digestive tract. Even though zebrafish larvae were able to survive at least 72 h in the presence of the human microbiota, we decided to perform this experiment during 48 h because to date obligate anaerobes are known to be able to survive during 48 h inside the zebrafish digestive tract[169]. Furthermore, maintaining the larvae 6 h in fresh sterile media ensured that the bacteria identified with the metabarcoding analysis were those that colonised the digestive tract. In fact, it is well established that food digestion lasts at least 6 h[172]. In addition, in other experiments performed in our laboratory we found that bacteria that do not colonise the digestive tract leave the intestine before 6 h[173]. In this experiment, we did not feed the larvae to avoid adding another variable.

Only 2% of the OTUs present in HUMAN samples were found in HCONV, supporting the view that only a small subset of the human intestinal microbiota is able to colonise the zebrafish digestive tract[149]. This proportion is low in comparison with experiments performed in mice[164], possibly because mice gut conditions are more similar to the human niche. Despite zebrafish lives in an aquatic habitat, the oxygen concentration in the gut might be higher than estimated. Another remarkable difference between zebrafish and mammals is the habitat temperature; human body is maintained at 35-37ºC (Celsius degree) while zebrafish are incubated at 27-28ºC. Thus, the temperature might be one of the most limiting conditions for the human intestinal bacteria to colonise and grow inside the zebrafish gut, since in our experiment certain obligate anaerobes were able to colonise. In addition, in previous studies, using 5-7 dpf axenic zebrafish colonised with one [153] or several microorganisms[169, 9, 149] a CFU of $10^4$ - $10^5$ (CFU/larvae) was achieved inside the zebrafish. Furthermore, the slurry analyzed in human samples is diluted several times to prepare the colonisation inoculum. Therefore, if we compare the high microbial load present in the human feces with the small number of bacteria found in the larvae, it should be noted that it is not uncommon for only a few species of microorganisms to colonise the fish. Nevertheless, to resolve this premise more studies are necessary.

The largest difference in terms of composition was the relative abundance of Proteobacteria in HCONV as compared to HUMAN, in particular *Escherichia* contributed up to 95% in HCONV. Rawls *et al.*[9] described that environmental factors could operate to select a subset of the input community prior to entry in the recipient fish digestive tract. Similarly to our results, the group of microorganims that increased in their experiments were gamma proteobacteria, particularly the Escherichia-Shigella group. Additionally, these authors did not

found Bacteroidetes, or obligate anaerobes in the output community presumably due to their restricted oxygen tolerance, since the larval and adult zebrafish gut have higher levels of oxygen that might exclude strictly anaerobes[9].

In our experiment, we tried to maintain anaerobic conditions as strictly as possible and favored the stability of the obligate anaerobes and other fastidious microorganisms for example by supplementing the larvae medium with vitamins and other ingredients. This procedure could be key to manage human or mammals microbiota since it is mostly composed of obligate fastidious anaerobes. By handling fecal samples under anaerobic conditions and using oxygen reduced and supplemented media, we have successfully recovered several OTUs related to obligate anaerobes inside zebrafish (*A. muciniphila, F. prausnitzii, Eubacterium spp., Roseburia spp., Bifidobacterium spp., Dorea longicatena*).

Despite the large differences in the composition found between HUMAN and HCONV, a remarkable result of our experiment is the large diversity of representative bacterial families from the human gut that were able to colonise the zebrafish larvae. Similarly to previous studies, both microbial communities were more similar at a higher taxonomic level[9].

Archaea had a very low contribution in all samples, because the primers only amplify a small amount of archaea sequences. In both HUMAN and HCONV this domain was represented by the *Methanobrevibacter* genus, belonging to *Methanobacteriaceae* family. The most representative OTU was affiliated with *M. smithii*, a methanogenic Archaea which have been specifically linked to altered metabolism and weight gain in the host[174].

From bacteria domain, 6 out of 9 phyla were found in HCONV samples; Firmicutes, Bacteroidetes, Verrucomicrobia, Proteobacteria, Actinobacteria and *Cyanobacteria*. Among the phyla detected, Firmicutes was the most abundant in HUMAN samples, which gathers 19 families, 12 of them also recovered in HCONV (e.g. *Ruminococcaceae, Lachnospiraceae, Veillonellaceae, Acidaminococcaceae*). Those families include *Faecalibacterium prausnitzii, Eubacterium spp*. or *Roseburia spp*., butyrate producing bacteria that colonise the mucus layer, and consequently increase the butyrate bioavailability for colon epithelial cells[162,175]. These bacteria have gained attention during the last years because of their contribution to gut homeostasis by preserving gut barrier functions and exerting immunomodulatory and anti-inflammatory properties. *Eubacterium rectale, F. prausnitzii, and/or R. intestinalis* concentration markedly decreased in intestinal bowel disease[176,177,178] and colorectal cancer patients[179,180]. Another relevant genus from Firmicutes phylum in HUMAN samples is *Blautia spp*., which was also recovered in HCONV, According to Xu *et al.*, *Blautia spp*. may be involved in relieving inflammation, insulin resistance and obesity in high fat diet rats [181]. Finally, the genus

*Oscillibacter* was also present in both samples, this genus has been associated with high fat diet obesity and diabetes[182,183].

The second most abundant phylum in Human was Bacteroidetes. In this group, four families from the 6 present in HUMAN samples were recovered inside the HCONV digestive tract, including *Prevotellaceae, Porphyromonadaceae, Staphylococcaceae* and *Bacteroidacea*. *Prevotella* members have been positively associated with high intake of carbohydrates and simple sugars and negatively with high intake of protein and fat of animal origin in humans[184,28,185]. The genus *Bacteroides*, which have significant effects on human health, has been most notably related to carbohydrate fermentation and catabolism of polysaccharides[186]. Differerent families of Actinobacteria phylum were also detected in HCONV samples. B. breve, *B. longum* and *B. thermophilum*, members of *Bifidobacteriaceae,* are the most valuable species belonging to this phylum, whose population inside the human digestive tract is suggested to have anti-obesogenic and anti-diabetic potential and its balance is correlated with a healthy status[27]. Moreover, the most appreciate member of the Verrucomicrobia phylum *Akkermansia muciniphila*, was found in 2 out of 6 samples in HCONV. It is the only cultivable member of Verrucomicrobia phylum and supposed to potentially have anti-inflammatory properties[187]. This means that *A. muciniphila* could be a target of study using zebrafish as animal model.

In a previous study, Roeselers *et al.*, studied the microbiota of different animals, including zebrafish from different laboratories, as well as environmental and human samples[125]. Analyzing the microbiota by tRFLP and 16S sequencing, the five most abundant families found in zebrafish (*Aeromonadaceae, Fusobacteriaceae, Enterobacteriaceae, Vibrionaceae and Shewanellaceae*) were not present in HUMAN samples (only *Enterobacteriaceae*). So, we can conclude that HUMAN related families that colonised the zebrafish larvae in our experiment were not members of the zebrafish natural microbiota. The host provides a basic niche that the microbial community expands and changes by its physical presence and metabolic activities. In addition to host habitat factors, dynamics within microbial communities interact with the host habitat to shape the final community[9]. However, an environmental and negative control might be necessary.

To finish, we should note that 23 OTUs appeared only in at least half of HCONV samples; 10 of them related to human intestinal microbiota that were not detected in HUMAN samples. At phyla level idem occurred; Chloroflexi, Parcubacteria, and Saccharibacteria phyla were not detected in HUMAN samples. This might be due to the sequencing method that is limited to a few thousand reads per sample, so members in low proportion in the HUMAN microbial community may not be detected even if they are present. Moreover, in this experiment OTUs

appearing in at least the half of the HCONV samples have been took into account to avoid random results. This threshold might be high and for that reason some taxons that might be able to colonise are not considered in the results. However, it may improve the intraspecific variation among HCONV samples, being a refinement because conventionally raised zebrafish larvae colonisation is not homogeneous[122].

Nevertheless, in HUMAN samples all OTUs have been considered in order to not misplace any taxon, even appearing in only one sample. Other 13 detected OTUs in HCONV samples were correlated to different environments. This might be attributable to the sterilization method that kill the bacteria but the DNA is still maintained in the medium. The addition of an environmental control and a mock community should circumvent this problem. However, this opens a field to the use of germ-free animals, because the axenity tests in general are done by culturing in general media (classical methods) or by 16S qPCR[136]. There is a great controversy about these techniques since some of them do not consider the non-cultivable viable microorganisms, and others do not take into account the DNA of dead bacterial. Some techniques might combine different methods to not detect dead bacteria, but they are expensive and time consuming[188]. To overcome this constraint, filtering the media and/or sterilizing with ultraviolet light to break the DNA should be taken into account for further studies.

In conclusion, in this research we have identified a large variety of human bacterial phylotypes that have the ability to successfully colonise zebrafish larvae. In addition, we have confirmed that zebrafish might be a good candidate as a model to study different human intestinal microbiota members in monoassociation or even in consortia. In this experiment, we tested the microbiota of only one donor; however, in the future it would be desirable to analyze samples for other humans because there is a huge interindividual variation that could help to identify additional human bacteria with the ability of colonise zebrafish. Our findings open the door to the study of diseases related to microbes such as obesity, Crohn’s disease, colorectal cancer, or Alzheimer among others, using zebrafish as an animal model. Additionally, host-microbe interactions, microbe-microbe interactions, new therapies, compound toxicity, or other basic and complex functions may also be studied in this model. This study opens a wide field in which more research is needed. Many improvements might be added in the future to this research. The absence of food in the present experiment can be expected to make it harder for some microbes to colonise. Therefore, it could be hypothesized that a greater number of human OTUs would have appeared in the zebrafish gut if the fish had been fed a diet. Moreover, Increasing the experimental time may provide information of bacterial colonisation, adaptation and metabolic behavior to the new environment.

Chapter 3

# High-fat diet consumption induces microbiota dysbiosis and intestinal inflammation in Zebrafish larvae

**PUBLICATION**

**Arias-Jayo, N., Abecia, L., Alonso-Sáez, L., Ramirez-Garcia, A., Rodríguez, A., Pardo, M.A. High-fat diet consumption induces microbiota dysbiosis and intestinal inflammation in Zebrafish.**

**CONFERENCE**

*European congress on obesity*
Poster and oral communication. “High fat diet causes an emerging inflammation and dysbiosis in zebrafish” Arias, N., Ramirez-Garcia, A.; Rodriguez, A., Galera, A., Pardo, M.A. (Portugar 2017)

*SEGHNP*
Poster and oral communication. “Estudio de la inflamación intestinal y disbiosis causadas por la ingesta de una dieta alta en grasa” Rodriguez, A., Arias, N., Ramirez-Garcia, A., Martin, G., Pardo, M.A.
*Awarded

## 1. Abstract

In this chapter, the effects of a high-fat diet (HFD) after 25 days on the intestinal microbiota and inflammation in zebrafish was tested. Microbial composition of HFD fed animals was compared to controls by 16S rRNA sequencing and qPCR. The expression level of several genes related to inflammation was tested. Furthermore, microscopic assessment of the intestine was performed in both conditions. The consumption of the HFD resulted in microbial dysbiosis, characterised by an increase in the relative abundance of the phylum Bacteroidetes. Moreover, an emerging intestinal inflammation via NF-κB factor activation was confirmed by the overexpression of several genes related to signalling receptors, antimicrobial metabolism and the inflammatory cascade. The intestinal barrier was also damaged, with an increase of goblet cell mucin production. This is the first study performed in zebrafish suggesting that the consumption of a diet enriched with 10% fat changes the intestinal microbial community composition, which was correlated with low-grade inflammation.

## 2. Introduction

The Overweight and obesity are defined as abnormal or excessive fat accumulation and a low-grade systemic inflammatory tone in the presence of a positive energy balance, representing a major risk factor for a number of chronic diseases including cancer, cardiovascular diseases, diabetes and premature mortality[189]. The consumption of a HFD is one of the main factors contributing to the development of obesity[190]. Human and animal studies have shown that both HFD and obesity are associated with changes in the gut microbiota, reducing the abundance and diversity of microorganisms[42, 191] and impacting both immunological and metabolic functions of the host [192, 193]. To date, studies performed in zebrafish describe microbial community changes or inflammation developed by the consumption of a high-fat, high-protein diet[121, 141, 194] or a high-cholesterol diet (HCD)[148]. To our knowledge, this is the first study relating HFD consumption to microbial changes and inflammation in zebrafish.

Studies performed in rodents have shown that the prolonged exposure to a HFD can alter the intestinal microbiota and perturb immune homeostasis, inducing intestinal inflammation[195]. These alterations lead to the activation of an innate immunity-mediated chronic low-grade inflammation known as meta-inflammation (metabolically triggered inflammation)[196], suspected to be chronically activated and modulated by pro-inflammatory cytokines. These molecules are likely to play a key role in metabolic disease pathogenesis which

develops locally, but becomes systemic through the release of numerous pro-inflammatory mediators into the blood stream[197].

Diet is an important modulator of the intestinal microbiota in humans and other animals[190, 198 142] and associated with impairments in epithelial integrity and barrier function [199]. The intestinal mucosa is the first barrier where fat is absorbed and metabolised and might therefore be involved in responses triggered by dietary lipids[148]. Both resident antigen-presenting cells and intestinal mucosal epithelial cells are equipped with PRR, such as TLR and NOD-like receptors (NLR). They detect PAMPs, for instance lipopolysaccharides (LPS), flagellin or peptidoglycans[200], and protect the organism from harmful pathogens, thereby promoting repair, regeneration and immune homeostasis of the intestine[201]. Recent findings have demonstrated that fatty acids and cholesterol are able to attach to those receptors, leading to inflammation[202] via stimulating inflammatory-signalling cascades, such as the IκBα kinase/nuclear factor-KB (IKK/ NF-κB), the endoplasmic reticulum (ER), the stress-induced unfolded protein response (UPR) and the NOD-like receptor P3 (NLRP3) inflammasome pathway[203].

The zebrafish has an increasing recognition as an excellent animal model for studying human metabolic or inflammatory diseases due to its similarity to humans in terms of organs and genomic content, its highly conserved biochemical and physiological pathways and the availability of a complete genome sequence. Despite the differences between the zebrafish and mammal microbiota composition, dominated by Proteobacteria in zebrafish and by Firmicutes and Bacteroidetes in mice and humans, the responses to microbial colonisation are similar[71]. The microbiota also plays a crucial role in immunity and host response to pathogens in zebrafish[111, 204, 205].

Moreover, pathways regulating microbial recognition and activation of the innate immune response are also greatly conserved[206]. In addition, zebrafish maintenance and manipulation are cheaper than in rodent models[132, 207, 208]. In the present work, we described for the first time the correlation between intestinal microbiota dysbiosis and inflammation in zebrafish, induced by the consumption of a high-fat diet over the period of 25 days.

# 3. Materials and methods

## 3.1. Zebrafish husbandry and experimental diets

Zebrafish embryos were obtained as described in chapter 1. Embryos were collected directly from the breeding tanks immediately after fertilisation and transferred to 2-L fish tanks. At five days dpf, 200 larvae were equally separated into two tanks and subjected to the control and the HFD. Both were maintained in 500 mL of isowater ($CaCl_2$ 294 mg.L$^{-1}$, $MgSO_4$ 123 mg.L$^{-1}$, $NaHCO_3$ 64.7 mg.L$^{-1}$, LCl 5.7 mg.L$^{-1}$) at 27°C for one month (30 dpf). Concentrations of nitrate, nitrite and ammonium were tested weekly (data not shown). All experimental procedures were approved by the regional animal welfare body (NEIKER-OEBA-AZTI14-005).

The control diet consisted of a commercial diet for zebrafish larvae (ZF Biolabs), prepared in Milli-Q water (1g in 100 ml water)[136] and autoclaved (Supplementary material, Table 1). The HFD consisted of the autoclaved control diet supplemented with cocoa butter to reach a fat content of 10% (w/w)[146]. Zebrafish larvae were fed three times per day during the experimental period, increasing the amount of food over time to facilitate normal zebrafish development[209]. At 30 mins after feeding, the remaining food was removed. Additionally, at the end of the day, half of the medium was replaced by fresh isowater.

## 3.2. Bacterial DNA extraction and quantification

After 30 dpf, five control larvae and five HFD larvae were immersed in a bath of sterile 0.01% Tween20 (Merck) and subsequently in two consecutive baths of sterile isowater in order to remove any bacteria from the skin[137]. Then, each larva was placed in a sterile Eppendorff vial and immediately frozen in liquid nitrogen. Samples were stored at −80°C until further use. Genomic DNA was extracted from frozen samples using a QIAamp DNA Mini Kit (Qiagen LTD, West Sussex, UK), following the manufacturer's instructions. The DNA concentration and purity were analysed using a Nanodrop (Thermo), measuring the absorbance at 260 nm and the A260/A280 ratio, respectively.

## 3.3. Analysis of the microbial community composition

Characterisation of the microbial community composition was performed on an Illumina Miseq Platform by sequencing the V3-V4 16S rRNA region, as described in chapter 1. Furthermore, the total number of bacteria per sample was quantified by qPCR, as previously described[210]. Alpha diversity measures were calculated in Mothur and R (3.4.0): number of observed species (SOBS), species richness (Chao1), community evenness (Simpson´s evenness)

and diversity (Shannon). Beta diversity was analysed from a Bray Curtis distance matrix in a 3-dimensional non-multimetric scaling plot (NMDS). Differences between treatments were determined with AMOVA (Mothur). Comparisons at different taxonomical levels were compared using the Kolmogorov-Smirnov test. Discriminatory analysis between treatments at the OTU level was performed with the LEfSe command implemented in Mothur. Pearson correlation among genes, phylotypes and goblet cell counts was accomplished using the Corrplot and Hmisc R packages.

All sequences from this study are available from the European Nucleotide Archive (ENA) (PRJEB23882).

### 3.4. Gene expression analysis by real-time PCR (qPCR)

To study gene expression levels, groups of 20 larvae were sampled at 30 dpf. Three replicates per diet were analysed in two independent experiments. At the end of the experimental period, zebrafish larvae were immediately frozen in liquid nitrogen and stored at -80°C until being processed. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The quantity and quality of RNA samples were determined by capillary electrophoresis, using an Agilent 2100 Bioanalyzer (Agilent Technologies); only RNA samples with an RNA integrity number (RIN) of at least 8.5 were used [211].

A reverse transcription reaction was performed with the Taqman Reverse Transcription Kit (Applied Biosystems), using synthesised cDNAs from the RNA samples containing 20 ng of RNA per assay. The mixture was incubated at 25°C for 10 min and at 48°C for 30 min, and the enzyme was inactivated at 95°C for 10 min. Changes in mRNA expression of the genes related to the innate immune system (*ASC, CASPA, IL10, IL1B, IL22 TNFA, NFKB* and *MYD88*), host-microbe interactions (*TLR4, TLR5, TLR2, MMP9, MPO, NOD1* and *DEF1*) and metabolic activity (IAP) were monitored by qPCR, using β-*actin* (*ACT*) and *elongation factor 1* (*EF1*) as reference genes to normalise the results. The primer sequences are listed in table 1. Quantitative PCR was carried out with a Light Cycler 480 sequence detection system (Roche Diagnostics). Each reaction was performed in a 10 μL solution containing 300 nM primers, 5 μL 2x Brilliant III SYBR Green qPCR Master Mix (Agilent) and 10 ng of cDNA template, as previously described[134]. The qPCR efficiency was maintained between 90 and 110%.

