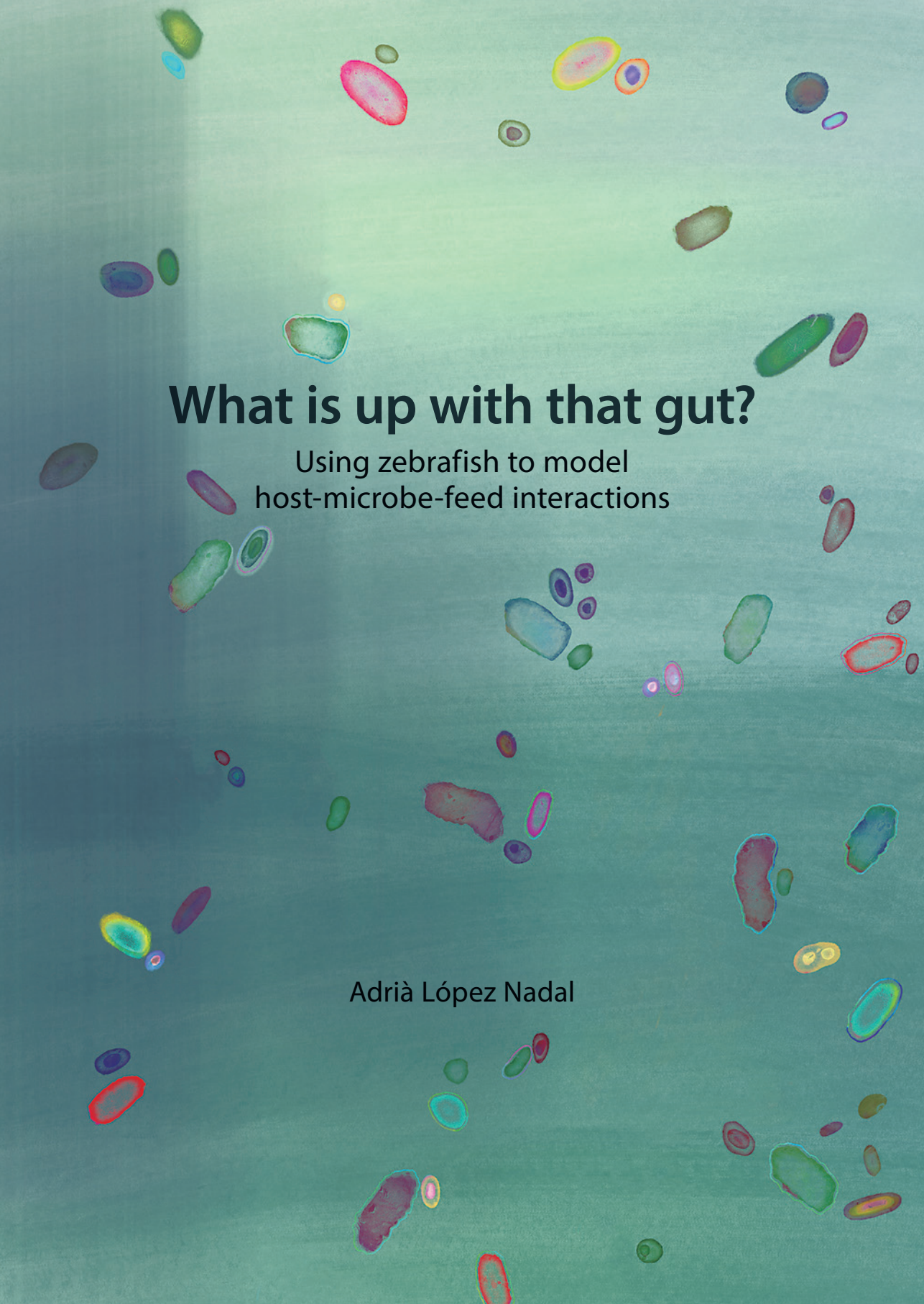


What is up with that gut?

Using zebrafish to model host-microbe-feed interactions

Adrià López Nadal

Propositions

1. Zebrafish provide an informative animal model to pre-screen antinutritional factors present in aquafeeds.
   This thesis
2. Neutrophil recruitment is a more accurate cellular hallmark of feed-derived gut inflammation than macrophage recruitment.
   This thesis
3. Scientific collaboration is based on dependency and thus contradicts with the PhD goal of becoming an independent researcher.
4. The unwritten rule of having four publishable chapters carries more weight than the written formal requirements for completion of a PhD thesis at Wageningen University.
5. The loneliness one experiences during the PhD challenges one's mental health.
6. Even discriminating majorities benefit from human rights fought for by minorities.
7. Individual freedom of expression leads to a more polarized society.

Propositions belonging to the thesis, entitled
"What is up with that gut? Using zebrafish to model host-microbe-feed interactions"
Adrià López Nadal, 17th of March 2023

Thesis committee

Promotor

Prof. Dr Geert Wiegertjes
Professor of Aquaculture and Fisheries
Wageningen University & Research

Co-promotors

Dr Sylvia Brugman
Associate Professor, Host-Microbe Interactomics Group
Wageningen University & Research

Dr Detmer Sipkema
Associate Professor, Laboratory of Microbiology
Wageningen University & Research

Other members

Prof. Dr Tinka Murk, Wageningen University & Research

Dr Trond M. Kortner, Norwegian University of Life Sciences, Ås, Norway

Dr Phil Elks, Sheffield University, United Kingdom

Dr Matthew Owen, Skretting Norway, Stavanger

This research was conducted under the auspices of the Graduate School Wageningen Institute of Animal Sciences

What is up with that gut?

Using zebrafish to model host-microbe-feed interactions

Adrià López Nadal

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 17 March 2023
at 1:30 p.m. in the Omnia Auditorium.

Adrià López Nadal

What is up with that gut? Using zebrafish to model host-microbe-feed interactions, 272 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2023)
With references, with summary in English

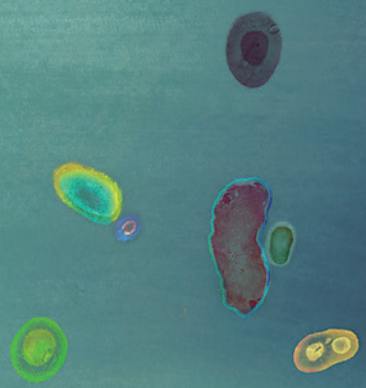
ISBN: 978-94-6447-537-1
DOI: https://doi.org/10.18174/583665

"A Ángel, y a su entendimiento de las moléculas"

Table of content

Chapter 1

General Introduction



The fish gastrointestinal tract

The key feature of the gastrointestinal tract (GIT) is the ability to digest foodstuffs to make them suitable for absorption by various transport mechanisms in each of the gastrointestinal sections (Bakke, Glover and Krogdahl, 2010). In fish, the GIT accounts for a great percentage of the fish metabolic rate and it is adapted for efficient capture of the nutritional value of the food intake (Karasov and del Rio, 2020). Many studies have compared gut sizes and digestive enzymes activities among naturally occurring herbivorous and carnivorous fishes to identify specializations of the GIT for different diets (AL, 1947; Zihler, 1981; Ribble and Smith, 1983; Kramer and Bryant, 1995; Elliott and Bellwood, 2003; German and Horn, 2006; Horn *et al*., 2006). Herbivorous fishes tend to have a longer GIT to retain food longer than carnivorous fishes, which allow higher nutritional intake from less digestible plant-based food (Ribble and Smith, 1983). Furthermore, herbivores fishes ingest more carbohydrates and consequently possess more carbohydrate-degrading enzymes in the digestive tract (e.g. amylase, α-glucanase) than carnivorous fishes (German and Horn, 2006; German *et al*., 2010).

Anatomically, the fish GIT can be subdivided into four topographical regions which from the anterior to the posterior part are (Harder, 1975): the headgut composed by mouth and pharynx where feed gets mechanically processed; the foregut comprised of oesophagus and stomach where digestion of feed by the commensal host bacteria begins; the midgut that accounts for the greatest proportion of the gut length and is where the digestion continues and absorption takes place and the hindgut which includes the rectum. There is high heterogeneity in the GIT morphology across fish species. Most of the fish present a stomach and pyloric caeca (blind sacs that increase the absorptive capacity of the GIT) in the anterior gut but about 20% of the fish species lack a true stomach (Wilson and Castro, 2010) among which the teleost families (bony fish) Gobiidae (goby) and Cyprinids (including carp and zebrafish) (**Figure 1**). The absence of stomach is often compensated by well-developed pharyngeal teeth and secretory glands in the oesophagus and in the anterior gut (Kapoor and Khawna, 1993; Stevens and Hume, 2004).

The first observations regarding fish gut anatomy were made more than 2000 years ago by Aristotle: *"with fish the properties of the gut are similar to other vertebrates (...). The whole length of the gut is simple, and if it have a reduplication or kink it loosens out again into a simple form*" (Aristotle 345BC *Historium Animalia*), transcribed in (Gill, 1911). In the last decades, the advances in technology and laboratory techniques favoured the study of the fish gut from a whole organ point-of-view to specific gut tissues. Recent research focused on differentiating cell types (sub)populations in the gut and on uncovering moelcular pathways associated to gut function in health and disease (Wilson and Castro, 2010).

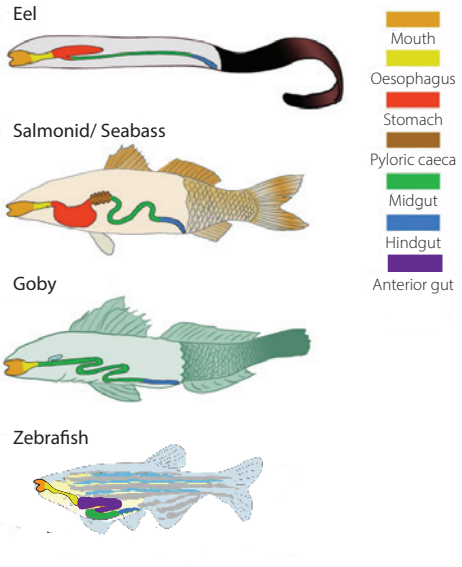


Figure 1: Heterogeneity in the fish gastrointestinal tract (GIT). In the presence of stomach there are three main shapes reported: Y-shaped, typically from predatory fishes such as eels; U-shaped, commonly found in omnivores and carnivorous fishes such as seabass and salmonids; and straight, found in some fresh-water species as well as marine fishes such as goby. Zebrafish lack stomach and digestive functions occur in the anterior gut. Illustration adapted from Egerton *et al.*, 2018.

The main function of the gut is to finalize the digestive process started in the stomach (or in the anterior gut in stomachless fish) and to absorb the nutrients. The digestion is mainly performed by bacteria that reside in the lumen of the fish gut and the main role of these bacteria is to make the nutrients available to the enterocytes. The fish gut occupies a large place in the coelomic cavity: the hollow fluid-filled space that contains the internal organs. The fish gut presents folds (either primary, one-fold; secondary, two-folds or tertiary, three-folds) of the mucosa (Al-Hussaini, 1946) that enlarge the apical surface of the epithelium maximizing nutrient absorption (Wilson and Castro, 2010).

The gut wall is formed of four concentric layers which from the lumen to the peritoneum are: 1) the *tunica mucosa* formed by the mucosal epithelium and the *lamina propria* is a vascularized connective tissue containing nerves and leukocytes; 2) the submucosa (not present in teleosts -bony fish- and agnathans -jawless fish-) which is an additional connective tissue layer; 3) the *tunica muscularis* consisting of circular and longitudinal layers of either striate or smooth muscle and 4) the *tunica serosa* delimited by the mesothelial cells and connective tissue containing blood vessels (Grosell, Farrell and Brauner, 2010).

Gut-associated terminology

Apical: side of the cell membrane that faces the lumen.
Basal: side of the cell membrane that faces the underlying connective tissue.
Commensal bacteria: Naturally-occurring non-harmful bacteria for the host.
Enterocyte: columnar absorptive cell of the gut epithelium.
Epithelium: protective cell layer that marks the border between the outside (lumen) and the inside (tissue) of the gut.
Lumen: cavity or channel that makes up the inside of the bowel.
Microvilli: microscopic cellular membrane protrusions that increase the surface area to maximize nutrient absorption.
Mucosal fold (villi): surface-enlarging finger-like projection that extends into the lumen of the gut.
Tight junction: protein complexes (containing claudins, desmosomes, occludins and cadherins) that seal the space between the enterocytes in the gut epithelia.

Which cells are present in the fish gut?

The gut epithelium consists of a monolayer of cells. These cells are connected laterally towards their apical ends by tight junctions to reduce the diffusion from the lumen to the lamina propria. Despite the physical barrier several substances and particles can cross the epithelium. Lipophilic substances passively diffuse over cell membranes while hydrophilic substances are generally actively taken up by membrane-bound transport proteins. Large hydrophilic substances (among which bacteria and viruses) are too large to go through the tight junctions and are often transported by active transcytosis: endocytosis at the apical side and exocytosis at the basal side through the epithelial cells (Jutfelt, 2011). The cells in the intestine differentiate from pluripotent stem cells located at the base of the villi, which are constantly being renewed. Most of the fish have several types of cells in the intestinal epithelium, mainly classified into:

i) enterocytes or absorptive cells are tall and narrow with elongated nuclei located towards the basal side (Ezeasor and Stokoe, 1981). At the apical side, the cell membrane of the enterocytes is packed with microvilli and collectively constitute the brush border. In certain tilapia species the brush border contributes to more than 90% of the total intestinal surface area (Frierson and Foltz, 1992). The brush border creates the microenvironment containing the necessary enzymes for the breakdown and absorption of the nutrients previously digested by the gut bacteria (Kuz'Mina and Gelman, 1997). At the basal side of the enterocytes Na^+/K^+-ATPase enzymes are responsible for the ion regulation and for the

production of the energy required for transepithelial transport and for nutrient uptake (Wilson and Castro, 2010).

ii) goblet cells or mucus-producing cells are cup-like shaped with the nucleus located at the narrow basal side. The main function of goblet cells is to synthetize and secrete mucus into the lumen. As a matter of fact, goblet cells secrete mucin glycoproteins, the major macromolecular components of the mucus from their apical side (Ma, Rubin and Voynow, 2018). By creating this layer of mucus, the bacteria are not in direct contact with the epithelial cells. Thus, the mucus plays an important role maintaining gut homeostasis (Dao and Le, 2021). Moreover, goblet cells contain antibacterial peptides that protect the epithelial surface from pathogens (Jutfelt, 2011).

iii) endocrine cells are found throughout the intestinal epithelium in all fish and together with the pancreas constitute the gastroenteropancreatic endocrine system (Holmgren and Olsson, 2009). Endocrine cells have in their cytoplasm secretory vesicles and can be classified by their relative position within the epithelium (open-type when the apical side extend towards the lumen and closed-type when does not) or classified based on the secretory granule morphology: size, shape and content. The substances secreted have neuroendocrine effects and include gastrin, cholecystokinin or serotonin among others (Holmgren and Olsson, 2009).

iv) immune cells are involved in protecting the host against pathogens and they are classified by their nature and functionality. The fish gut harbors two main populations of immune cells: lamina propria leukocytes, including macrophages, granulocytes (among which neutrophils, eosinophils, basophils), rodlet cells, T cells and plasma cells and intra-epithelial lymphocytes, mainly T cells and B cells located in between epithelial cells. The immune cells, their functionality and subtypes are examined in detail in the section below "Identification of the immune cells in the zebrafish gut".

The "bugs" in the fish gut

Besides the host cells, the fish gut harbours millions of bacteria which are collectively named microbiota. The mucosal layer of the fish gut is in direct contact with the environment and is a contact point between the microbes and the host. The mucus provides a carbon source for the commensal bacteria that subsequently prevent the outgrowth of invading pathogens (Hansen and Olafsen, 1999; Merrifield and Rodiles, 2015). Microbial colonization of fish starts upon hatching when larvae take in the chorion-associated bacteria that is bound by glycoproteins on the egg surface (Larsen, 2014). The surrounding bacteria colonize the fish gut when the larvae starts engulfing water (Hansen and Olafsen, 1999). Microbes establish symbiotic relationships with their hosts influencing development, digestion, nutrition, disease resistance and immunity (Romero, Ringø and Merrifield, 2014).