**Table 1. Gene expression primer list.** (* refers to this work)

| Gene | Short | Forward primer | Reverse primer | Ref |
|---|---|---|---|---|
| **Caspase recruitment domain** | *ASC* | GCTTCCTCTAAGGCTGTAAGCAA | GATGAAGTTTACCTTACCTTGGAGAAAGCA | 148 |
| **Caspase 1a** | CASPA | ACTTCATTTCTCTGATGTCGTGCA | CATGCCGGTAAGATTTGGTGT | * |
| **Interleukin-1 β** | *IL1B* | CATTTGCAGGCCGTCACA | GGACATGCTGAAGCGCACTT | 212 |
| **Interleukin 10** | *IL10* | ATATTTCAGGAACTCAAGCGGG | ACTTCAAAGGGATTTTGGCAAG | * |
| **Interleukin 22** | *IL22* | TGAGGGAGGGTCTGCACAG | GCATGGCTCATAAGCACTTGTT | * |
| **Tumor necrosis factor** | *TNFA* | ACCAGGCCTTTTCTTCAGGT | GCATGGCTCAAAGCACTTGTT | * |
| **Nuclear factor-kB** | *NFKB* | AGAGAGCGCTTGCGTCCTT | TTGCCTTTGGTTTTTCGGTAA | 134 |
| **Myeloid differentiation primary response 88** | *MYD88* | CACAGGAGAGAGAAGAGTCACAG | ACTCTGACAGTAGCAGATGAAAGCAT | * |
| **Matrix metallopeptidase 9** | *MMP9* | TTGGCTTCTGTCCCAGTGAG | TTAGGGCAGAATCCATACTT | 213 |
| **Myeloid-specific Peroxidase** | *MPO* | CAATGGCCCGCATAATCTG | GCGAAAAGGATCTCTGGGAACT | 212 |
| **Nucleotide-binding oligomerization domain 1** | *NOD1* | TGAGACAAACAGCGAGGACG | GCAGATATGCCGTTAGCCGT | * |
| **Toll like receptor 4** | *TLR4* | GGGAAGTCAATCGCCTCCA | ACGGCTGCCCATTATTCCT | 134 |
| **Toll like receptor 5** | *TLR5* | CGAATCTCTTCAGCACCCTC | CTGGCACACGTCACATCCTC | * |
| **Toll like receptor 2** | *TLR2* | GGAAGGTGGCACTAAGAGCCT | TGATCGGTCGTGGAGGAGTT | 212 |
| **Defensin 1** | *DEF1* | CTTTACTTGGGACCATTAGGCTG | TTCAGTTCTCAAAGAAAATGTGATACAC | * |
| **Intestinal alkaline phosphatase** | *IAP* | ATGGGAGTGTCCACGGTTTCAG | CGATGCCAACAGACTTTCCTTG | 111, |
| **Β-actin** | *ACT* | TGCTGTTTTCCCCTCCATTG | TTCTGTCCCATGCCAACCA | 212 |
| **Elongation factor 1** | *EF1* | GCCAACTTCAACGCTCAGG | AGAGATCTGACCAGGGTGGTTC | 215 |

To study gene expression levels, groups of 20 larvae were sampled at 30 dpf. Three replicates per diet were analysed in two independent experiments. At the end of the experimental period, zebrafish larvae were immediately frozen in liquid nitrogen and stored at -80°C until being processed. Total RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The quantity and quality of RNA samples were determined by capillary electrophoresis, using an Agilent 2100 Bioanalyzer (Agilent Technologies); only RNA samples with an RNA integrity number (RIN) of at least 8.5 were used[211].

### 3.5. Evaluation of intestinal mucus secretion and intestinal tract histology

The intestinal mucus secretion was visualised with a Nikon SMZ1000 stereomicroscope, staining whole larvae with alcian blue as previously described[215]. Briefly, at 30 dpf, five larvae per treatment were analysed and processed with "Color Deconvolution" plug of the imageJ software v1.47 (National Institutes of Health, NIH) to obtain the staining area ($mm^2$)[216].

In addition, five larvae of each treatment were fixed in 10% (v/v) neutral buffered formalin, dehydrated and paraffin-embedded. Subsequently, 4-μm sections were stained with hematoxylin and eosin as well as with alcian blue. Slides were analysed under a microscope by a blind pathologist. Histological damage was calculated using the following criteria: For mucopolysaccharide secretion, every goblet cell per intestinal crest was counted. Mucosal architecture and cellular infiltration were scored as follows: normal, moderate or extensive damage, respectively 0, 1 or 2; and normal, moderate and transmural infiltration, respectively 0, 1 or 2. The scores for the last two criteria were then summed with a maximum possible score of 4, as previously described[217, 218].

### 3.6. Statistics

The p-values were calculated with a T-test and a non-parametric Kolmogorov-Smirnov test, using the software package Statgraphics v16.1.17 (StatPoint Technologies, Inc.). All p-values were adjusted via Bejamini Hochberg correction[219]. A p value $< 0.05$ was considered statistically significant.

## 4. Results

### 4.1. The effect of the high-fat diet consumption on intestinal microbiota

To evaluate the effect of the diet on zebrafish microbiota, the microbial community composition of five larvae fed each experimental diet was analysed and described by metabarcoding at 30 days post-fertilisation (dpf). Compared to the control samples, HFD did not affect the microbial community alpha-diversity, calculated by different diversity indices such as community richness (measured by observed OTUs (SOBS) and Chao1 (Chao)) (Figure 22 a). Community evenness and diversity, tested by Simpson´s evenness and Shannon indices, were not affected by the HFD, although they tended to decrease. Beta diversity analysis revealed that the overall composition of the microbial community did not show significant differences

due to the HFD, as control and HFD samples were clustered together in the NMDS (AMOVA test, $P = 0.31$; Figure 22 b).

However, there were significant changes in community phylotypes. Overall, 31 phyla were characterised in both diet conditions, of which Proteobacteria was the most abundant one (59 and 66% of the sequences in control and HFD groups, respectively). However, Bacteroidetes were significantly increased ($p < 0.01$) in HFD samples (from 2 to 16% of the total sequences; Figure 23 a and b). The increase of Bacteroidetes due to the HFD was reflected at the family level in the enrichment of the *Cytophagaceae* family ($p < 0.01$), *Flectobacillus,* the *NS11-12 Marine Group* ($p < 0.01$) and the genus *Runella* ($p < 0.05$; Figure 23 c). Furthermore, a tendency to increase in the Proteobacteria phylum was observed. In contrast, Firmicutes and Actinobacteria tended to decrease. Moreover, the number of total bacteria from the same larvae extracted DNA was quantified by qPCR, showing that the consumption of a HFD over the period of 25 days did not significantly modify the number of total bacteria ($p = 0.60$; Figure 22 c).

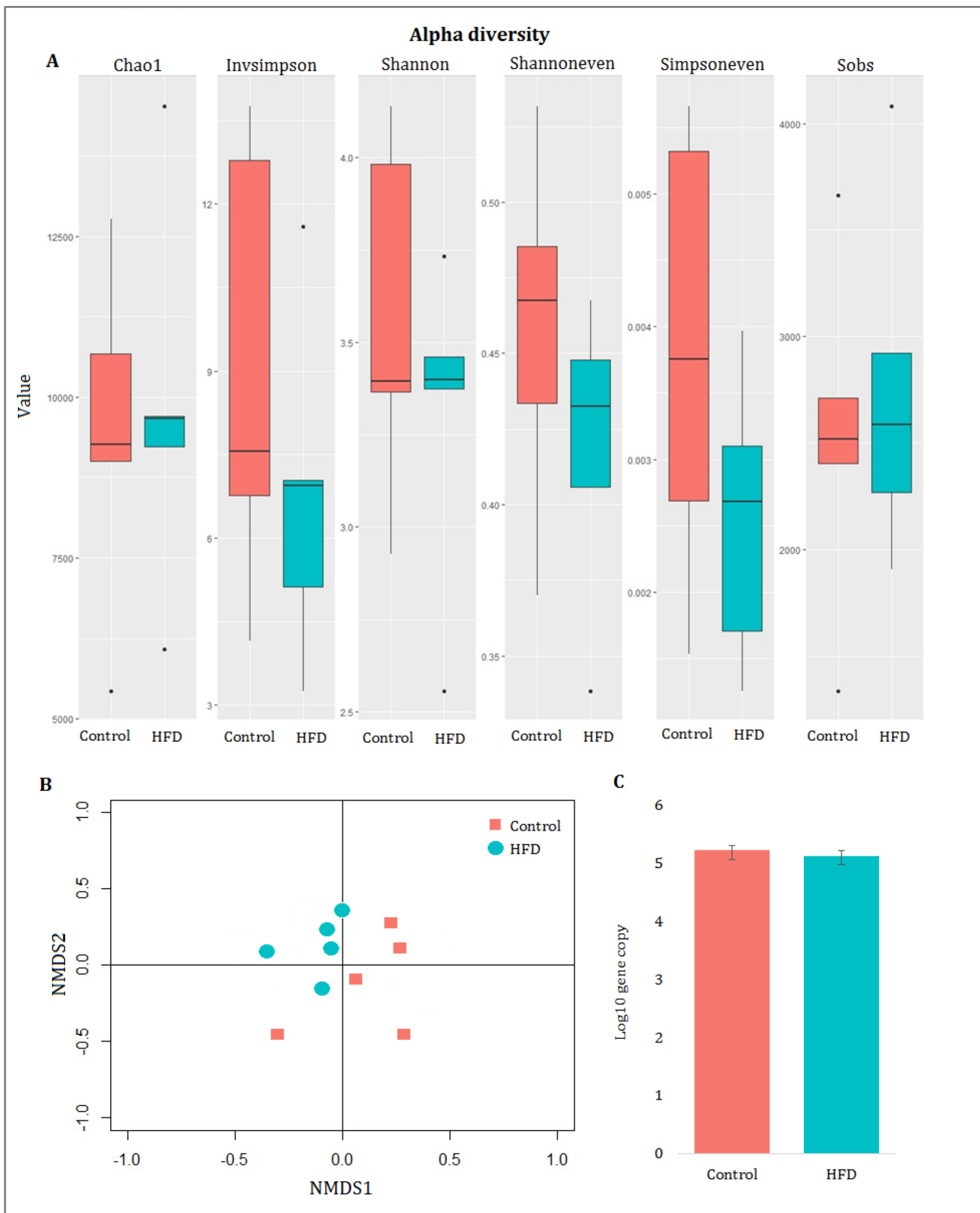



**Figure 22. Alpha and beta diversity in control and HFD samples.** A: Estimates of alpha-diversity represented in boxplots. B: Non-metric multidimensional scaling plot (using Bray Curtis dissimilarity) of Control and HFD samples. Lower stress = 0.115; $R^2$ = 0.894; p = 0.317, calculated via the AMOVA test. C: Total number of bacteria (log 16S rRNA gene copies/larva) in control and HFD zebrafish larvae (n = 5). Results represent the mean values of each condition in the vertical bars. The error bars indicate the standard error of the mean.

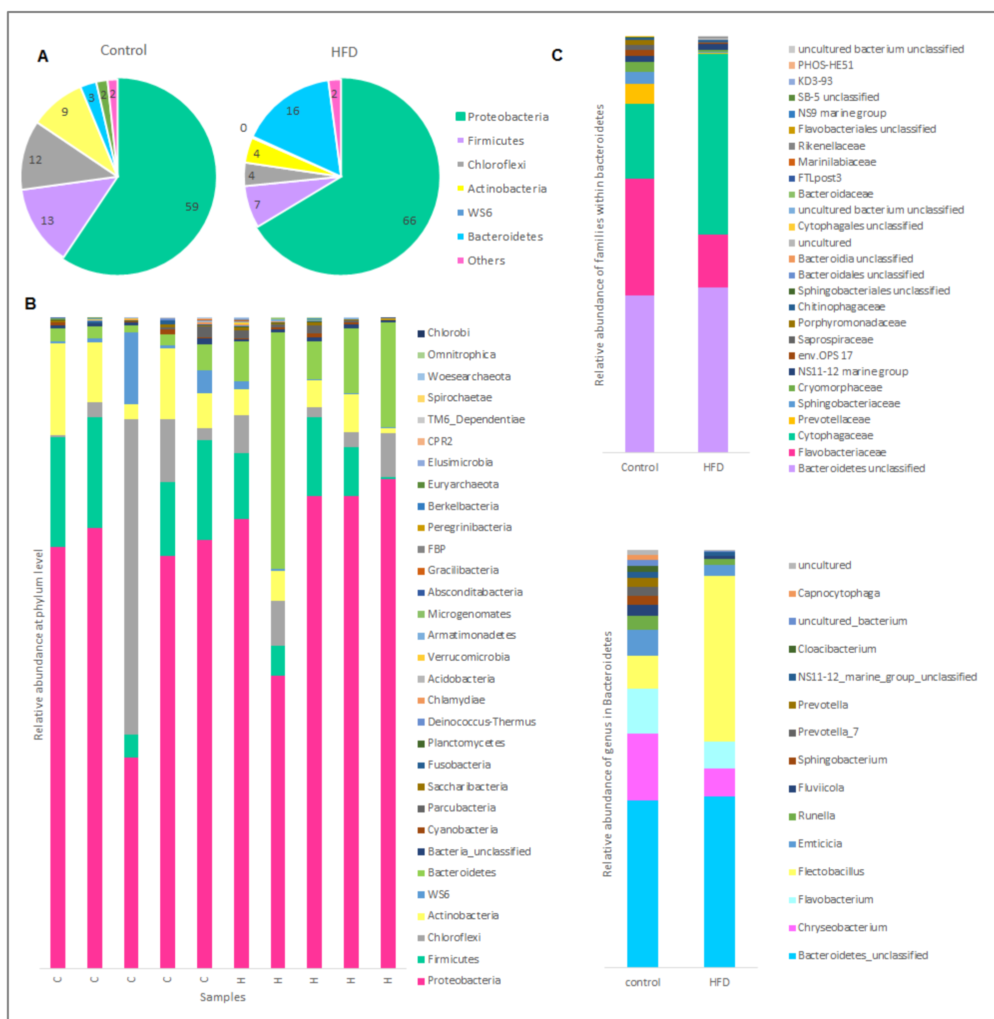



**Figure 23. Relative abundance of phylum, families and genus in control and HFD.** A: relative abundance at phylum level in larvae fed a control diet and the HFD, represented in Pie chart, and in a stacked bar chart (B). C: Relative abundance of Bacteroidetes at family and genus level.

In addition, Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify microbial OTUs that differed significantly between control and HFD groups. Twenty-one OTUs were significantly enriched or decreased in HFD samples. The OTUs affiliated to *Flectobacillus, Runella, Flavobacterium* and the *NS11-12 Marine Group*, Bacteroidetes phylotypes and to *Acidovorax*, *Rhizobiales* and *Acinetobacter,* Proteobacteria phylotypes, increased in HFD samples. In turn, OTUs affiliated to Proteobacteria, *Rhodobacteraceae, Meganema* and *Rhizobiales* phylotypes, Actinobacteria, *Mycobacterium* phylotype, and to Firmicutes, *Finegoldia* phylotype, were depleted (Figure 24). The OTU 16, related to the genus *Flectobacillus*, a member of the phylum Bacteroidetes, was the most enriched OTU in HFD samples.

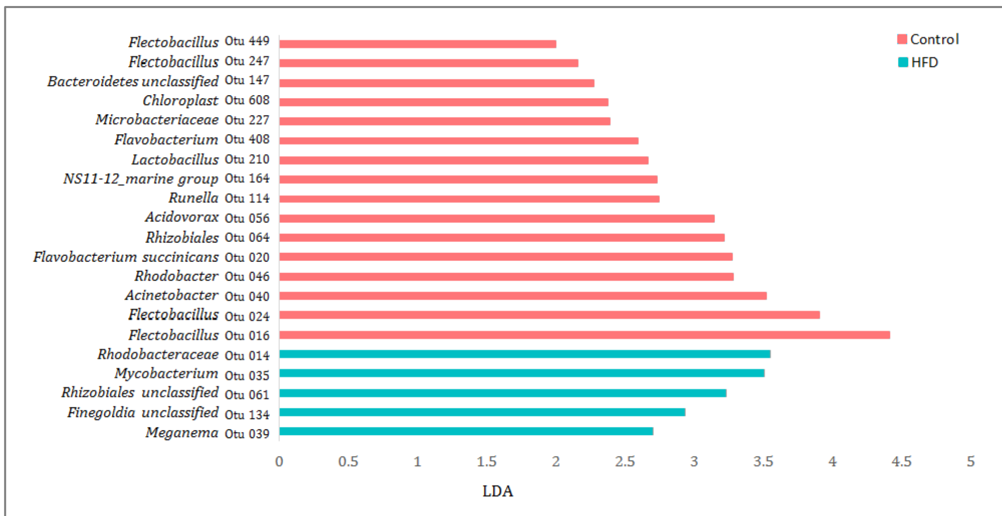



**Figure 24. Linear discriminant analysis (LDA) effect size (LEfSe).** OTUs and associated OTU taxonomy significantly enriched in HFD and in control samples. Differences were considered statistically significant at $p < 0.05$.

## 4.2. The effect of the high-fat diet consumption on immune system

We assessed the expression of 16 genes related to the zebrafish immune system by qPCR at 30 dpf (n = 6) to test the effect of the HFD on the immune system. Specifically, *NOD1*, *TLR2*, *4* and *5* are host receptors involved in the first contact and interaction with microbiota; *ASC* and *CASPA* are essential parts of the NPLR3 inflammasome; *NFKB, IL10, IL22 and MYD88* take part in cellular signalling and the triggering of inflammation; *IL1B* and *TNFA* are cytokines associated to the inflammatory cascade; and finally, *IAP, Defensin 1*, *MMP9* and *MPO* are antimicrobial peptides secreted by the host in response to microbial attacks.

Compared with the control, the expression levels of 12 of the genes studied were markedly upregulated in HFD samples. The HFD consumption induced an immune response mediated by *IL1B* activation by the canonical NF-κB pathway, as *MYD88, NFKB* and finally *IL1B* were significantly up-regulated in HFD samples. Conversely, the NLRP3 inflammasome was not affected in HFD-fed animals, as *CASPA* and *ASC* gene expressions did not change. Moreover, *TNFA* and *IL22*, proinflammatory cytokines, were also enhanced in HFD samples. Changes in the microbial composition, induced by HFD consumption, also increased the expression of *TRL2*, *TLR5* and *NOD1* genes, receptors implicated in host-microbe interactions, and *IAP*, *MPO*, *MMP9* and *Defensin 1*, genes related to the secretion of antimicrobial peptides as a defence strategy (Figure 25).