Overall the most abundant phyla in the fish gut are Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes (Sullam *et al*., 2012; Givens *et al*., 2015; Merrifield and Rodiles, 2015; Tran, Wang and Wu, 2017). Bacterial communities from intestines of several fish species clustered together and separately from environmental samples. Within the intestinal microbial cluster different gut bacterial communities exist depending on trophic level (herbivores, carnivores, or omnivores), habitats (saltwater, freshwater, estuarine, or migratory fish) and sampling methods (Sullam *et al*., 2012). Microbiota composition fluctuates depending on the naturally occurring fish diet (reviewed in Egerton *et al*., 2018) and diet shapes the gut microbiota already from the first feeding onwards (Reid *et al*., 2009; Lauzon *et al*., 2010; Stephens *et al*., 2016). As a matter of fact, omnivorous fish have a more variable diet than carnivorous fish and show higher bacterial diversity in their guts (Givens *et al*., 2015). Previous research showed that depending on the naturally-occurring diet fish harboured different microbiota composition: Fusobacteria were increased in carnivorous fish while Acidobacteria and Cyanobacteria in filter feeders (Liu *et al*., 2016). In another sutudy, Fusobacteria were increased in the guts of omnivores, Bacteroidetes in the guts of marine herbivores and Firmicutes in the guts of carnivorous fish (Sullam *et al*., 2012). Taking all these observation together, there is a clear effect of diets in shaping the fish gut microbial community (Ringø *et al*., 2016) although more research is warranted to properly understand the contribution of these bacteria on feed digestion and host gut health. Besides diet, other host factors modulate the gut bacterial composition. The development of the fish (Stephens *et al*., 2016) as well as the maturity of the immune system influences the gut microbial composition. The moelcular mechanisms of host-microbe-immune interactions in zebrafish are examined in the **General Discussion** (**chapter 8**) in this thesis. Overall, the symbiotic processes between host and bacteria are highly conserved among fish species and partly depend on environmental factors such as diet and natural habitat as well as host factors like immune status and metabolites produced by the gut bacteria (Sullam *et al*., 2012).

Short-chained fatty acids (SCFAs) are metabolic by-products resulting from the anaerobic fermentation of fibers by the gut microbiota. During the last decades, an increasing number of studies have addressed the important role of SCFAs in the interactions with the host, impacting gut homeostasis and host health overall (reviewed in van der Hee and Wells, 2021). The most abundant SCFAs in the fish gut are acetate, propionate and butyrate (Clements, Gleeson and Slaytor, 1994; German, 2009; Hao, Wu, Jakovlić, *et al*., 2017; Hao, Wu, Xiong, *et al*., 2017). The concentrations of SCFA increase towards the distal intestine becoming the highest towards the hindgut (Mountfort, Campbell and Clements, 2002). SCFA synthesis depends on the microbial composition and the environmental conditions as well as on the fish naturally occurring diet: SCFAs concentration is the highest in carnivorous fish followed by omnivorous fish and lowest

in herbivorous fish (Smith, Wahl and Mackie, 1996; Clements *et al.*, 2014; Hao, Wu, Jakovlić, *et al.*, 2017). Accumulating evidence claims intestinal-associated health benefits for the host by the microbiota harboured in their guts and their metabolites, such as SCFAs. Recently, several studies have addressed the supplementation of SFCAs in fish feed and reported growth-promoter, immune-boosting and anti-oxidant effects (reviewed in Hoseinifar, Sun and Caipang, 2017; Abdel-Latif *et al.*, 2020). SCFAs can diffuse into the host cells or interact with receptors, among which G-protein coupled receptors which recently have been suggested to be conserved between fish and mammals (Petit and Wiegertjes, 2022).

The zebrafish as a model for fish gut health

Zebrafish (*Danio rerio*) are teleosts cyprinids. Cyprinids constitute the largest family of vertebrates and are stomachless fish with toothless jaws (Nelson, Grande and Wilson, 2016). At the end of the sixties, the molecular biologist George Streisinger used zebrafish and applied forward genetics to investigate molecular mechanisms in a vertebrate model (reviewed in David Jonah Grunwald, 2002). Zebrafish research started mainly to study developmental biology and due to zebrafish unique characteristics rapidly became a very popular choice as a vertebrate model for many other fields. Zebrafish excelled as a model to study immunological processes due to the high presence (~70%) of human orthologous genes in the zebrafish genome (Howe *et al.*, 2013). Adult zebrafish are small (<5 cm) and easy to hosted in aquaria. Zebrafish lifecycle is properly characterized and they reach sexual maturity around 2-3 months of age (**Figure 2A**). The sexually matured females can potentially provide 200 to 300 eggs per week (Hensley and Leung, 2010; Lawrence, 2011). Under laboratory conditions, zebrafish lay eggs all-year around (Clelland and Peng, 2009). Hatching occurs around the 3rd day post fertilization (dpf) (Kimmel *et al.*, 1995) and the larval stage takes up to 2 weeks approximately (5mm standard length -SL-, **Figure 2B**) (Singleman and Holtzman, 2014) followed by a metamorphosis involving the absorption of larval fins into the body, the gut tube drops more ventrally and the development of scales starts (Ledent, 2002). The juvenile stage is the result of that process and starts around 28 dpf until 45 dpf when fish are considered to be adults. Zebrafish can reproduce from approximately 3 months until 42 months of age although the average lifespan is around 36 months (Gerhard and Cheng, 2002), **Figure 2A**.

Zebrafish develop *ex-utero* and are transparent in the first weeks of life allowing the study of the organism development from a very early life stage (reviewed in Yoder *et al.*, 2002). Moreover, several transgenic fish lines have been engineered and together with embryo's transparency allowed the study of specific (immune) cell populations *in vivo*. For instance, neutrophil biology is visualized *in vivo* based on the expression of the

neutrophil-associated enzyme myeloperoxidase (*mpx*) and by using a fluorescent microscope (Renshaw *et al.*, 2006). Furthermore, the fact that the zebrafish genome is well-annotated facilitated the generation of knock-out zebrafish lines, some of which contributed to uncover immune gene functions (reviewed in Yoder *et al.*, 2002). In the lasts years, the groundbreaking discovery of CRISPR-Cas technique (Hwang *et al.*, 2013) replaced other genome editing techniques like Zinc finger (reviewed in Urnov *et al.*, 2010) nuclease or TALENs (Bedell *et al.*, 2012). Nowadays, gene knockout can be performed in a relatively easy way leading to the generation of multiple knock-out lines (Albadri, del Bene and Revenu, 2017). Zebrafish intrinsic biological characteristics and the unique research tools developed placed these small cyprinids as a model to study biological and immunological processes such as gut health (reviewed in **chapter 5**).

The development of the gut in zebrafish

The zebrafish gut tube formation starts at mid- to late-somite stages when a layer of epithelia becomes the primitive gut endoderm. The temporal sequence of the GIT formation begins from the anterior part followed by the hindgut and the midgut (Wallace and Pack, 2003). Zebrafish do not present submucosa (absent in all teleosts) and the *tunica mucosa* connects directly with the *tunica muscularis* and that one to the outer *tunica serosa*. The epithelial monolayer of the zebrafish lacks intestinal crypts and presents fingerlike protrusions with decreasing size towards the posterior gut called folds (villi) (Wallace and Pack, 2003). The zebrafish GIT is divided in separate sections: the mouth, the esophagus, three gut segments (anterior, middle and posterior) and the cloaca (anus). In the anterior gut we find the intestinal bulb that functions as a reservoir comparable to the stomach (Wallace and Pack, 2003). The uptake of nutrients occurs in the anterior gut. The anterior gut presents high presence of digestive enzymes but no gastric glands and thus the pH in the zebrafish gut does not go below 7.5 in homeostatic conditions (Nalbant *et al.*, 1999). Nutrient uptake diminishes towards the posterior gut segment while ion transport, water reabsorption, fermentation processes and immune functions take place in the mid and posterior segments (Wallace and Pack, 2003; Wallace *et al.*, 2005). Epithelial renewal occurs when stem cells at the base of the villi proliferate and favor epithelial cells movement towards the tip of the villi to undergo apoptosis. This process occurs every 5 to 7 days in the anterior gut and 7 to 10 days in the mid intestine (Wallace *et al.*, 2005) and is dependent on the gut microbiota (Rawls, Samuel and Gordon, 2004; Bates *et al.*, 2006).

Identification of immune cells in the zebrafish gut

The identification of (innate) immune cells is possible *in vivo* due to the transparency of the zebrafish in the first weeks of life and the multiple transgenic cell reporter lines engineered.