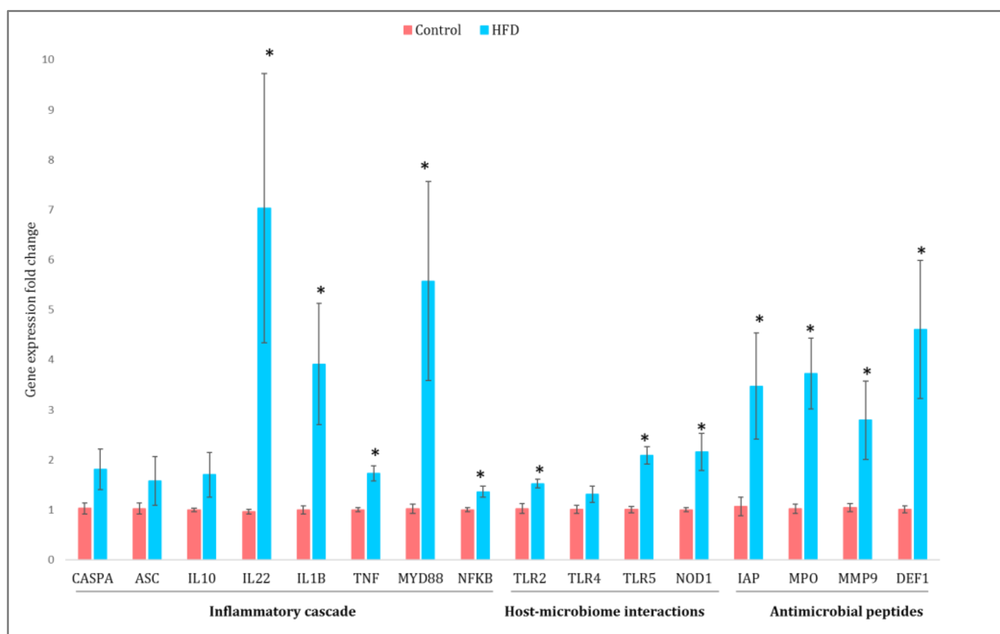
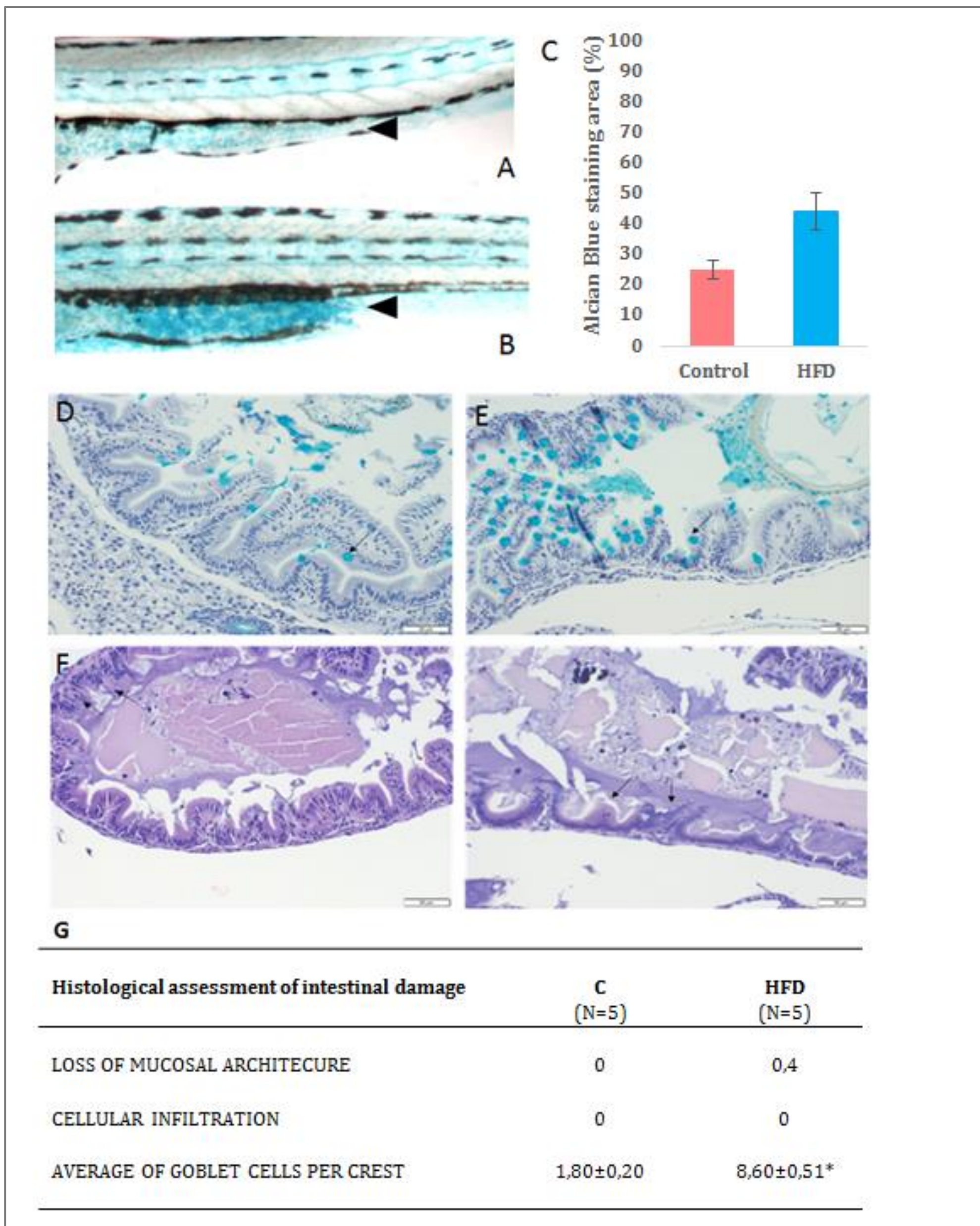



**Figure 25. Gene expression fold change analysis after 25 days of diet exposure (30 dpf) in control and HFD larvae.** Error bars indicate the standard error of the mean. Differences were considered statistically significant at $p < 0.05$ (*).

## 4.3. The effect of the high-fat diet consumption on intestinal barrier

Alcian blue staining of five whole-mount larvae at 30 dpf revealed a statistically significant increase in mucus production due to HFD consumption ($p < 0.05$; Figure 26, A and B). Furthermore, to reinforce these observations, alcian blue was also used to stain histological preparations of larvae intestines in order to observe mucus-producing goblet cells. A statistically significant increase in goblet cells was observed in HFD-fed animals at 50 µm, based on histological preparations ($p < 0.01$; Figure 26, C), counting an average of 8.6 compared to 1.8 goblet cells per crest in HFD and control animals, respectively (Figure 36, E and F). Moreover, a moderate loss of mucosal architecture was found in the intestinal epithelium of two out of five HFD-fed larvae (n = 5) with a score of 1, observing apical brush border cell fusion (Figure 26 D and G). We observed no infiltration of inflammatory cells, irrespective of the diet.




| Histological assessment of intestinal damage | C (N=5) | HFD (N=5) |
|---|---|---|
| LOSS OF MUCOSAL ARCHITECURE | 0 | 0,4 |
| CELLULAR INFILTRATION | 0 | 0 |
| AVERAGE OF GOBLET CELLS PER CREST | 1,80±0,20 | 8,60±0,51* |

**Figure 26. Histology of Control and HFD larvae.** A: and B: Alcian blue-stained control larva and HFD larva. Arrows indicate goblet cells stained blue. C: Percentage of alcian blue-stained area in control and HFD larvae, represented as the mean of three replicates; error bars indicate the standard error of the mean. Differences were considered statistically significant at $p < 0.05$ (*). D and E: Goblet cells stained with alcian blue in control and HFD intestine histological preparations at 50 µm. Arrows show goblet cells. F: Histological damage in the HFD zebrafish intestine, hematoxylin- and eosin-stained at 50 µm. Arrows show enterocyte degeneration points. G: Histological assessment of intestinal damage. Values represent means ± standard error of the mean (SEM), with n being the number of larvae. Differences were considered statistically significant at $p < 0.01$ (**).

### 4.4. Correlation between microbial changes and inflammation induced by the high-fat diet consumption

We found a positive correlation among microbial groups, which shifted between diets, gene expression and goblet cell count (Figure 27). The major correlations were identified among *TNFA, MYD88*, *IL1B* genes and goblet cell count with Bacteroidetes. The increase in *TNFA and MYD88* gene expression levels was positively correlated to the enrichment of Bacteroidetes (genus *Cytophaga* and OTUs affiliated to *Flectobacillus* and *Flavobacterium* (16, 20, 408, 247 and 449); R = .66-0.79; $p < 0.05$) and Proteobacteria (OTUs 40, 46 and 64 affiliated to *Acinetobacter*, *Rhodobacter* and *Rhizobiales*; R = 0.65-0.88; $p < 0.05$). The up-regulation of the *NFKB* gene was correlated to the increase of Bacteroidetes-affiliated taxa, the genus *Cytophaga*, *Flectobacillus* OTU 247 and Bacteroidetes unclassified OTU 147. The most correlated gene, *IL1B,* was strongly associated to the Bacteroidetes genera *Flectobacillus, Emitricia*, *Runella*, *Cytophagaceae unclassified*, *UKL 13.3*, the *NS11-12 Marine Group* and the associated OTUs 16, 449, 114, 164, 480 and 147 (R = 0.70-0.93; $p < 0.05$). In addition, the overexpression of *IL1B* was also correlated to the Proteobacteria-affiliated OTUs 40 and 46 (R = 0.40-0.66; $p < 0.05$). The OTU 24, related to *Acinetobacter*, was the most correlated OTU in the experiment, with the major correlation coefficients being related to the genes *IL22*, *CASPA*, *IL10* and *ASC* (R = 0.90-0.96; $p < 0.05$).

Furthermore, the increase in goblet cells in HFD samples was significantly positively related with the enrichment of Bacteroidetes *(Runella*, *Pseudarcicella* and the *NS11-12 Marine group*, OTUs 20, 114 and 164; R = 0.65-0.93; $p < 0.05$) and to Proteobacteria (OTUs 40, 46, 64 and 56; R = 0.65-0.81; $p < 0.05$). Cellular receptors *TLR 2*, *5* and *NOD1* were positively correlated to *Cytophagaceae unclassified*, *Flectobacillus* and OTUs 24 and 247, related to *Acinetobacter* and *Flectobacillus* (R = 0.58-0.93; $p < 0.05$). No significant negative correlations were found. Significant correlations are represented in Figure 27.

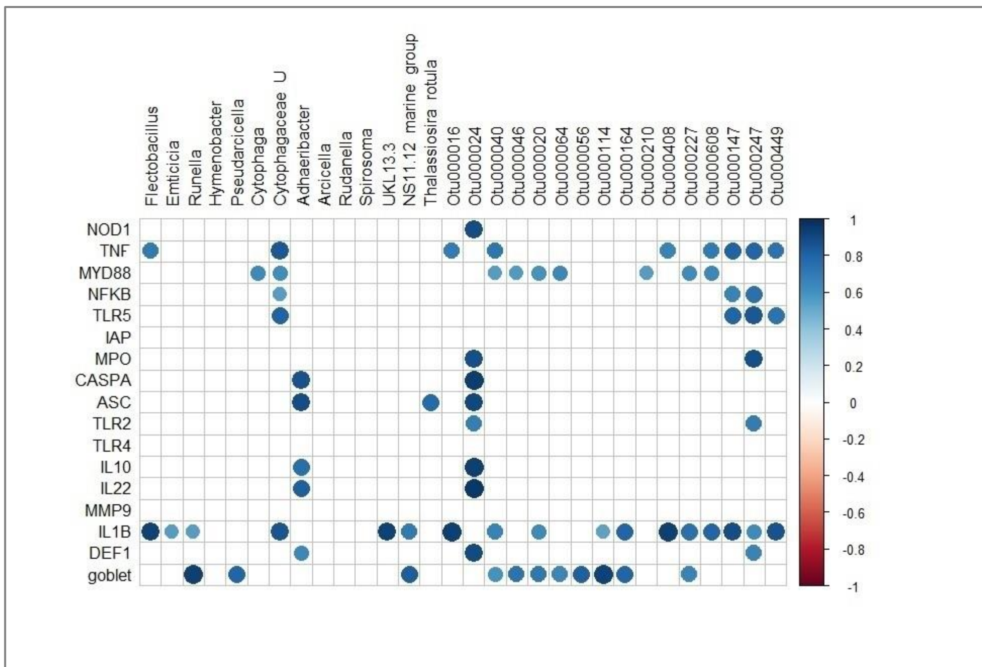



**Figure 27. Correlation plot.** Pearson correlations between gene expressed, goblet cell count and significantly different genera within Bacteroidetes and LEfSe OTUs are represented by coloured dots. Positive correlations are in blue. Only significant correlations are represented ($p < 0.05$).

## 5. Discussion

In the present study, we report that the consumption of a 10%-fat diet, enriched with cocoa butter (HFD), induces intestinal microbiota dysbiosis and inflammation in zebrafish larvae. This reformulated "cafeteria diet" has been described to induce obesity in rats[220] and body fat accumulation in zebrafish[146]. In addition, there is a strong evidence supporting that an overconsumption of nutrients initiates and triggers meta-inflammation, altering metabolic homeostasis[221]. Our results are in accordance with studies carried out in rodent models which showed that HFD consumption leads to intestinal inflammation, related to microbial dysbiosis[191]. Up to date, diet studies performed in zebrafish have focused on the effect of diets on other physiological aspects[121, 222]. Furthermore, most of these experiments were performed in adults[146].

In this work, we used zebrafish larvae to study the effects of a HFD on the intestinal microbiota and inflammation, taking into consideration that the larval adaptive immune system is not fully mature until 4-6 wpf and therefore does not interfere with the innate[223]; in addition, the transparency of the larvae allows whole-mount microscopy. The zebrafish anterior-mid and posterior gut segments are functional analogues of the mammalian small and large intestines.

In addition, most of the differentiated epithelial cell types found in mammals and the architectural organisation of the intestines are also conserved[97].

As previously shown, the gut microbiota can be directly or indirectly affected through diet in humans[32], mice[224] and zebrafish[121], which can in turn influence host physiology[225]. In this report, the consumption of a high-fat diet did not affect the total number of bacterial copies and the alpha and beta community diversity of 30 dpf zebrafish larvae, as previously described in reports studying the relationship between the intestinal microbiota and a high-fat diet or chemically-induced inflammation[121, 148, 226]. Although Wong *et al.*[121] did not reported alpha diversity changes with diets, they detected a significant dietary fat effect on the beta diversity at 35 and 75 dpf when HFD-fed larvae (28% fat) were compared to larvae fed a low-fat diet (10% fat). In this work, we enriched the control diet with cocoa butter for a final fat content of 10% to study the first changes in the microbiota, which did not yet affect the community diversity, but induced inflammation. For that reason, this point might be critical to revert dysbiosis and, consequently, inflammation. Thus, nutritional intervention from an early stage could lead to a difference in the gut microbiota assembly, with potential consequences on host physiology in the adult stage[121].

Although the HFD consumption likely did not alter alpha and beta diversity, it induced microbial changes at different taxonomic levels. The phylum Bacteroidetes was increased via the HFD. At the OTU level, the majority of the OTUs significantly enriched in HFD samples were affiliated to Bacteroidetes of the genera *Flectobacillus*, *Flavobacterium* and *Runella.* Some other increased OTUs were members of Proteobacteria, namely of the genera *Acinetobacter*, *Rhizobiales*, *Acidovorax* and *Rhodobacter*, the most abundant phyla in all zebrafish developmental stages[122]. Proteobacteria and Firmicutes tended to increase and reduce, respectively, as described by Qi He *et al.*[226]. They also related the overrepresentation of Proteobacteria and the lack of Firmicutes to the up-regulation of TNFA and, finally, to intestinal inflammation induced by chemicals. As expected, the diet-induced inflammation only slightly affected the microbiota and the immune system compared to chemicals. In contrast to our results, Wong *et al.* found that *Pseudomonas* and *Janthinobacterium* were indicative of HFD gut microbiota at 35 dpf[121]. The differences to our study in terms of larvae collection time point, the percentage of fat content and the fatty acids present in the diet might have led to a difference in developmental and physiological changes. These changes influence how different dietary fat levels impact the assembly of gut microbiota. Moreover, dietary fatty acids may directly affect the microbial selection through differential microbial capabilities for lipid metabolism and indirectly modulate host immunity and physiology[121].

Obesity-induced inflammatory mechanisms involve the activation of genes encoding for cytokines, chemokines and other immune inflammatory mediators through the activated transcription factors NF-κB and inflammasome, resulting in the proteolytic conversion of pro IL-1β to activated IL-1β[227]. Zebrafish possess both innate and adaptive immunity. In addition, key mediator-signalling proteins and cytokine[112] as well as the major mammalian blood cell lineages[206] have been identified in the zebrafish. The canonical NF-κB pathway and NLRP3 inflammasome are also conserved between zebrafish and mammals[115, 148].

In the present study, we observed the activation of the *IL1B* gene by the canonical NF-κB pathway, suggesting that in zebrafish larvae, IL-1β might be activated by the consumption of HFD. In our study, *IL1B* was activated via the canonical NF-κB pathway. This is in agreement with Landgraft *et al.*, who observed HFD-induced metabolic alterations in adult zebrafish (e.g. hyperglycemia and ectopic lipid accumulation in the liver and a metabolically unhealthy adipose tissue phenotype with adipocyte hypertrophy), accompanied by changes in the expression of marker genes such as *IL1B* [193]. Our results suggest that HFD consumption induced changes in the relative abundance of Bacteroidetes. Specifically, these changes enriched the genera *Flectobacillus*, *Cytophaga* and *Runella*, which may activate an inflammatory response mediated by IL-1β via the NF-κB pathway. Furthermore, shifts in Proteobacteria in HFD samples were correlated to the overexpression of the *TNFA* gene, as previously described in a chemically-induced inflammation[226]. The activation of *IL1B* via the NPLR3 inflammasome was not up-regulated. However, there was a strong correlation between Proteobacteria OTU 24, affiliated with *Acinetobacter*, *ASC* and *CASP1a;* NPLR3 components needed to activate this pathway. The increase of some Proteobacteria might be correlated with the activation of the inflammasome. Contrary to our research, in HCD-fed larvae, IL-1β activation involved the up-regulation of NPLR3 inflammasome components CASP1a and ASC[115], which suggests that a high-fat non-cholesterol diet, based on cocoa butter, may specifically induce the overexpression of IL-1β via NF-κB. However, more research is needed to elucidate this question.

In addition, to initiate the inflammation, the binding to cellular receptors of endogenous danger-associated molecular patterns (DAMPs) is required[228]. Orthologs of mammalian TLR and NLR have been identified in zebrafish[112]. Microbial signals such as LPS and lipid compounds are able to bind to these receptors, inducing expression[229, 230]. Dietary fatty acids affect the development of many human chronic diseases, in part mediated through the modulation of TLR[231]. Saturated fatty acids such as palmitate, stearate and oleate, the main components of cocoa butter, used to enriched the diet in the current study (HFD), can activate *TLR2* and *4,* stimulating the expression of *IL1B* via NF-κB[232]. As a result, other molecules involved in the inflammatory process, such as *MYD88* or *MMP9*[233], are also activated, as observed in our

experiment. These interactions are well described in diabetes[234] or obesity[232]. In the present study, the genes *TLR5*, *TLR2* and *NOD1* were overexpressed, correlating to the increase of Bacteroidetes and Acinetobacter. Altogether, these results suggest that the consumption of a high-fat diet activates the host immune response via the NF-κB pathway, initiated by cellular receptor stimulation through changes in the microbial community.

The inflammation process also triggers the secretion of a vast number of molecules by the host, which are required for the maintenance of the intestinal barrier function, a viscoelastic protective layer composed of mucins secreted by goblet cells[235], defensins (including Defensin 1)[139, 236], matrix metalloproteinases (MMPs)[213] and enzymes such as IAP[237]. As exposed earlier, TLR2 activation by dietary fatty acids not only induced the activation of cytokines, but also MMP9 secretion[232]. The increase in AMP (*MMP9*, *MPO*, *IAP* and *Defensin 1)* gene expression levels, as occurred in chemically and microbiologically induced intestinal inflammation[111, 139, 238], together with the increase in mucus production by goblet cells, correlated with the increase in Bacteroidetes and Proteobacteria. These results suggest that the host defence mechanisms are active and regulate the extracellular matrix, protecting inflammation induced by HFD consumption.

In contrast, cellular infiltration was not observed in histological preparations, although few cellular unions were destroyed. However, the *MPO* gene, a factor needed for neutrophil migration[239], was up-regulated, supporting the developing response to the barrier disruption by neutrophil expression. In agreement with our results, Progatzky *et al.*[148] described that the accumulation of neutrophils and macrophages around the intestine, together with inflammasome activation, resulted from HCD consumption, leading to intestinal inflammation.

Host physiological and morphological changes during development have significant effects on microbiota[69]. We suggest that the maturation of the innate immune system at 4-5 weeks post fertilisation, together with the microbial colonisation and evolution in zebrafish, might be related to some of these changes and powerfully affected by the diet. Therefore, the use of this diet-induced inflammation larvae model might be highly recommended to understand how dietary compounds interact with the microbiota and the host innate immune system, with the aim to mitigate the inflammation process. In the present work, we have achieved a dietary-induced inflammatory state, correlated to microbial changes, in 30 days, whereas dietary experiments in adults generally last 6-8 weeks[138, 146]. The easy and cheap maintenance, the reproducibility and the high number of individuals achieved per spawning confer the high potential of this model.