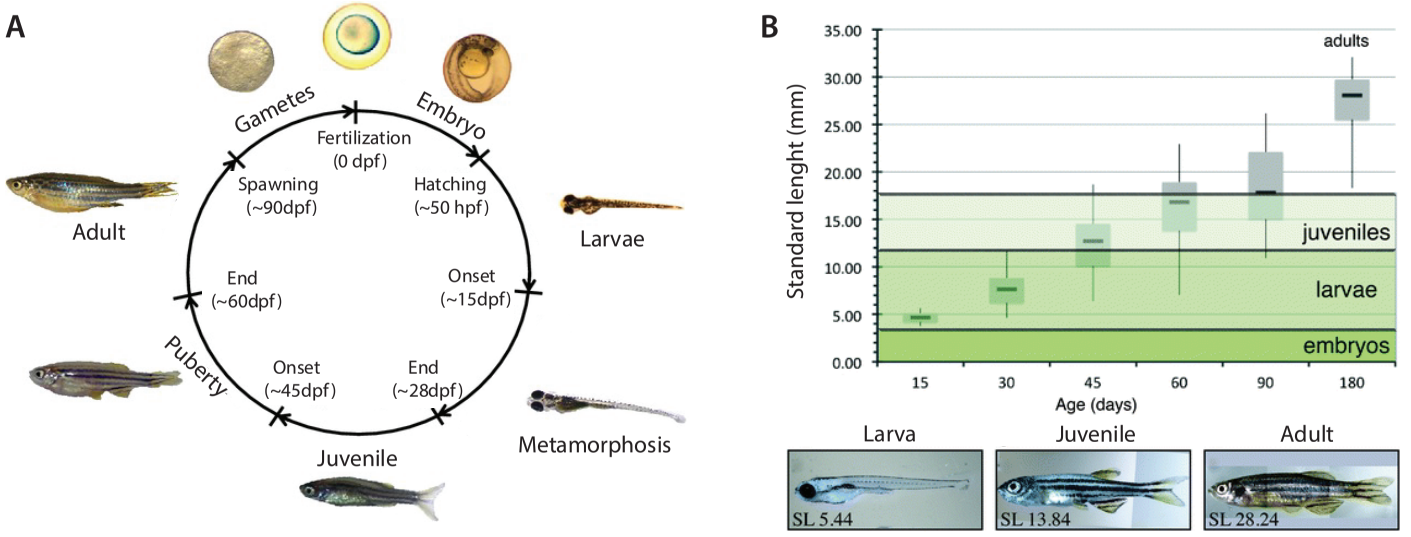


Figure 2: **(A)** Zebrafish life cycle (adapted from Ribas and Piferrer, 2014). **(B)** Zebrafish maturation from larvae to adults compared to their standard length (adapted from Singleman and Holtzman, 2014). Hpf is hours-post-fertilization and dpf is days-post-fertilization.

Neutrophils are the most abundant type of circulating leukocytes in zebrafish and are the first responders recruited of sites of injury, infection or inflammation (Lieschke *et al.*, 2001). Already at 30 hours post-fertilization (hpf) neutrophils are found in the zebrafish intestinal region, although are mostly present in the major blood vessels (Xu, Du and Wen, 2012).

The presence of macrophages was confirmed by *mpeg1* transgenic zebrafish at 28 hpf throughout the embryo including the gut area (Ellett *et al.*, 2011). Macrophages present high functional plasticity and can be classified as M1-type pro-inflammatory macrophages and M2-type anti-inflammatory/ regulatory macrophages (Hodgkinson, Grayfer and Belosevic, 2015). M1-type macrophages have a crucial role in host protection and kill pathogens by engulfment and production of toxic reactive intermediates, phagolysosome acidification and restriction of nutrient availability (Rieger, Hall and Barreda, 2010). Moreover M1-type macrophages produce lots of cytokines (innate immune molecules that signal an immune response) and chemokines (small cytokines that promote the recruitment of leukocytes) to initiate and amplify immune responses, among which INF-γ that induced Th1-type of immune response (reviewed in Wiegertjes *et al.*, 2016a). Contrarily, M2-type macrophages down-regulate the inflammation, preventing that becomes a chronic condition and repair the affected tissue by secreting IL-4/13 and inducing Th2-type of response. Interestingly, identification of macrophages subsets was reported by combination of transgenic zebrafish lines. Macrophages expressing *tnfa* acted like M1-type macrophages upon wounding and bacterial infection and were later converted into M2-type macrophages in the resolution phase of the inflammation (Nguyen-Chi *et al.*, 2015).

Eosinophils are granulocytic leukocytes that respond upon inflammatory or infectious agents such as environmental stressors or helminths and are identified with the eosinophil reporter *gata2:eGFP* (Balla *et al.*, 2010). Eosinophils are located under the epithelial cell layer of the zebrafish gut and under homeostatic conditions 2-3 eosinophils are found per villus but upon inflammation eosinophilic numbers dramatically increase (Brugman *et al.*, 2009; Witte *et al.*, 2014). Another granulocytic cell type reported in zebrafish are mast cells that are an excellent effector innate immune cell in hypersensitivity processes as well as for bacterial and parasite infections (Prykhozhij and Berman, 2014; Sfacteria, Brines and Blank, 2015). Mast cells are identified by the expression of *gata2*, *pu.1* and *cpa5* (Dobson *et al.*, 2008; Da'as *et al.*, 2012). *Cpa5*$^{+}$ cells are found in the blood circulation from 24 hpf and in the gut and gills in adult zebrafish (Dobson *et al.*, 2008).

Natural killer cells are nonspecific cytotoxic innate immune cells that spontaneously kill a variety of foreign targets (Fischer, Koppang and Nakanishi, 2013). Evidence of natural killer-like cells came from rag1-deficient fish (missing B and T cells) that harbored a lymphoid-like cell population expressing novel-immune-type receptors (NITRs) (Yoder *et al.*, 2004), non-specific cytotoxic cell receptor protein-1 and natural killer cell lysin while

lacking TCR and Ig (Petrie-Hanson, Hohn and Hanson, 2009). However, a definitive characterization of fish NK-like cells based on functional activities and surface membrane phenotype warrants more research.

Recently, due to single-cell transcriptional analysis innate lymphoid cells (ILCs) were identified in rag1-deficient zebrafish by the expression of *rorc*. ILCs produced a different repertoire of cytokines depending on the challenged exposed and correlated ILC1 response (*ifng1-1, infg1-2*), ILC2 response (*il4, il13*) or ILC3 response (*il17a/f3, il22*) (Hernández *et al*., 2018). Future studies will benefit from such characterization to understand the extent of the immunological responses of the immune system.

Dendritic cells have been identified as an antigen-presenting cell in the zebrafish intestine (Lugo-Villarino *et al*., 2010). Such cells expressed dendritic cell-associated genes like *il12*, *MHC class II invariant chain iclp1* and *csf1* as well as induced activation of T cell in an antigen-dependent manner. The construction of a *mhc2dab* transgenic zebrafish crossed with *cd45:DsRed* revealed that antigen presenting-like cells accounted for the 5% of the cell fraction in the gut (Wittamer *et al*., 2011).

The exact timing of the maturation of the adaptive immune system is a matter still being discussed within the scientific community. There is increasing evidence of adaptive immune cells migrating from the primary immune organs earlier than what was previously considered. As a matter of fact, intra-epithelial lymphocytes are reported in the zebrafish gut at 8 dpf (Trede, Zapata and Zon, 2001; Zapata *et al*., 2006) although the nature and function of these lymphocytes were not studied. Similarly, researchers detected by *in situ* hybridization TCR+ cells in the esophagus and intestine in 9 dpf-old zebrafish (Danilova *et al*., 2000). However, other studies using transgenic Tg(*lck:GFP*) T cell reporter zebrafish only detected positive cells after 21 dpf (Langenau *et al*., 2004; Brugman *et al*., 2014). In a later study using transgenic Tg(*cd4-1:mCherry*) T cell reporter zebrafish, $CD4^+$ T cells were detected outside of the thymus at 10 dpf (Dee *et al*., 2016) but their ability to contribute to the immune response was not assessed. Humoral responses (IgM) towards gram-negative bacterium could not be observed before 28 dpf (Lam *et al*., 2004) although later other isotypes of antibodies have been identified in zebrafish (IgZ) which transcripts have been detected already at 14 dpf (Hu, Xiang and Shao, 2010). Whether these immunoglobulins are functional in that early life stage of the zebrafish is a question that still remains unsolved. Taking all these data together, we can assume that for at least the first 10-14 dpf zebrafish rely exclusively on the innate immune system (reviewed in Brugman, 2016). In **Figure 3** the development of the innate and adaptive immune system in zebrafish is summarized. Overall, it is clear that zebrafish have greatly contributed the understanding of the immune cell lineage, their function and migration towards the gut tissue.