In conclusion, the present study supports the assumption that in zebrafish, the consumption of a HFD over the period of one month leads to microbiota dysbiosis and intestinal inflammation. We suggest that the fatty acids present in a 10% fat diet, enriched with cocoa butter, could modify the intestinal ecosystem, changing the microbial composition and favouring the increase of Bacteroidetes and Proteobacteria. These bacterial changes activated the innate immune system, inducing an inflammation via NF-κB activation and the secretion of protective molecules by the host. Furthermore, the fatty acids present in the diet directly interacted with receptors present in the enterocytes. These interactions might damage the host intestinal mucosa, provoking shifts in the host immune system and physiology, thereby perpetuating and exacerbating the inflammation and driving and triggering an inflammatory response. This inflammatory state is a pivotal point to study the changes in the microbial composition that might lead to the development of a meta-inflammation in diet-induced obesity and metabolic diseases. We therefore demonstrated that zebrafish larvae are a reproducible and adequate model to test nutritional interventions in early stages; this model allows us to study the effects of a particular diet on microbiota composition and the innate immune system.

Chapter 4

# Host-microbiome interactions in response to a high-fat diet and fish oil supplementation in zebrafish adults

PUBLICATION | In preparation

## 1. Abstract

In this chapter the impact of a high-fat diet (HFD) and the possible beneficial effect of a commercial fish oil rich in docosahexaenoic acid (DHA), on the intestinal microbiome, immune system and lipid metabolism of zebrafish adults was analysed. First, the effect of both experimental diets on the lipid profile of whole fish and liver was analyzed by gas chromatography. Then, the modulatory effect of diets on intestinal microbiota, its metabolic profile and inflammatory stimulation were determined by 16S rRNA Miseq illumina sequencing and RNA expression level analysis. The dietary fat changed not only microbial community composition and metabolism, but also fish lipid profile, whereas commercial fish oil intake ameliorated the effect of the HFD. These results suggested that the HFD exerted changes on the intestinal microbial environment altering microbial composition and its metabolisms. Opposite, HFD supplementation with fish oil benefited the host reducing the changes provoked by the HFD.

## 2. Introduction

Obesity is a complex, multifactorial and chronic condition arising as a consequence of an abnormal accumulation of body fat, with a major negative impact on human health. Genetic, metabolic, environmental and behavioural factors are widely accepted as causal contributors[240]. Epidemiological studies have shown a positive relationship between dietary fat intake and obesity[241]. Diet may play an important role in inducing or preventing obesity by changing the structure and metabolism of the intestinal microbial community[242]. Diet-induced obesity is associated with a less abundant and less diverse gut microbiome[31], and with an impact on host immunological and metabolic functions[192]. Thus, any factor that alters the microbiota could play a pivotal role in regulating this inflammation. For that reason, dietary components that can exert beneficial effects on the gut microbiota will be crucial for the prevention and treatment of chronic disease[243].

Diets rich in polyunsaturated fatty acids (PUFAs) have been shown to positively influence immune function, blood pressure and cholesterol and triglycerides levels in animals and humans[244]. However, western diet is rich in n-6 PUFA and deficient in n-3 PUFA[245]. It is well recognised that long-chain omega-6 PUFA (n-6 PUFA) (linoleic and arachidonic acids) and omega-3 PUFA (n-3 PUFA) (linolenic, eicosapentaenoic and docosahexaenoic acids) play important and opposing roles in the modulation of inflammation[246]. Generally, n-6 PUFAs

promote inflammation, whereas n-3 PUFAs have anti-inflammatory properties[247]. Since n-6 and n-3 long-chain PUFAs compete for the same enzymes for their synthesis and metabolism, their ratio in body tissues determines the profile of lipid mediators involved in the inflammatory response[243]. N-3 PUFA compete with n-6 PUFA reducing the production of n-6 PUFA-derived inflammatory eicosanoids, down-regulate key enzymes that synthesize lipid mediators, and inhibits the expression of inflammatory cytokines such as NF-κB[248]. Although the consumption of an omega-3 rich diet has beneficial effects on host health, the gut microbial changes associated with omega-3 fatty acids are poorly understood[249, 250]. Kaliannan *et al.* in their study conclude that n-3 PUFA supplementation reverts the gut dysbiosis and metabolic endotoxemia linked to western diet consumption[243].

In recent years the interaction between the intestinal microbiota and host metabolism has inspired broad interest[243]. Although rodent models have greatly contributed to our understanding[251] experiments using zebrafish have increased in number. As previously mentioned, zebrafish digestive organs and metabolic pathways are highly conserved, and zebrafish microbiota also plays a crucial role in immunity and host response to pathogens[205]. Pathways regulating microbial recognition and activation of the innate immune response are also highly conserved[252]. Moreover, zebrafish lipid metabolism is very similar to that in humans in terms of intestinal fat absorption, transport, and storage[143]. Diet-induced obesity in zebrafish shares common pathophysiology pathways with mammalian obesity[253]. Due to these similarities, the ease of their genetic manipulation and their economic potential, zebrafish are increasingly being used as models of human diseases[254].

# 3. Materials and methods

## 3.1. Zebrafish husbandry

Wild-type adult zebrafish were bred and maintained in the AZTI Zebrafish Facility following standard conditions described in chapter 1. All experimental procedures were approved by the regional animal welfare body (NEIKER OEBA-2017-002).

## 3.2. Experimental diets

The control diet consisted of a commercial diet for zebrafish adults (Gemma Micro 300; Skretting). The HFD was prepared by enriching the control diet with 20% (w/w) cocoa butter [146]. To prepare the high-fat DHA-enriched diet (HFO) the same HFD was prepared and enriched to a final concentration of 3% (v/w) with a commercial fish oil (85% DHA) (v/w), provided by

Sendabio. Briefly, to prepare the experimental diets, cocoa butter was melted and mixed with the commercial diet. Then the mixture was frozen for 5 minutes to allow it to solidify. It was then cold ground to reduce the particle diameter to appear the same as the normal diet. The HFO diet was prepared following the same procedure, adding the commercial fish oil rich in DHA after melting the cocoa butter (Supplementary Material Figure 2).

The experimental diets were prepared weekly, dispensed in monodosis and stored at 4°C. The nutritional composition of all experimental diets and the stability of the DHA in HFO during storage were also analysed (Supplementary Material Table 2).

### 3.3. Experimental design

Seventy-five adult zebrafish were allocated to 3 tanks (Figure 28). Each tank was fed with approximately 20 mg of one of the experimental diets 3 times per day[255] for 6 weeks (Control, HFD, HFO). Water inflow to the tanks was paused for 1 h during feeding, and fish were allowed to consume the diet for 45 min. After that, the surplus was removed. Twice a week the tanks were cleaned, and half of the medium was removed. In addition, the nitrate, nitrite and ammonium parameters of the water were tested to ensure the medium quality (data not shown).

After 6 weeks 54 fish were anesthetised in ice and weighed. Each individual was weighed and blood glucose was measured with a glucometer (Contour plus, Bayer) as previously described[256]. After that they were euthanised in an ice bath[257]. The intestines and livers of 39 fish were surgically removed under a stereo microscope. Extracted organs were collected in Eppendorf tubes for DNA and RNA extraction and for lipid profile analysis by fatty acid methyl esters (FAMEs). Remaining fish (n=15) were directly collected for lipid profile analysis after euthanasia (Figure 28). All samples were frozen directly in liquid nitrogen. Samples for lipid profile analysis were collected in 1 mL of ice-cold water and 10 µL of 100 mM ethylenediaminetetraacetic acid (EDTA) in order to stop enzymatic reactions. All samples were stored at -80°C until they were analysed.

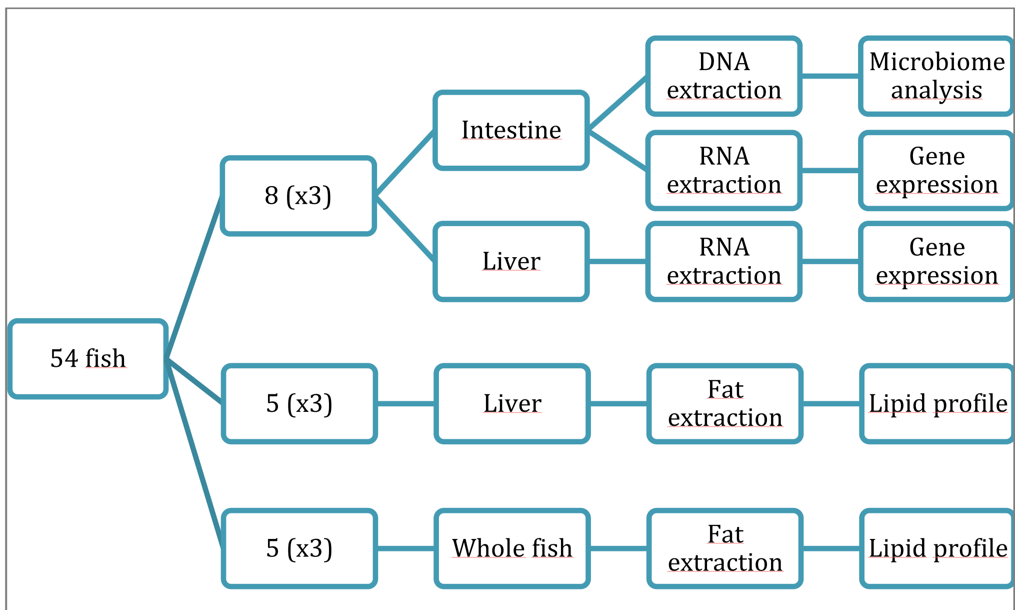



**Figure 28. Workflow of the sample collection and analysis.**

### 3.4. Lipid profile analysis

The lipid profile of whole zebrafish and zebrafish liver was determined following a protocol previously developed in our laboratory[258]. Briefly, total lipid content was extracted following the Folch lipid extraction method[259] with some modifications. Samples were homogenised with a pellet pestle (ultra turrax, Ika), and lipids were extracted using 2:1 chloroform:methanol solution partitioned with water, followed by phase separation, organic phase collection, dehydration and evaporation. Lipids were weighed and re-suspended in organic solvent (2:1 chloroform:methanol) to a final concentration of 1 mg/mL. Fatty acid identification was performed using gas chromatography analysis. Lipids were directly transesterified to generate FAMEs for detection by GC-FID. Analysis was performed using a gas chromatographer (Agilent 6850) equipped with a FID. Separation was performed in a 30 m x 250 µm x 0.25 µm column (DB-WAX, Agilent, USA). FAMEs were identified by comparison with cis and trans FAME standard retention times (Sigma). FAMEs were estimated as percentages relative to total peak area.

### 3.5. Microbiome analysis

Total DNA from the same 24 intestinal samples used for RNA extraction was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. DNA

concentration and purity were analysed measuring the absorbance at 260nm and A260/A280 ratio respectively with a Nanodrop (Thermo).

The microbial community composition of 8 fish per treatment (n=8) was characterised by sequencing the V3-V4 16S rRNA region using the primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21[155] in an Illumina Miseq Platform. Paired reads were merged using FLASH[156] after removing primer sequences with Trimmomatic. The rest of the bioinformatic analysis was performed using the Qiime (Quantitative Insights Into Microbial Ecology) software package (v.1.9.0)[50]. Sequences were clustered as operational taxonomic units (OTUs) of 97% similarity using UCLUST[260]. OTUs were checked for chimeras using the RDP 'gold' database and assigned taxonomy using the Greengenes database (version 4feb2011)[261]. Alpha diversity indices (Number of observed species, Chao1 and Shannon index) were calculated using the QIIME pipeline and displayed in R. Beta diversity metrics were analysed by weighted UniFrac and visualised in a principal component analysis plot (PCoA). The significances of grouping in the PCoA plot were tested and Analysis of Similarity (ANOSIM) with 999 permutations was employed. The significant fold changes of OTUs were performed in DESeq2 R package[262]. Pearson correlations among gut microbiota (OTUs that significantly differed among diets), the composition of the whole fish lipid profile, gene expression, and fat percentage were calculated and displayed in R (ggplot2 and corrplot packages).

In the current study, PICRUSt was used to obtain an overview of the metabolic features of the bacterial communities in zebrafish which had been fed experimental diets. The OTUs were associated with known bacterial genomes pre-calculated in PICRUSt, by first picking closed OTUs against the Greengenes 16S rRNA gene database (13.5) using QIIME[263]. The resulting OTU table was then normalised using the script normalize_by_copy_number.py and used for metagenome inference of Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs using PICRUSt. The difference in the predicted molecular functions of the bacterial communities was determined by Welch´s t-test using the Statistical Analysis of Metagenomic Profiles (STAMP) software package[264.]

In addition, raw sequences were deposited in the European Nucleotide Archive (ENA) under the project number PRJEB24592.

### 3.6. Gene expression analysis

Total RNA from the intestines and livers of 8 zebrafish per treatment (n=24) was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The quantity and quality of RNA samples were determined by capillary electrophoresis using an

Agilent 2100 Bioanalyzer (Agilent Technologies). Only RNA samples with a RIN of at least 8.5 were used. These values fulfil one of the requirements of a satisfactory qPCR experiment[211].

cDNAs were synthesised from the RNA samples in a reverse transcription reaction performed with TaqMan™ reverse transcription kit (Applied Biosystems) containing 20 ng of RNA per assay. Changes in mRNA expression of genes related to the innate immune system (*ASC, CASP1A, IL-1B, IL10, IL22 TNFA, NFKB, MYD88, MMP9, MPO* and *NOD1*), host-microbe interaction (*TLR4, TLR5, TLR22, DEF1* and *MUC2*), metabolic activity (IAP), insulin growth factors (*IGF1* and *IGF2*) and lipid metabolism (*FAS* and *PPARg*) were monitored by real-time qPCR using β-actin (*ACT*) and elongation factor 1 (*EF1*) as reference genes to normalised the results, as previously described in chapter 3. Primer sequences are listed in chapter 3 and supplementary material Table 3.

### 3.7. Statistics

Data are expressed as means +/- SEM. The p-values were calculated with a T-test and ANOVA test, using the software Statgraphics v16.1.17 (StatPoint Technologies, Inc.) and SPSS version 3.4 (IBM). All p-values were adjusted via Bejamini Hochberg correction[219] or false discovery rate (FDR). A p value $< 0.05$ was considered statistically significant.

## 4. Results

### 4.1. Effect of experimental diets on zebrafish glucose and lipid profile

At the end of the experiment, fish blood glucose levels were measured. Although no significant differences were identified, blood glucose levels tended to be higher in HFD samples, whereas in samples fed with HFO they tended to decrease. In addition, total body fat percentage (per weight) tended to increase in HFD- and HFO-fed animals compared to the control group.

In the current study, the fatty acid profiles of liver and whole fish fed with different diets was analysed by FAMEs (Table 2). A significantly increased level of palmitic (16:0) and stearic acids (18:0) was observed in HFD-fed animals compared with the control diet (20.7 versus 19.3% of palmitic and 10.3% versus 3.9% of stearic respectively, $p < 0.05$). In contrast, palmitic acid decreased in HFO-fed animals (17.8%, $p < 0.01$). The most abundant monounsaturated fatty acid (MUFA), oleic acid (16:1), was augmented by 55% in the HFD group compared to the control (17.2 versus 32.2, $p < 0.01$), while in the HFO samples it was 13% lower (27.9, $p < 0.05$).

For PUFA detected in whole fish, linoleic (18:2) and alpha-linoleic (18:3) acids accumulated in the HFD group ($p < 0.01$), while EPA (20:5) and DHA (22:6) fell by 1.6 and 1.4 times, respectively ($p < 0.01$). Conversely, EPA and DHA levels rose in HFO-fed animals 1.3 and 1.8 times respectively ($p < 0.01$).

Thus, the total SFA and MUFA were augmented in HFD-fed animals compared to control animals (32.5% versus 26.6%, and 36% versus 23.8%, respectively, $p < 0.01$), whereas animals fed HFO exhibited reduced levels (29.6% and 31.2%, respectively, $p < 0.01$). Interestingly, the total levels of PUFA and EPA + DHA were much lower in the HFD group (30.4% versus 48.2% and 10.34% versus 15.7% respectively, $p < 0.01$). In HFO samples PUFA rise (36.9% and 18.1% respectively, $p < 0.01$) (Figure 29).

In the liver, the only incremented SFA and MUFA in the HFD samples, compared to the control, were stearic (9.1% and 4.5% respectively, $p < 0.05$) and oleic (17%, 7.9% respectively, $p < 0.05$) acids, which, in contrast, decreased in the HFO group (6.3% and 8.1% respectively, $p < 0.05$). In PUFAs, DHA only changed in HFO-fed fish comparing to HFD, increasing its level (1.5% and 0.8% respectively, $p < 0.05$). Finally, the total PUFA content in HFD and HFO samples changed significantly, diminishing 1.25 times in HFD and conversely, increasing 1.26 times in HFO ($p < 0.01$). Results are summarised in supplementary material table 4.

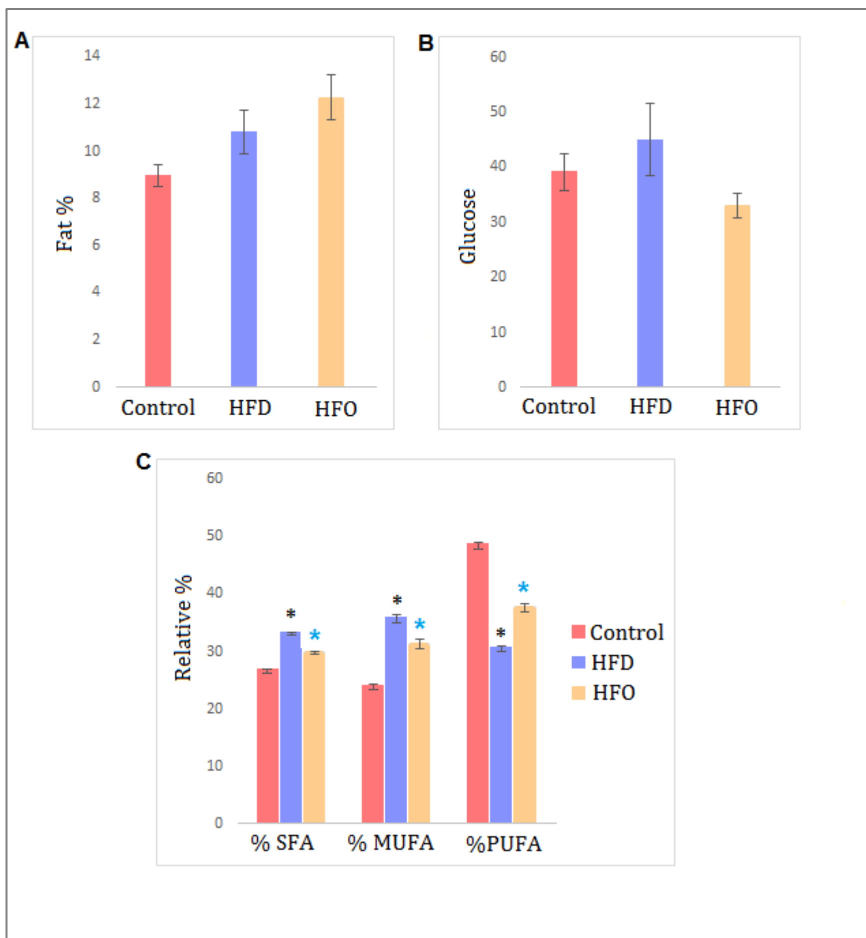



**Figure 29. Effects of HFD and HFO on fish.** A: total fat percentage, B: glucose level in blood and C: relative proportion of SFA, MUFA and PUFA, for the experimental diet groups (control, HFD, HFO) * represents statistically significant differences between control and HFD ($p < 0.05$), whereas * represent differences statistically significant between HFD and HFO ($p < 0.05$).

## 4.2. Changes in the intestinal microbial community in response to the high-fat diet and DHA supplementation

After 6 weeks of 3 different dietary interventions, the DNA from the intestine of 8 fish per diet (n=24) was extracted and sequenced by 16S RNA tagging for analysis of the gut microbial community. A total of 1639702 sequences of the V3–V4 region of 16S rRNA were collected from the 24 intestinal samples, of which the median sequence number was 72322 (25857 SD). All the sequences were clustered into 1402 OTUs at a 97% similarity level. The Venn diagram shows that 50% of the OTUs (699 OTUs) were common to all three diets (Figure

30 A). However, 11% of the OTUs (160 OTUs) were found only in HFD-fed groups (HFD and HFO), and 2% (28) corresponded to OTUs present only in the DHA-supplemented group (HFO).

The changes in bacterial richness (expressed by SOBS and Chao1) and diversity (expressed by the Shannon index) in response to the HFD and HFO diets were analysed. None of the diversity indices tested indicated significant change. These results suggest that the consumption of HFD and HFO did not affect the alpha diversity of the community (Figure 30 B). However, as demonstrated by unweighted UniFrac analysis of beta diversity, different sample clustering was observed between diet groups (ANOSIM $p=0.001$). This means that the consumption of a HFD changed gut microbiota composition significantly compared to the control group (ANOSIM Control-HFD $p<0.001$). In addition, there is a significant effect on the microbiota in response to DHA supplementation compared to HFD (ANOSIM HFD-HFO $p<0.001$) (Figure 30 C).

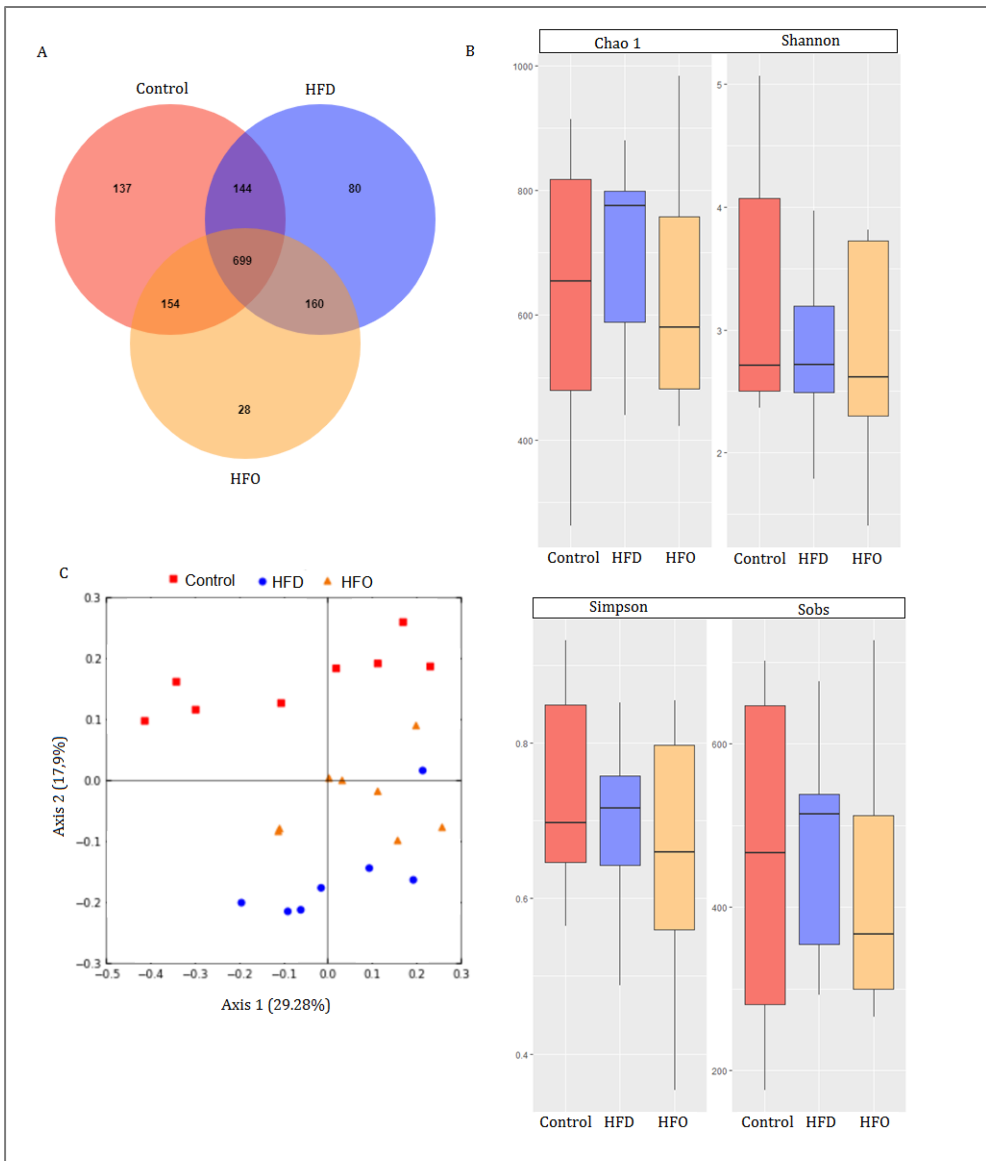



**Figure 30. Alpha and beta diversity index.** A: Venn diagram showing the unique and shared distribution of OTUs of control (red), HFD (blue) and HFO (orange) groups. B: SOBS and Chao1 index as estimators of the gut microbiota community richness, and Shannon index as community diversity estimator. C: Principal component analysis (PCoA) score plot based on unweighted UniFrac distance of control (red squares), HFD (blue dots) and HFO (orange triangles) ($p < 0.001$).

At phylum level, the most abundant phyla in all samples were *Fusobacteria* (45.5%, 44.5% and 64.5% in control, HFD and HFO samples, respectively), *Proteobacteria* (37.2%, 50% and 30.5%), *Cyanobacteria* (14%, 1.2% and 2.8%) and *Tenericutes* (1.8%, 3.1% and 1%) (Figure 31 A). At family and genus level significant differences were observed among the diets. Pseudomonadaceae increased in the HFD (0.8% vs. 26.7% in control and HFD samples, respectively) ($p<0.05$). In contrast, *Tenericutes* CK-1C4-19 decreased (1.6% and 0.1%,

respectively) (p>0.05) (Figure 31 B). At genus level, the most important changes were found in *Pseudomonas*, *Acinetobacter* and *Tenericutes ck1c4-19*. *Pseudomonas* and *Acinetobacter* significantly increased (p<0.03) (from 0.8% to 26.6%, *Pseudomonas*, and from 0.001% vs. 0.1%, *Acinetobacter*) with the consumption of a HFD. Conversely, *Tenericutes ck1c4-19* decreased (p<0.05) (from 1.6% to 0.1%). As a result, in the HFO group *Pseudomonas* and *Acinetobacter* tended to decrease (5.1% and 1% respectively), whereas *Tenericutes ck1c4-19* increased (1%) (Figure 31 C).

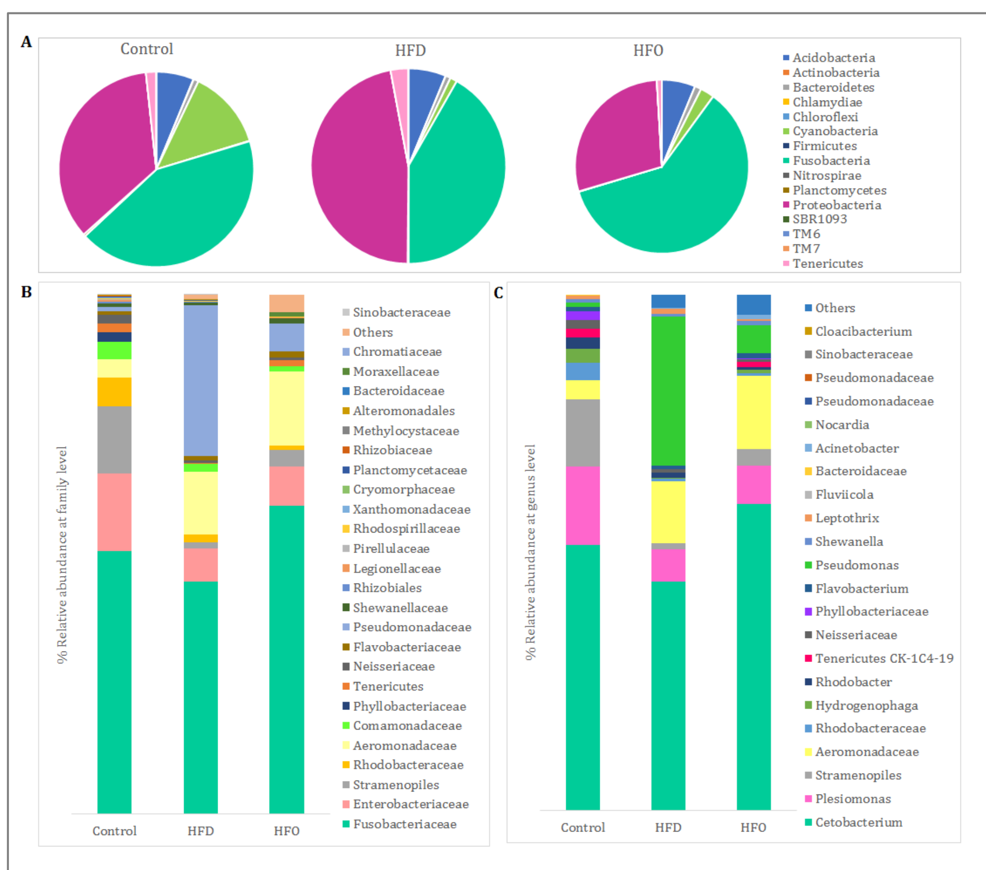
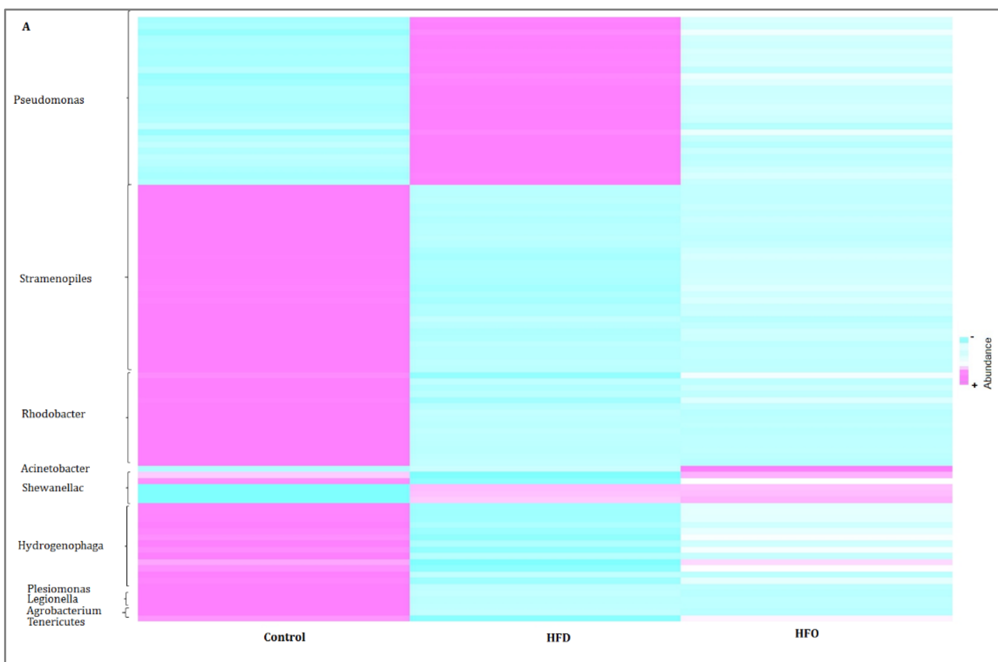



**Figure 31. Differences in relative abundance.** A: Pie chart representing the relative abundance of phyla in animals fed a control diet and the HFD and HFO. Stacked bar chart representing the mean in control and HFD B: families and C: genera.

In order to identify the specific bacterial taxa differentially affected by HFD and DHA supplementation, the significant fold-change at OTU level was calculated. Based on this analysis 97 OTUs were identified as key phylotypes responsible for the differences among the dietary

groups (Figure 32 A). In particular, HFD samples were enriched by 17 OTUs affiliated to *Pseudomonas*, 3 to *Shewanella* and 1 to *Acinetobacter*. In contrast, 1 OTU affiliated to *Tenericutes CK-1C4-19*, 24 to *Stramenopiles*, 23 to *Rhodobacter*, 2 to *Plesiomonas shigelloides*, 3 to *Legionella* and 2 to *Agrobacterium* were down-regulated in HFD-fed animals (Figure 32 B). When HFD was co-supplemented with DHA, 11 out of 17 *Pseudomonas*-affiliated OTUs, and one of the OTUs affiliated to *Legionella* decreased. In contrast, the unique OTU affiliated to *Tenericutes* increased in HFO. In addition, an increasing tendency was observed in OTUs affiliated to *Hydrogenophaga* and *Plesiomonas shigelloides* in HFO samples (Figure 32C).



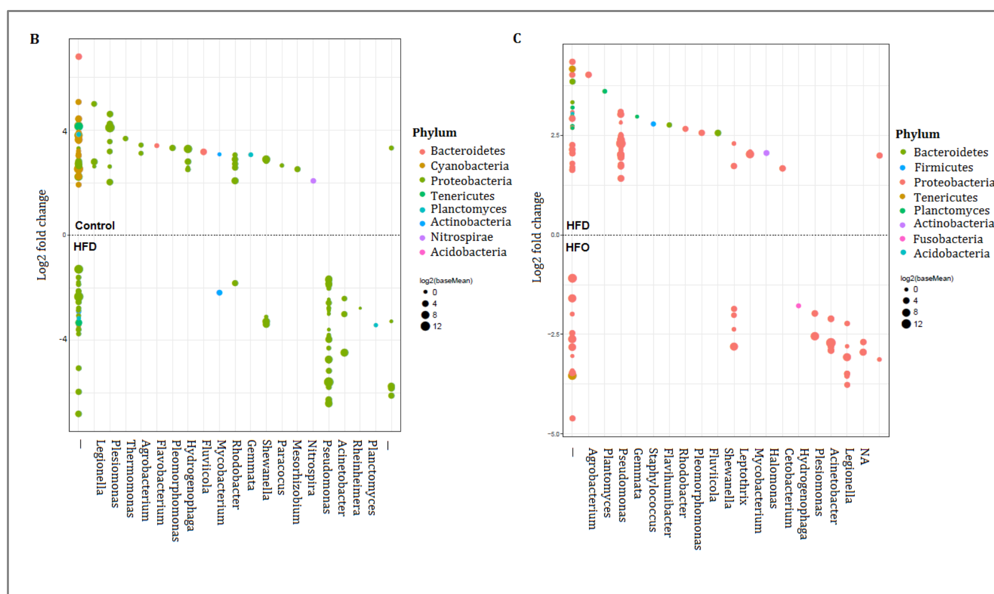



**Figure 32. Differences in OTU composition.** (A) Heatmap comparison based on the relative abundance of 97 key phylotypes identified from the experimental diets. OTUs were significantly different (q < 0.1 FDR) between control and HFD-fed-animals. B: Upper axis represents OTUs with a log2 fold positive difference for control relative to HFD while the lower y-axis is the negative fold difference of the control relative to HFD C: the difference of HFD compared to HFO. Each point represents a single OTU coloured by phylum and grouped on the x-axis by taxonomic genus level, size of point reflects the log2 mean abundance of the sequence data.

## 4.3. Microbial community predictive functions

The results of the predicted metabolic functions of the microbial communities were analysed in PICRUSt using level 3 categories. In animals fed the HFD, three pathways involved in amino acid metabolism, ABC transporters, and glutamatergic synapse were more dominant (Figure 33 A). Whereas for animals fed HFO, pathways concerning adiponectin signalling, transporters, lipid metabolism including linoleic and arachidonic acids and ABC transporters, and tryptophan metabolism were more prominent. Pathways related to the synthesis of ketone bodies, lysine-arginine-proline metabolism, bacterial chemotaxis, N-glycan biosynthesis, and peptidoglycan metabolism were regulated in an inverse manner in both HFD and HFO animals, suggesting a beneficial effect of DHA on those pathways (Figure 33 B).

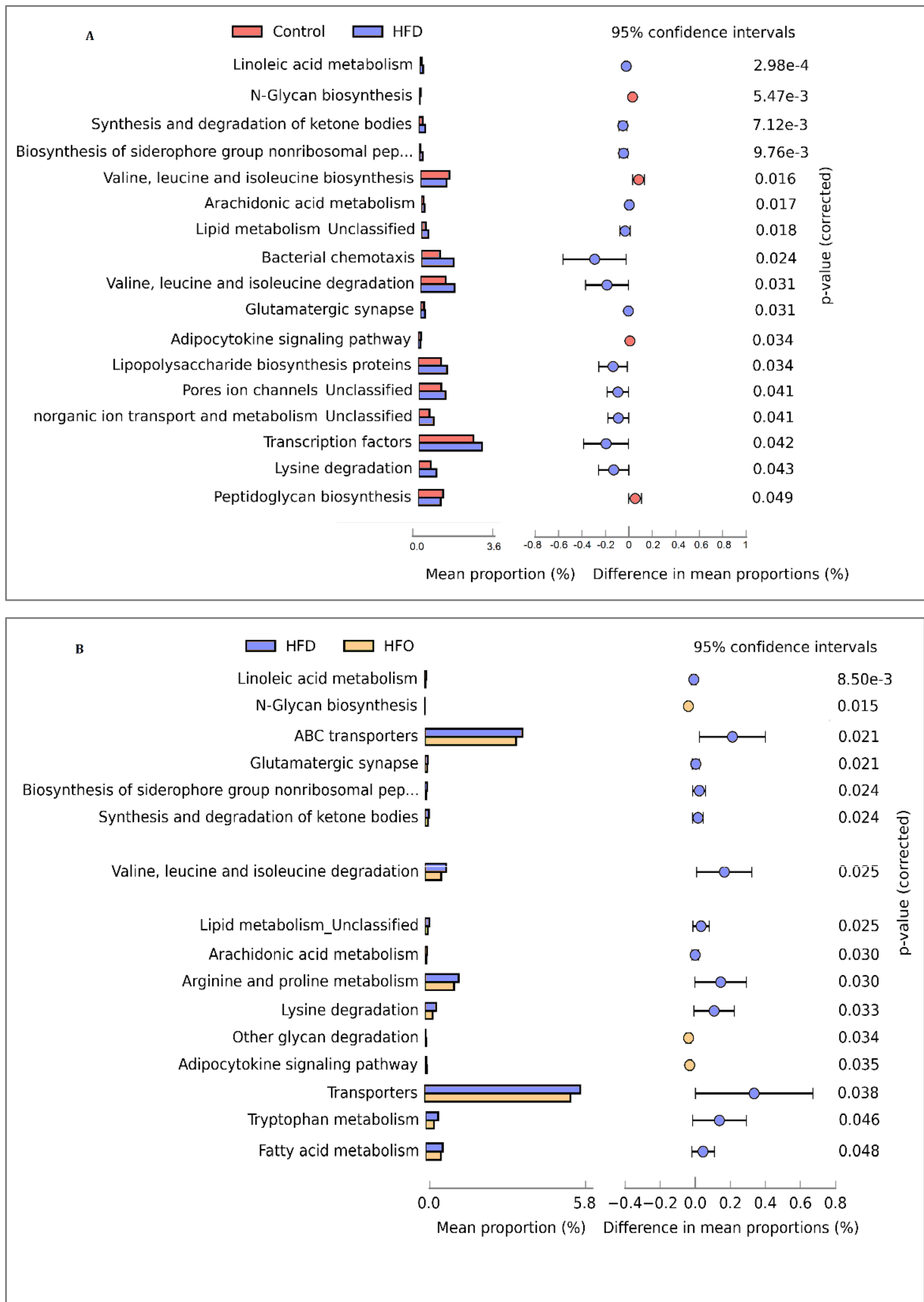



**Figure 33. PICRUST.** Phylogenetic Investigation of Communities by Reconstruction of Unobserved States of A: control vs HFD and B: HFD vs HFO samples. The mean proportion of each inferred metabolic pathway is represented in bars. The difference in mean proportion and the p value are also represented.