In zebrafish, contrarily than mammals, gut lymph nodes, Peyer's patchers and germinal centers are not identified so the exact place where APC and adaptive immune

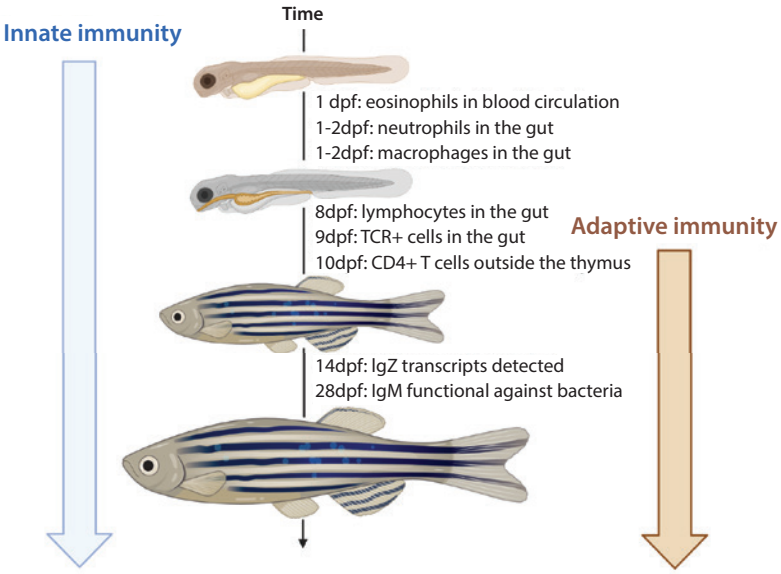


Figure 3: Development of the innate and the adaptive immunity in the zebrafish gut. Data taken from the following studies: Trede, Zapata and Zon, 2001; Lieschke *et al.*, 2001; Langenau *et al.*, 2004; Lam *et al.*, 2004; Zapata *et al.*, 2006; Hu, Xiang and Shao, 2010; Ellett *et al.*, 2011; Brugman *et al.*, 2014 and Dee *et al.*, 2016. Dpf is days-post-fertilization.

cells meet is still an unsolved matter (Lewis, del Cid and Traver, 2014). Recent research showed that the spleen is the major site for $CD4^+$ T and IgM^+ B cell proliferation upon infection that in rainbow trout, suggesting the existence of a semi-organized lymphoid tissue not described before in teleosts (Shibasaki *et al.*, 2019). Interestingly, lymphocytes aggregates (~56% $CD4^+$, 24% IgM^+, 16% $CD8a^+$ and 4% IgT^+) were found in the organized nasopharynx-associated lymphoid tissue (O-NALT) in rainbow trout. Moreover, intranasal vaccination triggered expansion of B and T cells suggesting that O-NALT may be involved in the affinity maturation in teleost (Garcia *et al.*, 2022). More research is warranted to uncover if such structured lymphoid tissues are present in the spleen, in the nasopharynx and in the gut of zebrafish.

Gut inflammation in the zebrafish model

Inflammation is a local temporary response initiated by the innate immune in response to an injury (cellular damage) or infection (pathogen). Acute inflammation is characterised by five different stages after which -if the inflammatory process it is not resolved- can potentially become a chronic condition (reviewed in Campos-Sánchez and Esteban, 2021). The five stages of an acute inflammatory process are:

1) Release of mediators: tissue-resident immune and non-immune cells release inflammatory mediators while the endothelial cells retract to increase blood flow (vasodilation and vascular permeability) associated with redness and swelling signs (reviewed in Hawiger and Zienkiewicz, 2019). Vasodilation and increase in permeability enables large and soluble molecules (mediators) such as pro-inflammatory cytokines (like *il1b, tnfa, il6*), chemokines, histamines, prostaglandins and others that facilitate the recruitment of leukocytes to the site of the inflammation (reviewed in Kolaczkowska and Kubes, 2013). The immune response towards inflammation is regulated by the activation of transcription factors (such as NF-kB, Mulero *et al.*, 2019) that encode the above-mentioned mediators and are functionally well conserved in vertebrates (Zou and Secombes, 2016).

2) Effect of mediators: zebrafish neutrophils are recruited at the site of inflammation and secrete chemokines to activate tissue-resident cells and recruit more leucocytes to amplify the inflammatory response. Resident cells release cytokines *tnfa* and *il1b* to induce expression of ligands for leukocyte integrins by endothelial cells and to stimulate the production of chemokines, such as C-X-C motif chemokine ligand 8 (*cxcl8*) and C-C motif chemokine ligand 2 (*ccl2* renamed to *ccl38.5*) among others that bind to neutrophils and monocytes respectively (Imitola *et al.*, 2004; Burhans *et al.*, 2018). Other mediators, such as *tnfb* orchestrate the activation of the clotting pathways that are also well conserved between fish and mammals: fish thrombocytes are nucleated cells and functionally equivalent to the mammalian platelets (Kim *et al.*, 2010; Khandekar, reviewed in Kim and Jagadeeswaran, 2012).

3) Cellular recruitment: cells from the surrounding tissues migrate to the site of inflammation. Zebrafish do not have structured lymph nodes nor bone marrow but present head kidney as a major haematopoietic and lymphoid organ instead: leukocytes migrate from the head kidney to the site of inflammation (Drummond and Davidson, 2010). The migration process is subdivided in: circulating leukocytes roll along the endothelium via union of selectins and their ligands, leukocytes adhere to the endothelium and lastly leukocytes extravasate towards the luminal site (reviewed in Ley *et al.*, 2007; and in Kolaczkowska and Kubes, 2013). Specifically in zebrafish parts of the leukocyte migration process are visualized *in vivo* due to the existence of several transgenic fluorescently labelled leukocyte reporters (Herbomel, Thisse and Thisse, 1999; Davis *et al.*, 2002; Herbomel and

Levraud, 2005). Firstly, neutrophils infiltrate and are recruited to the site of inflammation followed by the monocytes that differentiate to "classically-activated M1" pro-inflammatory macrophages: high antigen presenting capacities and expression of reactive oxygen species (ROS), *tnfa* and *il1b* (reviewed in Nathan, 2006; and in Medzhitov, 2008; and in Kolaczkowska and Kubes, 2013; Nguyen-Chi *et al.*, 2015). These innate immune cells promote a cascade of cytokines and effector molecules (ROS, proteases, myeloperoxidase, among others) to destroy the inflammatory agent so macrophages can phagocytize it.

4) Regulation of the inflammation: to avoid negative effects of prolonged inflammation the activation of the inhibitory mechanisms is a crucial step for balancing the inflammatory response (Nathan and Ding, 2010). Macrophages are progressively differentiated to "alternatively-activated M2" anti-inflammatory macrophages and the process includes stages where both markers of M1 and M2 polarization are present in these cells (Nguyen-Chi *et al.*, 2015; reviewed in Wiegertjes *et al.*, 2016). There is also a switching in products secreted towards anti-inflammatory cytokines (such as *il10*), prostaglandins or lipoxins, which cease the neutrophil influx in favour of monocyte recruitment (reviewed in Chandrasekharan and Sharma-Walia, 2015). Resident and recruited macrophages are responsible to phagocytise cellular debris as well as neutrophil leftovers and initiate the tissue repairing process (reviewed in Nathan, 2006; Medzhitov, 2008; and in Kolaczkowska and Kubes, 2013) although some presumably anti-inflammatory neutrophil subpopulation have a longer lifespan and are able to return to recirculation (Ellett *et al.*, 2015). By using *Tg(mpeg1:mCherry-F)^{ump2Tg}* to visualize macrophages and *Tg(tnfa:eGFP-F)^{ump5Tg}* to visualize *tnfa* expression, it was showed that pro-inflammatory macrophages (*tnfa+*, GFP+) leave the inflamed site and only anti-inflammatory macrophages (*tnfa-*, GFP-) remained until the regeneration of the tissue was completed (Nguyen-Chi *et al.*, 2017).

5) Reparation: the alternatively-activated M2 macrophages remaining at the site of the inflammation secrete growth factors to attract fibroblasts and increase the phagocytic activity of leukocytes as well as new vascularization (Richardson *et al.*, 2013). Experiments in mice showed that re-epithelization was independent of the inflammatory cells and that wounds closed faster in neutrophil-depleted mice (Dovi, He and DiPietro, 2003; Martin *et al.*, 2003). However, in zebrafish immune cells activated by an inflammatory event served as cues to promote tissue regeneration (reviewed in Kizil, Kyritsis and Brand, 2015). Zebrafish present higher regeneration capacities than their mammalian counterparts although the regeneration as well as the whole inflammatory process seems to be well conserved across vertebrates (reviewed in Campos-Sánchez and Esteban, 2021).

Models of intestinal inflammation in zebrafish

Due to zebrafish larvae research particularities, researchers developed several models to study intestinal inflammation. Most of the knowledge about zebrafish gut topography,

cell recruitment and expression changes upon intestinal inflammation comes from chemically-induced models (reviewed in Fleming, Jankowski and Goldsmith, 2010; Oehlers *et al*., 2013; Brugman, 2016; Xie, Meijer and Schaaf, 2021).