## 4.4. The effect of experimental diets on immunity

The expression of 19 genes related to the zebrafish immune system were analysed from the intestines of 8 individuals per treatment by qPCR. Additionally, genes associated with inflammation and with lipid and glucose metabolism were tested in 8 livers per diet group. None of the genes tested changed its expression in response to the experimental diets, suggesting that the HFD did not induced inflammation in zebrafish adults (Figure 34).

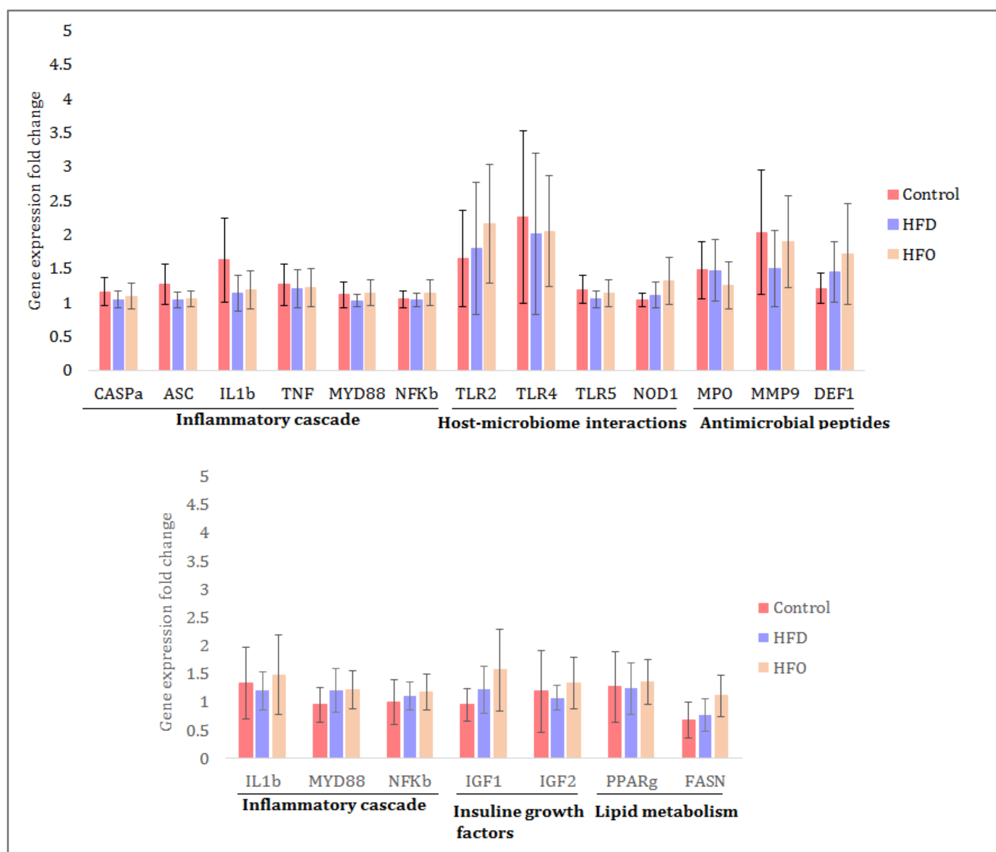



**Figure 34. Gene expression fold change analysis after 6 weeks of diet exposure in control, HFD and HFO groups in** A: intestine and B: liver. Error bars indicate the standard error of the mean.

## 4.5. Correlation between fatty acids and microbial changes

OTUs that significantly differed among diets were correlated with the fatty acid profiles of whole fish calculated using Pearson correlation analysis (Figure 35) The heatmap shows significant correlations among OTUs affiliated to Pseudomonas, Acinetobacter, Shewanella, Plesiomonas and Tenericutes CK-1C4-19 with the fatty acids related to different diets.

In the HFD-fed group, the increase of cocoa butter fatty acids (stearic, palmitic and oleic acids, SFA, and MUFA) and the decrease of PUFA, omega-3 and EPA+DHA was correlated with the augmentation of Pseudomonas- and Acinetobacter-affiliated OTUs, and in contrast, with the reduction of Tenericutes CK-1C4-19 and P. shigelloides OTUs. Interestingly, Pseudomonas OTUs were also positively correlated to the increase in blood glucose. In addition, 2 OTUs assigned to Shewanella were enriched in HFD samples, whereas 3 OTUs decreased. As in the groups mentioned above, both changes were correlated with the fat content. Conversely, the HFO-fed group exhibited a decrease in Pseudomonas, and an increase in Tenericutes CK-1C4-19 and 2 Shewanella OTUs, which correlated with the enrichment of PUFA, omega-3 and EPA+DHA, and with the reduction of cocoa butter fatty acids. Glucose level also correlated positively with the decrease in Pseudomonas in these samples.

The decrease in the relative abundance of Rhodobacter, Hydrogenophaga, Plesiomonas, 2 OTUs affiliated to Shewanella, and Legionella was not correlated with changes in the lipid profile.

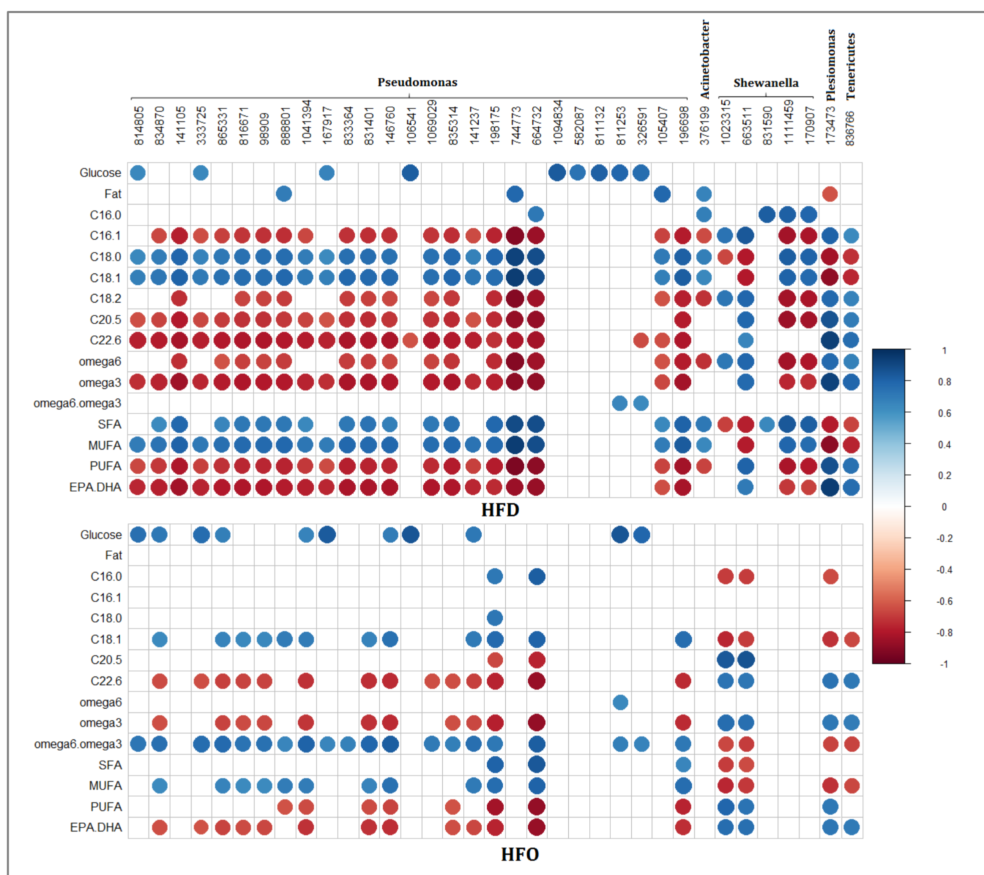



**Figure 35. Correlation plot showing Pearson correlations between fatty acid lipid profile and key phylotypes.** Blue dots are positive correlations, whereas red dots are negative correlations. Dot size is determined by the correlation grade. Only significant correlations are represented ($p < 0.05$).

## 4.6. Correlation between fat and gene expression

The differential expression of genes in the intestines and liver were compared to whole fish and liver lipid profiles in order to examine any correlation. Interestingly, the increase of fat in HFD samples was positively correlated to the increase of *TLR 2* and *4* expression. However, in HFO samples, there was a positive correlation between the decrease of stearic acid and *TLR 2* and *4* expression. In addition, in the liver, a negative correlation was observed between omega-3 liver parameters (C 22:6, total omega-3, and EPA+DHA ratio) and *FASN* (fatty acid synthase), *NFKB* and *MYD88* genes tested in the HFD group (Supplementary Material Figure 3).

## 5. Discussion

It is widely accepted that diet alters the gut microbiota diversity in humans and other animals[265]. In this chapter the ability of two experimental diets to induce changes in intestinal microbial composition, the immune system, fatty acid lipid profiles, and metabolic functions in adult zebrafish was investigated. HFD was formulated by enriching the fat content with cocoa butter (20%), composed of SFA and MUFA, as it has previously been demonstrated that 20% fat-increased HFD induces obesity, body fat accumulation and intestinal dysbiosis in zebrafish[146,121]. In contrast, the HFO diet was supplemented with a commercial fish oil in a concentration previously described as effective in zebrafish[266]. Diets rich in omega-3 have been shown to positively influence host health by maintaining microbial homeostasis in humans[249], enriching beneficial bacteria, and reducing other microbes. However, the bacterial phylotypes affected by the effect of omega-3 and the mechanism are not yet understood[250].

Numerous studies have demonstrated that the consumption of a HFD leads to intestinal microbial dysbiosis[190] [251], altering the community composition and reducing the diversity[32].In our study, no significant changes in community richness or evenness induced by the experimental diets were observed. This result is in agreement with Wong *et al.*[121], who did not report changes in alpha-diversity at any age in zebrafish fed a HFD (20% fat-enriched). Conversely, they relate community membership changes from 35 dpf to adulthood with dietary fat, as occurred in our study. In the results of our experiment, the PCoA plot showed a clear separation of control, HFD and HFO dietary groups suggesting that dietary fat content exerts an effect on the intestinal microbial community. Furthermore, our results indicate that fish oil supplementation may induce a positive effect on the microbial dysbiosis provoked by HFD consumption.

Besides changes in the overall gut microbial community structure, HFD consumption induced microbial alterations at different taxonomic levels. The dominant phyla for all diets were *Fusobacteria* and *Proteobacteria* in zebrafish adults, as previously described[122]. However, the difference in the source of fat chosen to formulate the HFD, the age of the fish, and the microbial community of the facilities make it difficult to compare our results directly to other studies that described zebrafish microbial changes induced by other formulations of HFD.

Ninety-seven specific bacterial taxa altered by the HFD and modulated by fish oil supplementation were identified. In addition, this change correlated with changes in host lipid profile and immunity. An increase in OTUs affiliated to *Acinetobacter*, as previously described

in obese human individuals consuming a HFD[267], and *Pseudomonas* was observed in HFD samples in zebrafish. *Pseudomonas* are widely distributed, constituting a part of the normal microbiota of the aquatic environment[268]. They are part of zebrafish core microbiota[125], occurring in an abundance of 0-2% in normal conditions of domesticated zebrafish, as in our control group. In a HFD environment, Wong *et al.* also found *Pseudomonas* OTUs were more abundant[121]. Additionally, the increase in both taxa was positively correlated with the increase in fat and cocoa butter fatty acids, and with the decrease of omega-3 related fatty acids. However, in HFO samples the fish oil exerted a positive effect on *Pseudomonas's* relative abundance, which correlated with the decrease in cocoa butter fatty acids and the increase in omega-3. A previous study corroborated the positive effect of a commercial omega-3 supplement on the growth of *Pseudomonas aeruginosa* in *Galleria mellonella*, a caterpillar model, and mice[269 270]. Dietary control of *Pseudomonas* by modulating omega-3 intake could represent an interesting target in nosocomial infections caused by this taxa, as some species are described as highly resistant, and frequently escape eradication by antibiotics, constituting a serious problem[271].

The HFD also produced an effect of reducing the relative abundance of *Tenericutes CK-1C4-19, P. shigelloides*, *Stramenopiles*, *Legionella*, *Rhodobacter* and *Hydrogenophaga*-related OTUs. Increases in *P. shigelloides, Rhodobacter* and *Legionella* have previously been associated with microbial changes due to the consumption of a high-protein diet (HPD) and a gluten-rich diet in zebrafish[140, 141]. Interestingly, Koo *et al.* reported that these changes were associated with a gluten rich diet, which contained less fat than the control, thus supporting our results[140]. Furthermore, Rurangwa *et al.* found that HPD-fed zebrafish positively associated with an increase in protein biosynthesis and amino-acid transport[47]. In our HFD group the digestion and absorption of carbohydrates and peptidase activity were reduced in comparison to the control group, in contrast to what occurred with HPD and gluten diets. In addition, changes in the above taxa did not directly correlate with the effect of dietary fat. This could be explained by the influence exerted by increases in microbes in response to a HFD. The intestine is a dynamic ecosystem where environmental changes result in microbial ecological niches driving shifts affecting the microbiome composition. The interactions between the microbiota and the niche environment are complex and diverse, but they are generally deterministic[7]. The increase in fat in the intestine favours the increase of *Pseudomonas*, in consequence reducing the relative abundance of other taxa, such as those mentioned above, and therefore modifying the gut environment. Bacterial interference occurs via exploitative competition where one organism

consumes the available resources, or via direct competition, characterised by an active secretion of competitor molecules (siderophores, secondary metabolites, or toxins)[272].

The relative abundance of the inferred functional capacities of the microbial communities showed that different pathways related to bacterial interactions were affected by the diets. Lysine, arginine and proline metabolism and siderophore biosynthesis were up-regulated in the HFD group. These pathways might be associated with *Pseudomonas* metabolic activity, which usually produce two siderophores, pyoverdine and pseudobactin, conferring them with a higher capacity to colonise and compete with the other microorganisms of the niche[273]. In addition, a bacterial chemotaxis pathway, previously associated with the bloom of *Proteobacteria* in mice[274], and pore ion channels were over-expressed in HFD-fed animals. This suggests that bacterial movement and communication skills may provide a growth advantage to *Pseudomonas*, which has the ability to form biofilms[275]. Conversely, the N-glycan biosynthesis pathway exhibited lower expression in HFD-fed animals, as previously found in obese children. In humans, N-glycan biosynthesis disorders are associated with metabolic diseases[276]. In HFO-fed animals, the bacterial chemotaxis pathway was down-regulated, whereas N-glycan biosynthesis was increased, suggesting a beneficial effect of the fish oil in modulating bacterial interactions.

In summary, the results suggest that dietary fat causes changes in the intestinal environment, enhancing the growth of *Pseudomonas* and *Acinetobacter* and reducing the abundance of other taxa. This change involves the augmentation of bacterial metabolism, which may enhance the growth and expansion of *Pseudomonas* and reduce the resources of the niche which are needed for the maintenance of the other microbes, such as *Tenericutes CK-1C4-19, P. shigelloides*, *Stramenopiles*, Legionella, *Rhodobacter* and Hydrogenophaga. Fish oil supplementation seems to alleviate these effects by decreasing the relative abundance of *Pseudomonas* and increasing *Tenericutes CK-1C4-19* and *Legionella,* thus helping to maintain intestinal homeostasis.

In addition, in the HFD-fed group, the increment in animal fat content correlated with the up-regulation of *TLR2* and *4* expression in the intestine, even though they were not overexpressed. TLR 2 is typically bound by gram-positive bacteria, whereas TLR4 is activated by gram-negative. It is well known that the increase of gram-negative bacteria, such as *Pseudomonas* in our experiment, in response to a HFD may increase the expression of these receptors, activating an inflammatory response. However, dietary fats are also able to induce the expression of those two receptors, as mentioned in chapter 3, specifically stearic and palmitic acids. Additionally, TLR4 can be also activated by lauric acid[277]. All these acids are

components of the cocoa butter used to formulate the HFD. It is known that SFAs trigger inflammation activating MyD88 adaptor protein which induces activation of NF-κB[277], whereas omega-3 and omega-6 are potent TLR4 inhibitors[278]. In the HFO-fed group, the presence of fish oil decreased the stearic acid level, which correlated positively with the decrease of *TLR 2* and *4* expression. Interestingly, the expression levels of *NFKB* and *MYD88* in the liver were negatively correlated with the increase of DHA in HFO samples. In addition, functional capacities for omega-6 metabolism were lower in HFO samples. The activity of linoleic and arachidonic acid metabolism depends on the omega-6/omega-3 ratio, so this indicates a positive effect on omega-3 increment induced by the fish oil. This result suggests that fish oil intake decreases inflammatory cytokine production by inhibiting the activation of NF-κB[279]. Dietary changes in the intestinal environment favour the increase of *Pseudomonas* and specific SFA, which are able to induce *TLR 2* and *4* expression. Fish oil may exert a beneficial effect on inflammation. As no inflammation was observed in this experiment more research is needed to clarify this question.

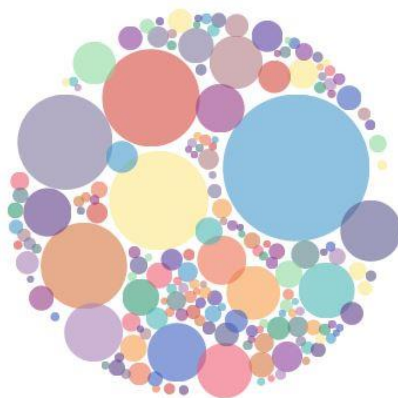
In the liver the expression of *IGF2*, and *FASN*, correlated negatively with the increase of DHA in the HFO group. IGF2 is a hormone associated with insulin and glucose uptake whose overexpression has been related to obesity and diabetes[280], whereas FASN relates to lipogenesis in hepatic tissue[281], and this expression level did not change in previous studies performed in adult zebrafish[282]. Functional pathways regulating lipid metabolism, obesity and diabetes were suggested to differentially express in HFD and HFO groups. In HFD samples metabolism related to transport, biosynthesis and degradation of branched-chain amino acids (BCAA) and ketone bodies, was heightened. ABC transporters, whose metabolism was increased, are essential for the maintenance of cell and tissue homeostasis as they drive the exchange of nutrients and metabolites, such as hormones, amino acids, ions, vitamins and lipids[283]. These transporters have been found to be overexpressed in obese Chinese children and adolescents[276]. Rurangwa *et al.* also correlated the heightened expression of transport metabolism in response to diet with the activation of immune response in zebrafish[141]. The up-regulation of the biosynthesis and degradation of BCAA (leucine, isoleucine and valine) and ketone bodies may play a role in obesity and diabetes in humans and rodents[284,285]. Fish oil may help to reduce this effect, as we found that BCAA metabolism was down-regulated in the HFO group. Previous studies in mice suggested that the reduction in isoleucine levels promote lipolysis via induction of lipolytic genes and by the suppression of lipogenesis in the liver[286]. Finally, in HFO samples adipocytokine signalling, related to adiponectin release which has anti-obesogenic and anti-diabetogenic properties, was increased, as previously described[287]. These results suggest that

changes produced in the intestinal microbiome by the HFD are associated with obesity and diabetes metabolic pathways. In contrast, fish oil supplementation seems to contribute to reverse this effect.

In recent years microbes have been increasingly recognised as having an important role in brain development and behaviour[288]. In the current study metabolic pathways associated with the homeostasis of the gut-brain axis have also been affected by HFD and fish oil supplementation. Glutamatergic synapse signalling, whose alterations may play a role in the acute regulation of food intake and energy balance[289], was up-regulated in HFD samples. In contrast, tryptophan metabolism was reduced in HFO samples. This metabolite participates in serotonin release, whose biosynthesis in the brain is regulated by gut microbiota[290]. This neurotransmitter is an important gastrointestinal regulatory factor, as its dysregulation has been associated with several diseases in human such as intestinal bowel disease and cardiovascular disease[290]. This suggest, that the microbial changes due to a HFD affect functions in other organs beside the intestine, where the microbiota plays a defined role. However, fish oil may palliate these effects.