The first chemically-induced model employed oxazolone intrarectally injected in adult zebrafish. The inflammatory response was characterized by intestinal infiltration of granulocytes (eosinophils), neutrophils and lymphocytes together with changes in the intestinal architecture among which bowel-wall thickening, loss in intestinal folds and depletion of goblet cells as well as increased expression of *tnfa, il1β* and *il10* and disrupted microbiota composition (Brugman *et al*., 2009). A second model of chemically-induced gut inflammation was developed by using 2,4,6-trinitrobenzebesulfonic acid (TNBS) in zebrafish larvae (Fleming, Jankowski and Goldsmith, 2010) and later on injected in adults (Geiger *et al*., 2013). In larvae, after 5 days (3-8 days post fertilization -dpf-) immersion in a solution containing TNBS the inflammatory response included disruption and disappearance of the villi, increased goblet cells numbers and TNF-α+ staining in the epithelium (Fleming, Jankowski and Goldsmith, 2010). Furthermore, another study exposing zebrafish larvae to TNBS reported increased intestinal leukocytes, increased proliferating cells (PCNA+ staining), increased NO production as well as increased expression of *il1b, tnfa, ccl20* and*, cxcl8a* (Oehlers *et al*., 2010, 2011). Adult zebrafish injected intrarectally with TNBS did not present changes in goblet cell number but the epithelial integrity was compromised by the emergence of ulcers, swelling and thickening of the bowel-wall and detachment of the villi together with neutrophil infiltration and increased expression of *il1b, il10* and *il8* (Geiger *et al*., 2013). Glafenine was used in larvae to induce intestinal inflammation that consisted of intestinal epithelial cell apoptosis and shedding into the gut lumen due to an increased endoplasmic reticulum (ER) stress from misfolded proteins (Goldsmith *et al*., 2013). Dextran sodium sulfate (DSS) was also employed in zebrafish larvae to further explore the chemically-induced inflammation traits. After 3 days (3-6 dpf) of DSS larval immersion, larvae showed an increase number of neutrophils in the gut, increased mucus quantity (although not number of goblet cells), increased number of proliferating cells (PCNA+ staining) and increased expression of *il1b, il23, mmp9, tnfa, ccl20, pcna* and *cxcl8a*.

Taking all these observation together we can summarize the hallmarks of an inflamed zebrafish gut by an increase number of recruited immune cells such as granulocytes, monocytes, neutrophils and lymphocytes (B and T cells only when the adaptive immunity is present); alteration of the goblet cells and proliferating cells although not consistently; compromised epithelial integrity by villi disruption; altered microbiota composition; thickening and swelling of the wall-bowel; lumen enlargement and increased expression of pro-inflammatory cytokines that may orchestrate some of these cellular changes (summarized in **Figure 4**).

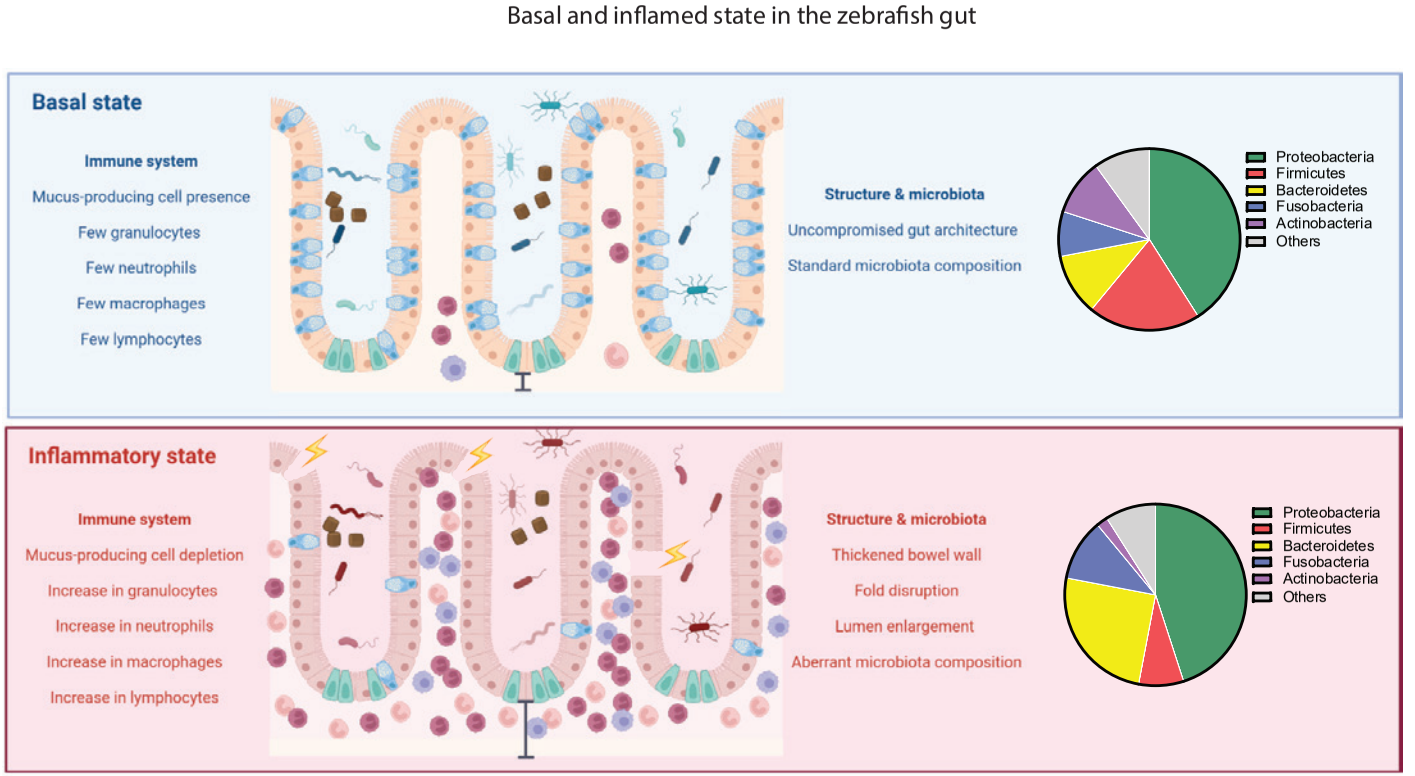


Figure 4: Zebrafish adult gut in basal conditions and in inflamed conditions, summary of the section "Gut inflammation from a zebrafish point of view" data of the microbial composition of the phyla was comparable across studies and it has been averaged from Roeselers *et al.*, 2011; Stephens *et al.*, 2016 and Arias-Jayo *et al.*, 2018.

Zebrafish as a model for feed-induced inflammation

Feed-induced models started emerging due to the necessity of inducing gut inflammation under physiologically relevant circumstances. For example, after assessing that soybean meal induced gut inflammation in aquaculture relevant species like salmonids and carp, soybean meal was utilized in zebrafish to visualize and untangle the etiology of the enteritis.

In salmonids, soybean meal-induced gut inflammation is characterized by infiltration of immune cells to the lamina propria, among which macrophages, neutrophilic granulocytes, eosinophils and IgM^+ cells contributing to the enlargement of the lamina propria and to an overall disruption of the gut epithelium architecture (Bakke-McKellep *et al.*, 2000; Urán *et al.*, 2008, 2009). Moreover, the transcriptomic profile of soybean meal-inflamed salmonids showed an increase in expression of genes and regulators of B and T cell function as well as cell repair and extracellular matrix remodelling (Sahlmann *et al.*, 2013). In carp, soybean meal induced gut inflammation by shortening of the mucosal folds, increased number of basophilic granulocytes, thickening of lamina propria and sub-epithelial mucosa, impaired uptake from the enterocytes with disruption of the microvilli and increased expression of pro-inflammatory cytokines (among which *tnfa* and *il1b*) in isolated intraepithelial lymphocytes (Urán *et al.*, 2008). Contrarily to salmonids, carp started recovering from the feed-induced gut inflammation from the fourth week of switching to the soybean meal diet (Urán *et al.*, 2008). The naturally occurring diet of fish employed in each study may determine the plasticity of the gut epithelium to respond to the inflammation-induced soybean: salmonids are carnivores while carps are omnivores and their digestive enzymes can optimally digest plant-based meals.

In zebrafish, soybean meal showed an increased number of neutrophils around the gut area *in vivo* as well as an increase of pro-inflammatory cytokines (*il1b, il8* and *il10*). In later studies, soybean meal has been fed to zebrafish to induce an inflammatory response and test the capacity of other feed ingredients to attenuate it. As an example, microalgae protected from a pathogenic challenge in soybean meal fed zebrafish larvae (Bravo-Tello *et al.*, 2017). Moreover, aloe vera added to a soybean meal diet prevented intestinal inflammation in zebrafish larvae (Fehrmann-Cartes *et al.*, 2019). It is known that soy saponin is an anti-nutritional component of soybean meal that interacts with cell membranes and promotes pore formation, vesiculation and membrane domain disruption (Augustin *et al.*, 2011) and thus may be the causing agent of the soybean meal-induced enteritis in fish. We employed saponin as a feed component that elicits an inflammatory response in the zebrafish larvae in this thesis (**chapter 2**, **chapter 3** and **chapter 4**).

On the other hand, several fish feed supplements have been promoted as immune-boosters by the aquafeed companies. For instance, butyrate a SCFA derived from the fibre

fermentation has been extensively supplemented to fish feeds. Butyrate, either as sodium butyrate or butyric acid promoted fish growth as well as intestinal health status by modulating the microbial communities, the expression of pro-inflammatory cytokines and the topography and structure of the intestinal epithelium (Abdel-Latif *et al.*, 2020). Nonetheless, several studies addressing the potential gut-health benefits of feed supplements were based on one or two readout parameters and more comprehensive and holistic research is needed to understand the complex effects and interactions of the feeds with the host immune system (reviewed in **chapter 5**). In **chapter 7** a multi-parametric study of butyrate and saponin-supplemented feeds is performed for a proper assessment of the fish gut health.

Thesis content and societal relevance

Fish consumption derived from aquaculture practices already surpassed wild-catch fish in 2016 and the raw materials to manufacture aquafeed diets need to become more sustainable economically and environmentally (FAO, 2020). Furthermore, aquafeeds must strengthen fish (gut) health optimizing gut microbiota and boosting the fish immune system. In order to understand the relationship of feed ingredients and their interplay with host gut health *in vivo* fish models are needed.

Therefore, the **two main aims** of this thesis were:

1) to set-up a reliable fish *in vivo* model to study the host-microbe-immune interactions in the context of feed (ingredients).
2) to create a toolbox of different parameters as readouts for feed-derived inflammation suitable to evaluate novel aquafeeds.

There are many different fish species and not all have the same gastrointestinal tract (GIT). Therefore, the general introduction (**chapter 1**) describes the particularities of the GIT of different fish and investigates why zebrafish are an excellent vertebrate animal model for many scientific disciplines. When it comes to the discipline of immunology, zebrafish stand out as an animal model for studying intestinal inflammation and gut health, with well-described cell types in the gut and hallmarks of gut inflammation derived from chemically-induced inflammation models (**Figure 4**). **Chapter 1** also introduces limitations in the evaluation of the health-associated effects of promising novel diets and the interest of aquafeed companies for immune-boosting supplements, leading to the formulation of the two main aims of this thesis.

In **chapter 2** the ability of soy saponin to elicit an inflammatory response in zebrafish larvae is described. This antinutritional factor of soybean meal has been known to elicit inflammatory responses in fish species relevant to aquaculture, but not well studied in zebrafish. In this chapter the inflammatory response is quantified by gene expression of several pro-inflammatory cytokines and by counting of neutrophils and macrophages in double transgenic zebrafish larvae. In this chapter, a first step is taken to study host-microbe-immune interactions by correlating fluctuations in microbial composition determined by 16S rRNA profiling with saponin-induced inflammation in zebrafish larvae.

In **chapter 3**, host-microbe-immune interactions upon saponin-induced inflammation in zebrafish larvae are examined in more detail. To this end, microbial imbalances in zebrafish larvae caused by exposure to oxytetracycline-treated adult gut content were assessed by 16S rRNA profiling. Subsequently, zebrafish larvae were challenged with saponin and their immune responses quantified by gene expression of pro-inflammatory cytokines and by counting of neutrophils and macrophages in double transgenic zebrafish larvae.

In **chapter 4,** changes in behavior and thermal preference of zebrafish larvae were examined as a readout for saponin-induced inflammation. To this end, a novel infrared camera-based live-tracking set-up was designed to record the movement of free swimming zebrafish larvae within an observation chamber comprising a selected range of temperatures. Saponin-induced inflammation and dexamethasone-driven anti-inflammatory effects were used to study immune responses quantified by gene expression of pro-inflammatory cytokines and chemokines, and by counting of neutrophils and macrophages in double transgenic zebrafish larvae. In addition, the behavior of *cxcl8a* knock-out zebrafish was studied in relation to thermal preference of zebrafish larvae.

Chapter 5 emphasizes the usage of zebrafish to study gut health and summarizes the current literature on studies with prebiotics, probiotics and other feed supplements in zebrafish. The review makes stresses that some feed supplements are administrated at very early developmental stages of zebrafish, when the immune system is not yet fully mature. This leaves unaddressed a whole range of potential actions of feed supplements. Finally, it is suggested that multi-parametric studies are key to provide a more holistic understanding on zebrafish gut health.

Designed as a case study, in **chapter 6** the potential of zebrafish to evaluate feed ingredients and their gut health effects was tested by using fucoidan from brown algae. Zebrafish larvae were exposed and adults supplemented with two different concentrations of fucoidan, and immune responses quantified by cytokine gene expression and correlated with fluctuations in microbiota composition determined by 16S rRNA profiling. Neutrophils and macrophages counts were quantified from double transgenic zebrafish larvae.

Designed as a holistic approach to immunologically-mature zebrafish (juveniles, 40 dpf), in **chapter 7,** novel butyrate and saponin-supplemented diets were used to study their effects on gut health. The multi-parametric approach included a comprehensive pipeline analysis of fish growth, gut microbial composition and taxa connectivity (16S rRNA profiling), host gut transcriptomics and unbiased gut quantitative histology. Moreover, the multi-parametric study was complemented with a quantification of *in vivo* neutrophils and macrophages from larvae exposed to saponin and butyrate. All datasets combined should allow for a proper assessment of the effects of feed supplements on fish gut health.

The general discussion (**chapter 8**) reflects on the achievement of the main aims of this thesis, and discusses the use of zebrafish as an animal model to study host-microbe-immune interactions in the context of feed ingredients and novel diets. Experimental designs are critically reviewed and follow-up experiments proposed. Read-outs of inflammation for zebrafish larvae, juvenile and adults are proposed for future studies and discussed are parameters such as gut microbiota, (innate) immune cell quantification and gene expression biomarkers. Finally, the zebrafish model is revisited to study host-microbe-immune interactions and to test feeds derived from novel protein sources that may contribute to more sustainable diets for our farmed fish which in turn will feed our future generations.

References

Abdel-Latif, H.M.R. *et al.* (2020) 'Benefits of Dietary Butyric Acid, Sodium Butyrate, and Their Protected Forms in Aquafeeds: A Review', *Reviews in Fisheries Science & Aquaculture*, 28(4), pp. 421–448. Available at: https://doi.org/10.1080/23308249.2020.1758899.

AL, H.A.H. (1947) 'The feeding habits and the morphology of the Alimentary tract of some Teleost living in the neighbourhood of the Mar. Biol. Stat. Ghar.' Publ. Mar. Biol. Stat. Ghardapa.

Albadri, S., del Bene, F. and Revenu, C. (2017) 'Genome editing using CRISPR/Cas9-based knock-in approaches in zebrafish', *Methods*, 121–122, pp. 77–85. Available at: https://doi.org/10.1016/j.ymeth.2017.03.005.

Al-Hussaini, A.H. (1946) 'The anatomy and histology of the alimentary tract of the bottom-feeder, Mulloides auriflamma (Forsk.)', *Journal of Morphology*, 78(1), pp. 121–153.

Arias-Jayo, N. *et al.* (2018) 'High-Fat Diet Consumption Induces Microbiota Dysbiosis and Intestinal Inflammation in Zebrafish', *Microbial Ecology*, 76(4), pp. 1089–1101. Available at: https://doi.org/10.1007/s00248-018-1198-9.

Augustin, J.M. *et al.* (2011) 'Molecular activities, biosynthesis and evolution of triterpenoid saponins', *Phytochemistry*, 72(6), pp. 435–457.

Bakke, A.M., Glover, C. and Krogdahl, Å. (2010) 'Feeding, digestion and absorption of nutrients', in *Fish physiology*. Elsevier, pp. 57–110.

Bakke-McKellep, A.M. *et al.* (2000) 'Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, Salmo salar L., with soybean meal-induced enteritis', *Journal of Fish Diseases*, 23(2), pp. 115–127. Available at: https://doi.org/10.1046/j.1365-2761.2000.00218.x.

Balla, K.M. *et al.* (2010) 'Eosinophils in the zebrafish: prospective isolation, characterization, and eosinophilia induction by helminth determinants', *Blood, The Journal of the American Society of Hematology*, 116(19), pp. 3944–3954.

Bates, J.M. *et al.* (2006) 'Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation', *Developmental Biology*, 297(2), pp. 374–386. Available at: https://doi.org/10.1016/j.ydbio.2006.05.006.