In conclusion, this research supports that the HFD feeding in zebrafish adults induces a negative effect on intestinal environment. These environmental alterations exert changes in the intestinal microbial community favouring the increase of *Pseudomonas* and *Acinetobacter*. The augmentation of these taxa induces the reduction of other microbes thus losing diversity and affecting the intestinal homeostasis. The intestinal microbes and the dietary fatty acids are able to bind the enterocytes TLR, activating the inflammatory response that characterised obesity. Furthermore, the bacterial metabolism which favours lipid transport, obesity, insulin release and gut-brain axis mediation are up-regulated by the HFD consumption. In contrast, it was demonstrated that the HFD supplementation with fish oil may exert a beneficial effect on these changes alleviating the negative impact induced by the HFD.

# General discussion



The zebrafish is a model organism widely used for basic research in multiple fields of study. In microbial research, it has been used to understand the pathogenesis of diverse microorganisms and interactions of microbes, such as pathogens *vs* probiotics[137]. The optical transparency of embryos and larvae constitutes a distinctive feature of zebrafish, allowing the visualisation of bacteria in vivo, previously labelled with fluorescence. It is also interesting to work with transgenic lines to observe the interaction of pathogens with the immune system. Moreover, from our perspective, this model allows us to go further than has previously been possible.

To determine the possible use of zebrafish as a feasible model for the study of host–microbiota interactions, two different types of study have been proposed herein. The first aimed to shed light on the possibility of human microbiota transplantation into GF zebrafish as a recipient, describing which species of the human flora are able to colonise the zebrafish digestive tract. The second was performed to gain an in-depth understanding of the impact of diet on the host and its intestinal microbiota.

In the first chapter of this thesis, a colonisation protocol was developed maintaining sterility and anaerobiosis. The success of this part was crucial for the subsequent studies because at that point all the colonisation approaches reported by other studies failed[149]. Our colonisation protocol was based on static immersion, a technique easier to carry out than microinjection. Toh *et al.* did not report differences in terms of colonisation success using the two techniques, recovering only 2 out of 30 strains used in the experiment inside the zebrafish digestive tract[149]. However, using our protocol, all the strains included in the study, which contained not only facultative anaerobes but also obligate anaerobes, were identified inside the zebrafish gut at 48 hpi.

It has previously been reported that facultative anaerobes are able to colonise and grow inside the zebrafish gut, occupying almost all the niche space and resources[9]. For this reason, Toh *et al.* decided to limit such microorganisms in their experiment[149]. In addition, the bacterial species proposed in both, Toh *et al.*'s experiment and our experiment are not a part of the normal flora of zebrafish, which may cause difficulty for their establishment and colonisation success in this hostile environment. For this reason, we tested the colonisation protocol not only in consortium, but also with each bacterial strain separately (monoassociation). In consortium, although all the strains included in the study were found inside the zebrafish intestine tract, in general monitoring showed lower counts. Facultative anaerobes in this consortium, such as *E. coli* and *E. faecalis*, were able to establish and grow with time, whereas the growth of the obligate anaerobes, *E. limosum* and *B. breve*, decreased and finally disappeared. The bacterial

interspecific competition for the niche resources might act as a limiting factor. In contrast, in monoassociation, the absence of interspecific competition allows the colonisation of the zebrafish gut, presenting higher counts for all the strains.

Taking into account these promising results, we designed the study presented in the second chapter. There, the purpose was to determine which human microbiota phylotypes were able to colonise the digestive tract of the zebrafish larvae. This objective represented a great challenge because, as far as I know, no-one has previously published these useful data. To date, previous studies transplanting mice intestinal microbiota into GF zebrafish larvae only recovered facultative anaerobes inside the output community[9]. As previously described, differentiated parts of the digestive tract, tissue cell types and microbial colonisation and functions are highly conserved from zebrafish to mammals. These features might make zebrafish a suitable model for the transplantation of human flora. Here we demonstrated that up to 60 human intestinal bacterial phylotypes, representing 2% of the OTUs present in humans, were able to colonise the digestive tract of GF zebrafish larvae.

One of the limitations encountered in achieving better colonisation results was the zebrafish intestine as a niche itself. Even though the structure and cell types are conserved, the length and size are very small in zebrafish. For this reason, the high microbial load present in the human slurry inoculum needed to be diluted to carry out the colonisation, losing microbial taxa in the dilution process. In addition, the niche environmental factors, such as oxygen concentration and temperature, are also distinctive features which interact with the microbes shaping the final community[9]. Microbial activity also plays an important role in the colonisation process. As a result, *E. coli* is able to multiply in the zebrafish gut, reaching the 95% of the counts in 48 h, whereas the relative abundance in the inoculum was 0.1%. *Escherichia coli* is a ubiquitous bacterium able to persist and replicate in water and soil environments. Thus, it is a faecal indicator of water quality[291]. An important strategy in our infection protocol that others have not reported was the enrichment of the larvae medium with vitamins and other ingredients to favour the growth of fastidious microorganisms. However, while these microbes might take advantage of these resources, other bacteria may also use them to grow more. The excessive growth of *E. coli* may lead to ecological resource consumption and space invasion, not permitting the establishment of other microbes which grow slower. Finally, not only are environmental factors important in an ecosystem, but also microbe–microbe interactions are decisive and unfortunately uncontrollable in most cases.

An alternative solution to this problem might be larvae feeding, allowing the entry of nutrients to the intestinal ecosystem and avoiding food limitation. However, the best option for

eradicating this difficulty would be *E. coli* growth limitation. Phage therapy is a very specific and safe technique which may help to control *E. coli* proliferation[292]. Furthermore, the lytic action of phages is completed in a few hours.

Despite all these limitations, humanised zebrafish might be used to study processes that do not extend over time. In addition, with knowledge of the species that are able to colonise the zebrafish gut, different SIHUMIs may be created. Furthermore, simplified models might be also used as a mock community for sequencing.

Another point of interest in this thesis was the study of host–microbiota interactions. As a matter of fact, we deepen into the knowledge of this fact in the third and fourth chapters. The humanised model did not allow experiments extended over time due to the complexity of the maintenance of stable microbiota and the axenic environment. Therefore, it was decided to continue the study with conventional zebrafish microbiota. The importance of the functions of the microbiota for the host, its modulation by diet and also the similarities of the zebrafish immune system to that of humans might make zebrafish a suitable model for studying host–microbiota interactions, although the microbiota in terms of species is different[135].

The obesity is considered the 21$^{th}$ century pandemic. For that reason, in our point of view understand the mechanisms underlying this metabolic disease it is highly important. HFD consumption lead to obesity in zebrafish and humans, related to the intestinal microbiota dysbiosis and inflammation[265]. Lipid metabolism and immune system pathways are also conserved between zebrafish and mammals. Therefore, here we studied the intestinal microbial community, and immune system modulation by the consumption of a diet rich in fat in zebrafish larvae and adults.

As previously described, the microbiota of zebrafish larval and adult stages differs in composition and are therefore not comparable. This has already been described by Wong *et al.*[121], demonstrating that the diversity of the microbiota in the larval stage is greater than in adulthood. In relation to this, in the third and fourth chapters, we also observed that the microbiota composition at taxonomic levels at both ages was different (Figure 36). The microbiota of the larvae is dominated by Proteobacteria and Firmicutes. In contrast, in the adulthood Fusobacteria is the predominant phylum, followed by Proteobacteria and Cyanobacteria. In larval samples greater number of OTUs than in adults were identified. In addition, of all the OTUs presented in both samples, only 37 are shared between both states, which is called "core microbiota"[125] (Figure 36).

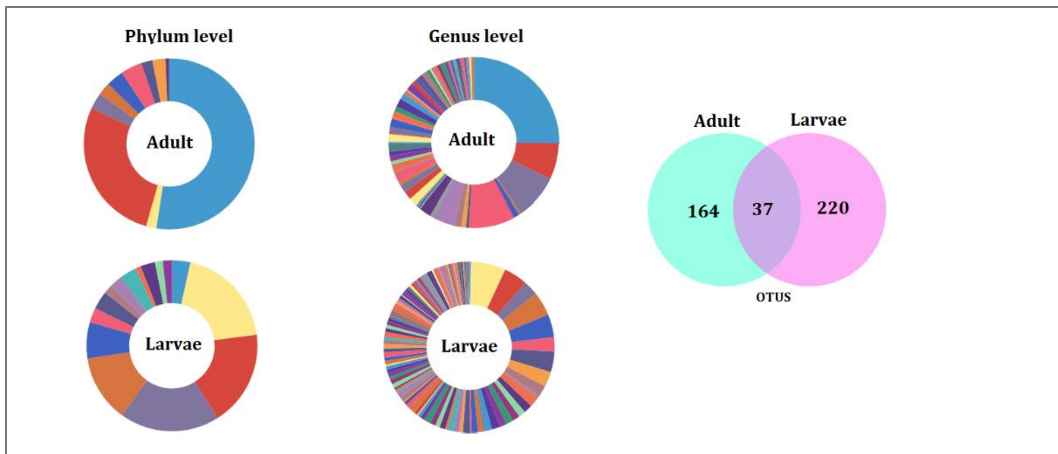



**Figure 36. Relative abundance at phylum and genus level in adult and larval control sample and Venn diagram showing the number of OTUs in adult and larval control samples and OTUs shared between them.**

The consumption of a HFD did not change alpha diversity neither in larvae nor in adults. This, could be explained by the changes produced by the diet not being as pronounced as in other cases such as in an IBD, in which some taxa disappear and therefore there is a significant loss of diversity[293].

As previously mentioned, the larvae microbiome resembles the environment because it is nourished by the environmental microbiota[121]. In addition, the immune system is not completely developed, as the adaptive immune response is not completely formed until 6 wpf[223]. Thus, as seen in chapter 3, changes in the microbiota in the larval stage induced by the consumption of the HFD, led to the immune response activation via the canonical NF-κB pathway. In addition, a host response to protect the intestinal barrier was also detected by the secretion of AMP and the increase of goblet cells. The transparency of the zebrafish embryo and larvae allows whole-mount microscopy, which is one of the main advantages for carrying out the studies at larvae state[97]. Moreover, zebrafish spawning produces a high number of embryos, however, many of them die in the first month of life. For that reason, the larvae maintenance is more delicate than in adulthood. In addition, it is difficult to surgically remove the organs, and in most of cases it is necessary to make pools to obtain the quantity of tissue needed for the subsequent analysis.

The HFD in this thesis, for both larval and adult experiments, was formulated by adding cocoa butter to the diet recommended for each developmental stage. This ingredient was concretely chosen due to the high content of SFA and MUFA and the relationship of its fatty

acids with obesity[220]. It is more difficult to carry out dietary studies with fish than with mice because in the aquatic environment the dose that each individual eats cannot be controlled. For that reason, the study of the lipid profile that we carried out in the fourth chapter of both, diet and animals is of importance to identify which fatty acids are responsible for the changes identified. However, there are some limitations in carrying out this technique in larvae since a huge amount of fat is needed and therefore a large number of individuals[258]. Thus, it has only been carried out in adults.

In adults, the control diet contained higher percentage of fat than in the larvae. Although the manufacturer does not detail the fat composition, the lipid profile analysis shows that the fatty acids that compose this diet are especially SFA and MUFA. The manipulation and maintenance of adults, are simpler than in larvae. Additionally, the extraction of all the organs is feasible, thus obtaining several samples for the same individual with enough tissue to analyse each individual separately and to correlate changes in different organs. In this study no inflammation associated with microbial changes was detected in adults. This result might be due to that fish could already be inflamed because of the high content of saturated fats in the normal diet that the fish had been eating throughout their adult stage. To clarify this issue, it would be desirable to carry out the experiment with fish that have been fed a quality diet from the juvenile stage. Nevertheless, this would extend the experiment over time. Although no inflammation was observed, the increase in SFA and MUFA derived in intestinal environment and microbial changes. In fact, the heightening in saturated and monounsaturated fatty acids in both the diet and the fish represented important changes in the microbial community and correlated to TLR expression. The growth of some taxa and the increase in cocoa butter fatty acids (stearic and palmitic acid) in the intestine correlated with *TLR2*, *4* and *NFKB* expression. This suggests that there is an activation of these receptors and the NF-κB-mediated inflammatory pathway with the microbial changes and dietary fatty acids.

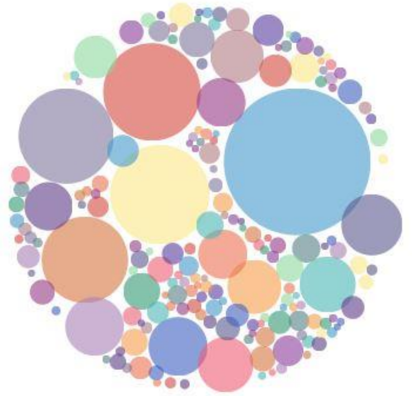
In addition, thanks to advances in sequencing techniques, the study of the composition of the bacterial community and metabolic prediction from the same DNA sample are possible. In this regard, changes in the intestinal environment favoured the growth of certain bacteria such as *Pseudomonas*, which increased in abundance. These bacteria are highly ubiquitous and are powerful colonisers, capable of forming biofilms. They took advantage of the resources of the ecosystem, consuming them, expanding and also limiting the growth of other bacteria-secreting compounds such as siderophores. Thus, other bacteria were affected in terms of a reduction in their abundance. In addition, the metabolic activity of the microbiota of the

samples fed with the HFD was also related to processes previously observed in relation to obesity and diabetes[294].

The effect of fish oil rich in DHA on the lipid profile and intestinal microbiota of adults was also studied. PUFA-rich diets exert a beneficial effect on host health, playing an important role in modulating inflammation and intestinal microbial composition[250]. In this study it was noted that the supplementation of the HFD with this oil helped improve the changes in both the lipid profile and the microbial community, reversing to a certain extent the variations exerted by the HFD. These results are of importance not only for humans, but also for aquaculture species. The dietary composition alters the gut microbiota and consequently other aspects of fish health. Non-beneficial microbial changes may favour the growth of pathogens in aquaculture facilities, which could affect the fish and contribute to economic losses. In addition, understanding how dietary nutrients affect fish health and body composition is of importance as humans are ultimately the consumers of aquaculture fish.

Summarizing, the current study demonstrated the usefulness of the zebrafish animal model for host-microbiome studies. Dietary modulation of intestinal microbial community, inflammation and lipid accumulation is comparable to that in humans, despite the community composition differs in terms of phylotypes. Additionally, to study the effect of a particular molecules on specific strains of human microbiota, zebrafish represents a suitable model, as this study reveals several species of the human flora that colonise and compete inside the zebrafish digestive tract.

# Conclusions



1. Obligate anaerobes, which are the most representative microbes of the human flora, are able to colonise and compete inside the intestine of axenic zebrafish larvae in monoassociation and in consortia. The consortium created from collection strains is the first effective SIHUMI described in zebrafish.

2. Several species of the human flora colonise and compete inside the zebrafish digestive tract. Among them, highly important members from human flora related to eubiotic and dysbiotic state, such as *A. muciniphila, F. prausnitzii* or *Bifidobacterium spp*, have been identified for the first time as active colonisers of zebrafish model following the protocol proposed.

3. Humanised zebrafish has demonstrated to be a powerful model at least during the first 48 hours of colonisation. Thus, it represents a rapid and cost-effective system for the preliminary screening of rapid-action molecules in an alternative vertebrate model.

4. A 10% fat enrichment in the diet induces changes in zebrafish larvae gut microbiota, increasing Bacteroidetes and Proteobacteria, which damage the intestinal barrier, provoking the activation of immune inflammatory pathways via NF-κB and the secretion of protective molecules by the host.

5. In adult zebrafish, the consumption of a diet enriched in fat by 20% led to dysbiosis in intestinal microbial composition and metabolism and lipid profile changes correlated with the fat accumulation. In addition, predictive inferred metabolism suggests that the HFD consumption may induce obesity, diabetes and gut–brain axis effects in zebrafish adults, as occurs in humans.

6. HFD supplementation with fish oil rich in DHA exerts a beneficial effect on microbial dysbiosis, microbial metabolism and fish lipid profiles in zebrafish adults.

7. Zebrafish at both larvae and adult states, is a suitable model to study host-microbiome interactions. The larvae model, is appropriate for the study of microbiota-immune system interactions, whereas the adult model is useful to study the relationship between the microbiome and the dietary nutrients metabolization.

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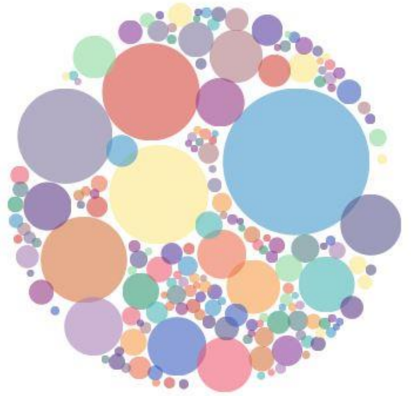

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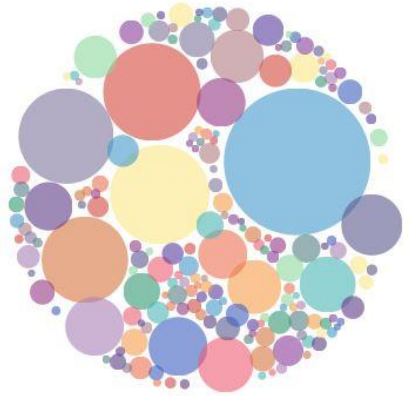
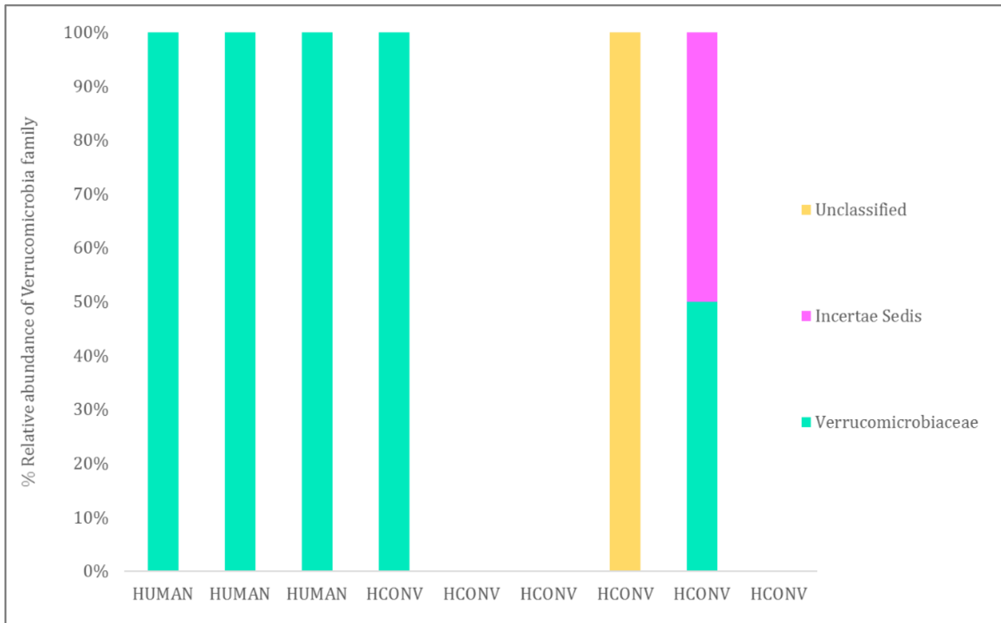
# Annex

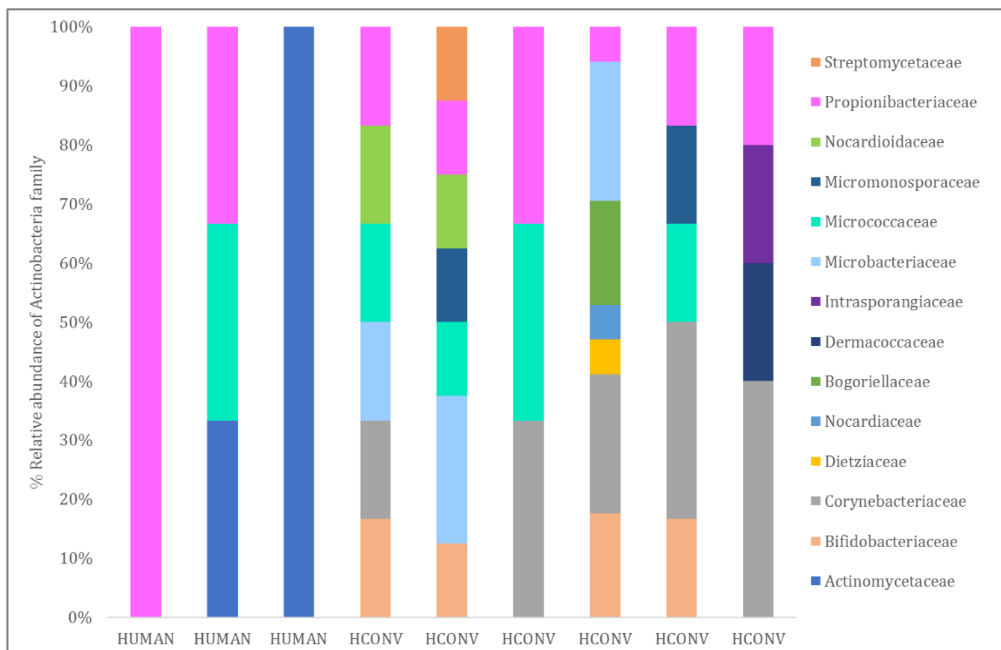
Supplementary material

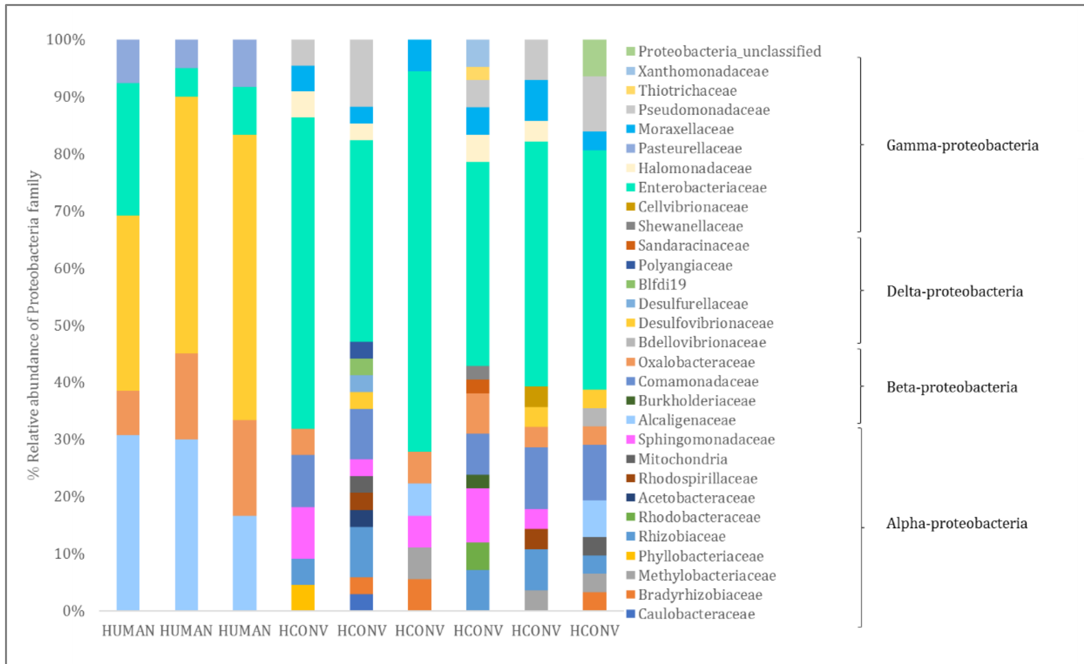


## Chapter 2

*Zebrafish axenic Larvae Colonisation with Human Intestinal Microbiota*






**Supplementary Figure 1. Relative abundance of Verrucomicrobia, Actinobacteria and Proteobacteria at family level in human and HCONV samples represented in stacked bar chart.**

## Chapter 3

*High-fat diet consumption induces microbiota dysbiosis and intestinal inflammation in Zebrafish*

**Supplementary table 1. List of ingredients of ZF biolabs diet.**

| Ingredients | ZF biolabs (%) |
|---|---|
| Lipids | 8 |
| Proteins | 56 |
| Ash | 13 |
| Water | 8 |
| Fiber | 1.4 |
| Calcium | 2 |
| Sodium | 2.5 |
| Phosphorus | 1.5 |

# Chapter 4

*Host-microbiota interactions in response to a high-fat diet and fish oil supplementation in zebrafish adults*

**Supplementary table 2. Experimental dietary fatty acid composition: Control, HFD, Sendabio DHA, HFO and HFO diet after one week of storage at 4◦C (*).**

| Fatty acids | Control | HFD | Sendabio DHA | HFO | HFO* |
|---|---|---|---|---|---|
| Butiric C4:0 | 0.00 | 0.00 | 0 | 0.00 | 0.00 |
| Caproic C6:0 | 0.00 | 0.00 | 0 | 0.00 | 0.00 |
| Caprilic C8:0 | 0.00 | 0.00 | 0 | 0.03 | 0.05 |
| Capric C10:0 | 0.00 | 0.00 | 0 | 0.02 | 0.04 |
| Lauric C12:0 | 0.00 | 0.00 | 0 | 0.02 | 0.00 |
| Miristic C14:0 | 2.37 | 0.72 | 0 | 0.80 | 0.40 |
| Miristoleic C14:1 | 0.00 | 0.00 | 0 | 0.00 | 0.00 |
| Palmitic C16:0 | 13.65 | 22.83 | 0,06 | 21.17 | 20.42 |
| Palmitoleic C16:1 | 2.75 | 0.85 | 0,05 | 0.89 | 0.55 |
| Margaric C17:0 | 1.14 | 0.26 | 0 | 0.25 | 0.23 |
| Estearic C18:0 | 4.01 | 26.21 | 0,22 | 22.10 | 27.08 |
| Oleic C18:1 | 16.50 | 28.27 | 0,04 | 24.30 | 27.59 |
| Linoleic C18:2 | 14.56 | 9.70 | 0,03 | 10.93 | 4.03 |
| Linolenic C18:3n6 | 0.00 | 0.05 | 0 | 0.05 | 0.00 |
| Linolenic C18:3n3 | 0.00 | 0.05 | 0 | 0.05 | 0.00 |
| Estearidonic C18:4 | 1.31 | 0.21 | 0,05 | 0.12 | 0.28 |
| Arachidic C20:0 | 0.33 | 0.88 | 0,00 | 0.77 | 0.87 |
| Eicosenoic C20:1 | 4.62 | 1.06 | 0,00 | 1.11 | 0.70 |
| Arachidonic C20:4n6 | 1.09 | 0.25 | 0,45 | 0.28 | 0.22 |
| Eicosatetraenoic C20:4n3 | 0.37 | 0.11 | 0,21 | 0.10 | 0.07 |
| EPA C20:5 | 7.44 | 1.71 | 2,29 | 1.86 | 1.35 |
| Behenic C22:0 | 0.22 | 0.24 | 0 | 0.25 | 0.18 |
| Erucic C22:1 | 6.54 | 1.16 | 0 | 1.20 | 1.00 |
| Clupanodonic C22:5 | 1.26 | 0.26 | 2,08 | 0.64 | 0.47 |
| Lignoceric C24:0 | 0.00 | 0.13 | 0,35 | 0.14 | 0.12 |
| DHA C22:6 | 19.91 | 4.12 | 83,30 | 11.87 | 13.96 |
| % SFA | 21.72 | 51.26 | - | 45.55 | 49.38 |

| % MUFA | 30.42 | 31.33 | - | 27.51 | 29.85 |
|---|---|---|---|---|---|
| % PUFA | 47.87 | 17.41 | - | 26.94 | 20.77 |

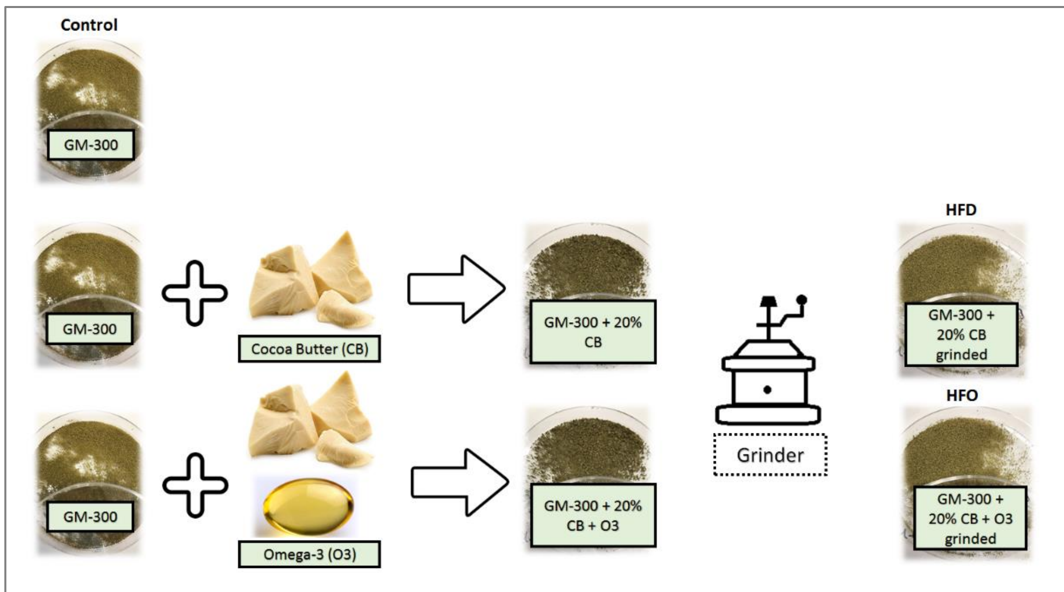
**Supplementary table 3. Gene expression primer list.**

| Gene | Short | Forward primer | Reverse primer | Ref |
|---|---|---|---|---|
| **Insuline growth factor 1** | IGF1 | GCATTGGTGTGATGTCTTTAAGTGTA | GTTTGCTGAAATAAAAGCCCCT | [295] |
| **Insuline growth factor 2** | IGF2 | GAAACACGAACAACGATGCG | AGTACTTCACATTTATGGTGTCCTTG | [295] |
| **fatty acid synthase** | FASN | CGCTTGTCCTACTTCTTTGATTTCA | TTCCAGTGCCAGCAGACTAGAG | [296] |
| **peroxisome proliferator activated receptor** | PPARg | GGCATGTCACACAACGCG | CCTTCTCAGCCTGCGGC | [258] |

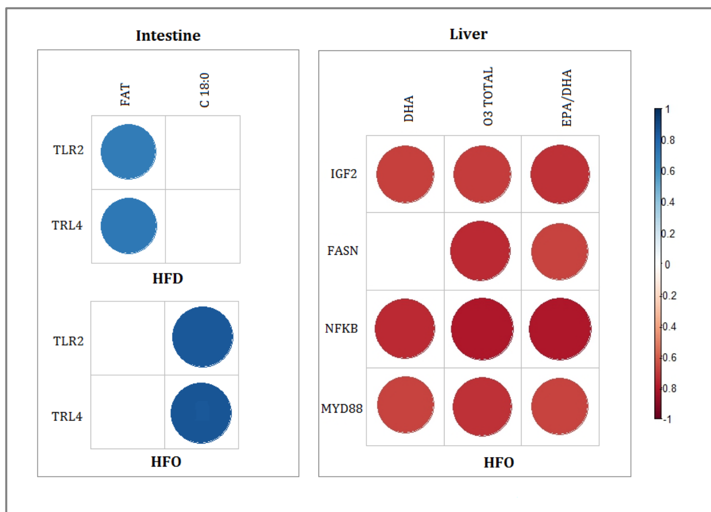
**Supplementary table 4. Median of the total content of whole zebrafish and liver fatty acids.** The results are expressed as a median. MAD*2 is the median absolute deviation. Differences were considered statistically significant at p < 0.05 (*) and p < 0.01 (**).

| Zebrafish Fatty Acids % | Control | MAD*2 | HFD | MAD*2 | HFO | MAD*2 | p C-HFD | p HFD-HFO |
|---|---|---|---|---|---|---|---|---|
| **C10:0** | 0.0000 | 0 | 0.0000 | 0 | 0.0159 | ±0.003 | | ** |
| **C12:0** | 0.0571 | ±0.002 | 0.0339 | ±0.002 | 0.0372 | ±0.006 | ** | |
| **C14:0** | 1.9536 | ±0.041 | 1.0349 | ±0.052 | 0.9667 | ±0.048 | ** | |
| **C14:1** | 0.0532 | ±0.017 | 0.0349 | ±0.009 | 0.0323 | ±0.005 | ** | |
| **C16:0** | 19.6873 | ±0.134 | 20.7509 | ±0.503 | 17.8604 | ±0.932 | * | ** |
| **C16:1** | 2.6552 | ±0.174 | 1.7294 | ±0.041 | 1.5238 | ±0.096 | ** | * |
| **C17:0** | 0.4427 | ±0.027 | 0.3152 | ±0.073 | 0.2901 | ±0.04 | ** | |
| **C18:0** | 3.9202 | ±0.590 | 10.3515 | ±0.245 | 10.1854 | ±0.397 | ** | |
| **C18:1** | 17.8948 | ±0.223 | 32.2668 | ±2.5 | 27.9423 | ±1.924 | ** | ** |
| **C18:2** | 26.3517 | ±1.311 | 16.7897 | ±1.845 | 15.6457 | ±1.813 | ** | |
| **C18:3** | 3.0229 | ±0.197 | 1.7803 | ±0.043 | 1.7730 | ±0.209 | ** | |
| **C18:4** | 0.3104 | ±0.045 | 0.2967 | ±0.071 | 0.2426 | ±0.126 | | * |
| **C20:0** | 0.1144 | ±0.012 | 0.1499 | ±0.021 | 0.1703 | ±0.004 | ** | * |
| **C20:1** | 2.0123 | ±0.104 | 1.1773 | ±0.045 | 1.2418 | ±0.045 | ** | |
| **C20:4n6** | 0.8666 | ±0.157 | 0.6380 | ±0.125 | 0.6400 | ±0.155 | ** | |
| **C20:4n3** | 0.6148 | ±0.026 | 0.3395 | ±0.008 | 0.3677 | ±0.037 | ** | |
| **C20:5** | 3.8185 | ±0.197 | 2.3197 | ±0.091 | 3.1472 | ±0.364 | ** | ** |
| **C22:0** | 0.0833 | ±0.007 | 0.0690 | ±0.005 | 0.0785 | ±0.005 | ** | |
| **C22:1** | 1.1157 | ±0.222 | 0.7712 | ±0.082 | 0.7721 | ±0.023 | ** | |
| **C22:5** | 1.0692 | ±0.08 | 0.6400 | ±0.044 | 0.8469 | ±0.131 | ** | ** |
| **C24:0** | 0.0000 | 0 | 0.0000 | ±0 | 0.0279 | ±0.001 | | |
| **C22:6** | 11.7808 | ±2.139 | 8.1919 | ±0.927 | 15.3734 | ±0.735 | ** | ** |
| **Omega 6 (total)** | 27.2688 | ±1.255 | 17.4582 | ±2.040 | 16.2082 | ±1.658 | ** | |
| **Omega 3 (total)** | 20.6307 | ±2.015 | 13.1289 | ±1.235 | 21.0938 | ±1.821 | ** | ** |
| **Omega 6/Omega3** | 1.3218 | ±0.181 | 1.2684 | ±0.145 | 0.7688 | ±0.079 | | ** |
| **% SFA** | 26.6115 | ±1.308 | 32.5039 | ±0.996 | 29.6622 | ±0.867 | ** | ** |
| **% MUFA** | 23.8402 | ±0.358 | 36.0264 | ±2.025 | 31.3864 | ±1.769 | ** | ** |
| **% PUFA** | 48.2329 | ±0.528 | 30.4813 | ±1.633 | 36.9647 | ±0.779 | ** | ** |
| **EPA + DHA** | 15.7276 | ±2.427 | 10.3427 | ±1.264 | 18.1833 | ±1.790 | ** | ** |

| Liver<br>Fatty Acids % | Control | MAD*2 | HFD | MAD*2 | HFO | MAD*2 | p<br>C-HFD | P<br>HFD-HFO |
|---|---|---|---|---|---|---|---|---|
| **C10:0** | 0.028 | 0.031 | 0.013 | 0.027 | 0 | 0 | | |
| **C12:0** | 0.017 | 0.035 | 0.018 | 0.009 | 0 | 0 | | |
| **C14:0** | 0.048 | 0.070 | 0 | 0 | 0 | 0 | | |
| **C14:1** | 0.553 | 0.306 | 0.515 | 0.096 | 0.224 | 0.1146 | | |
| **C16:0** | 12.258 | 8.905 | 14.664 | 1.689 | 8.155 | 4.10 | | * |
| **C16:1** | 0.787 | 0.751 | 0.733 | 0.354 | 0.304 | 0.176 | | |
| **C17:0** | 0.323 | 0.192 | 0.180 | 0.04 | 0.151 | 0.036 | | |
| **C18:0** | 4.504 | 1.662 | 9.142 | 3.159 | 6.370 | 2.985 | * | * |
| **C18:1** | 7.94 | 1.732 | 17.089 | 2.76 | 8.173 | 5.224 | * | * |
| **C18:2** | 8.953 | 7.204 | 7.486 | 4.2874 | 3.757 | 2.626 | | |
| **C18:3** | 0.925 | 0.407 | 0.734 | 0.399 | 0.465 | 0.083 | | |
| **C18:4** | 24.572 | 20.216 | 19.658 | 9.203 | 27.054 | 7.224 | | |
| **C20:0** | 0.095 | 0.097 | 0.092 | 0.005 | 0.088 | 0.042 | | |
| **C20:1** | 1.353 | 0.937 | 0.892 | 0.068 | 1.018 | 0.160 | | |
| **C20:4n6** | 0.961 | 0.174 | 1.100 | 0.275 | 1.000 | 0.376 | | |
| **C20:4n3** | 0.599 | 0.087 | 0.420 | 0.060 | 0.544 | 0.074 | * | |
| **C20:5** | 1.967 | 1.457 | 1.760 | 0.375 | 2.081 | 0.6722 | | |
| **C22:0** | 0 | 0 | 0.031 | 0.063 | 0 | 0 | | |
| **C22:1** | 0.294 | 0.519 | 0.312 | 0.125 | 0.129 | 0.14 | | |
| **C22:5** | 0.9347 | 0.446 | 0.833 | 0.156 | 1.552 | 0.9972 | | * |
| **C24:0** | 8.94 | 4.069 | 9.157 | 3.275 | 10.798 | 5.5554 | | |
| **C22:6** | 10.931 | 8.01 | 8.974 | 5.522 | 5.223 | 2.7582 | | |
| **Omega 6 (total)** | 14.288 | 1.989 | 13.700 | 3.830 | 15.978 | 8.5396 | | |
| **Omega 3 (total)** | 0.732 | 0.157 | 0.7569 | 0.389 | 0.326 | 0.060 | | |
| **Omega 6/Omega3** | 2.009 | 0.709 | 1.742 | 0.091 | 1.282 | 0.285 | | |
| **% SFA** | 18.141 | 9.78 | 24.189 | 5.865 | 16.459 | 4.399 | | * |
| **% MUFA** | 9.931 | 2.18 | 19.102 | 3.316 | 9.798 | 5.75 | | |
| **% PUFA** | 51.290 | 3.09 | 40.741 | 1.527 | 51.607 | 2.606 | ** | ** |
| **EPA + DHA** | 10.951 | 3.99 | 11.616 | 3.694 | 13.378 | 4.662 | | |




**Supplementary Figure 2. Experimental diets design schema.**




**Supplementary Figure 3. Correlation plot showing Pearson correlations between fatty acid lipid profile and gene expression in the intestine and liver.** Blue dots are positive correlations, whereas red dots are negative correlations. Only significant correlations are represented ($p < 0.05$).