Bedell, V.M. *et al.* (2012) 'In vivo genome editing using a high-efficiency TALEN system', *Nature*, 491(7422), pp. 114–118. Available at: https://doi.org/10.1038/nature11537.

Bravo-Tello, K. *et al.* (2017) 'Effect of microalgae on intestinal inflammation triggered by soybean meal and bacterial infection in zebrafish', *PLoS ONE*, 12(11), pp. 1–13. Available at: https://doi.org/10.1371/journal.pone.0187696.

Brugman, S. *et al.* (2009) 'Oxazolone-Induced Enterocolitis in Zebrafish Depends on the Composition of the Intestinal Microbiota', *Gastroenterology*, 137(5), pp. 1757-1767.e1. Available at: https://doi.org/10.1053/j.gastro.2009.07.069.

Brugman, S. *et al.* (2014) ' T Lymphocyte–Dependent and –Independent Regulation of Cxcl8 Expression in Zebrafish Intestines ', *The Journal of Immunology*, 192(1), pp. 484–491. Available at: https://doi.org/10.4049/jimmunol.1301865.

Brugman, S. (2016) 'The zebrafish as a model to study intestinal inflammation', *Developmental and Comparative Immunology*, 64, pp. 82–92. Available at: https://doi.org/10.1016/j.dci.2016.02.020.

Burhans, M.S. *et al.* (2018) 'Contribution of adipose tissue inflammation to the development of type 2 diabetes mellitus', *Comprehensive Physiology*, 9(1), p. 1.

Campos-Sánchez, J.C. and Esteban, M.Á. (2021) 'Review of inflammation in fish and value of the zebrafish model', *Journal of Fish Diseases*, 44(2), pp. 123–139.

Chandrasekharan, J.A. and Sharma-Walia, N. (2015) 'Lipoxins: nature's way to resolve inflammation', *Journal of inflammation research*, 8, p. 181.

Clelland, E. and Peng, C. (2009) 'Endocrine/paracrine control of zebrafish ovarian development', *Molecular and cellular endocrinology*, 312(1–2), pp. 42–52.

Clements, K.D. *et al.* (2014) 'Intestinal microbiota in fishes: what's known and what's not'. Wiley Online Library.

Clements, K.D., Gleeson, V.P. and Slaytor, M. (1994) 'Short-chain fatty acid metabolism in temperate marine herbivorous fish', *Journal of Comparative Physiology B*, 164(5), pp. 372–377. Available at: https://doi.org/10.1007/BF00302552.

Da'as, S.I. *et al.* (2012) 'The zebrafish reveals dependence of the mast cell lineage on Notch signaling in vivo', *Blood, The Journal of the American Society of Hematology*, 119(15), pp. 3585–3594.

Danilova, N. *et al.* (2000) 'Immunoglobulin variable-region diversity in the zebrafish', *Immunogenetics*, 52(1), pp. 81–91.

Dao, D.-P.D. and Le, P.H. (2021) 'Histology, goblet cells', in *StatPearls [Internet]*. StatPearls Publishing.

David Jonah Grunwald, J.S.E. (2002) 'Hearwaters of the zebrafish - emergence of a new model vertebrate', *Nature Reviews Genetics*, 3, pp. 717–724.

Davis, J.M. *et al.* (2002) 'Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos', *Immunity*, 17(6), pp. 693–702.

Dee, C.T. *et al.* (2016) 'CD4-transgenic zebrafish reveal tissue-resident Th2-and regulatory T cell–like populations and diverse mononuclear phagocytes', *The Journal of Immunology*, 197(9), pp. 3520–3530.

Dobson, J.T. *et al.* (2008) 'Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination', *Blood, The Journal of the American Society of Hematology*, 112(7), pp. 2969–2972.

Dovi, J. v, He, L. and DiPietro, L.A. (2003) 'Accelerated wound closure in neutrophil-depleted mice', *Journal of leukocyte biology*, 73(4), pp. 448–455.

Drummond, I.A. and Davidson, A.J. (2010) 'Zebrafish kidney development', in *Methods in cell biology*. Elsevier, pp. 233–260.

Egerton, S. *et al.* (2018) 'The gut microbiota of marine fish', *Frontiers in microbiology*, 9, p. 873.

Ellett, F. *et al.* (2011) 'mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish', *Blood*, 117(4), pp. e49–e56. Available at: https://doi.org/https://doi.org/10.1182/blood-2010-10-314120.

Ellett, F. *et al.* (2015) 'Defining the phenotype of neutrophils following reverse migration in zebrafish', *Journal of leukocyte biology*, 98(6), pp. 975–981.

Elliott, J.P. and Bellwood, D.R. (2003) 'Alimentary tract morphology and diet in three coral reef fish families', *Journal of Fish Biology*, 63(6), pp. 1598–1609.

Ezeasor, D.N. and Stokoe, W.M. (1981) 'Light and electron microscopic studies on the absorptive cells of the intestine, caeca and rectum of the adult rainbow trout, Salmo gairdneri, Rich', *Journal of Fish Biology*, 18(5), pp. 527–544.

FAO (2020) *The State of World Fisheries and Aquaculture 2020*. Sustainabi. Rome.

Fehrmann-Cartes, K. *et al.* (2019) 'Anti-inflammatory effects of aloe vera on soy meal-induced intestinal inflammation in zebrafish', *Fish & shellfish immunology*, 95, pp. 564–573.

Fischer, U., Koppang, E.O. and Nakanishi, T. (2013) 'Teleost T and NK cell immunity', *Fish & shellfish immunology*, 35(2), pp. 197–206.

Fleming, A., Jankowski, J. and Goldsmith, P. (2010) 'In vivo analysis of gut function and disease changes in a zebrafish larvae model of inflammatory bowel disease: a feasibility study', *Inflammatory bowel diseases*, 16(7), pp. 1162–1172.

Frierson, E.W. and Foltz, J.W. (1992) 'Comparison and estimation of absorptive intestinal surface areas in two species of cichlid fish', *Transactions of the American Fisheries Society*, 121(4), pp. 517–523.

Garcia, B. *et al.* (2022) 'A Novel Organized Nasopharynx-Associated Lymphoid Tissue in Teleosts That Expresses Molecular Markers Characteristic of Mammalian Germinal Centers', *The Journal of Immunology*, 209(11), pp. 2215–2226. Available at: https://doi.org/10.4049/jimmunol.2200396.

Geiger, B.M. *et al.* (2013) 'Intestinal upregulation of melanin-concentrating hormone in TNBS-induced enterocolitis in adult zebrafish', *PLoS ONE*, 8(12). Available at: https://doi.org/10.1371/journal.pone.0083194.

Gerhard, G.S. and Cheng, K.C. (2002) 'A call to fins! Zebrafish as a gerontological model', *Aging cell*, 1(2), pp. 104–111.

German, D.P. (2009) 'Do herbivorous minnows have "plug-flow reactor" guts? Evidence from digestive enzyme activities, gastrointestinal fermentation, and luminal nutrient concentrations', *Journal of Comparative Physiology B*, 179(6), pp. 759–771.

German, D.P. *et al.* (2010) 'Evolution of herbivory in a carnivorous clade of minnows (Teleostei: Cyprinidae): effects on gut size and digestive physiology', *Physiological and Biochemical Zoology*, 83(1), pp. 1–18.

German, D.P. and Horn, M.H. (2006) 'Gut length and mass in herbivorous and carnivorous prickleback fishes (Teleostei: Stichaeidae): ontogenetic, dietary, and phylogenetic effects', *Marine Biology*, 148(5), pp. 1123–1134.

Gill, Theo. (1911) 'A New Translation of Aristotle's ``History of Animals''', *Science*, 33(854), pp. 730–738.

Givens, C.E. *et al.* (2015) 'Comparison of the gut microbiomes of 12 bony fish and 3 shark species', *Marine Ecology Progress Series*, 518, pp. 209–223.

Goldsmith, J.R. *et al.* (2013) 'Glafenine-induced intestinal injury in zebrafish is ameliorated by μ-opioid signaling via enhancement of Atf6-dependent cellular stress responses', *Disease models & mechanisms*, 6(1), pp. 146–159.

Grosell, M., Farrell, A.P. and Brauner, C.J. (2010) *Fish physiology: the multifunctional gut of fish*. Academic Press.

Hansen, G.H. and Olafsen, J.A. (1999) 'Bacterial interactions in early life stages of marine cold water fish', *Microbial ecology*, 38(1), pp. 1–26.

Hao, Y.T., Wu, S.G., Jakovlić, I., *et al.* (2017) 'Impacts of diet on hindgut microbiota and short-chain fatty acids in grass carp (Ctenopharyngodon idellus)', *Aquaculture Research*, 48(11), pp. 5595–5605.

Hao, Y.T., Wu, S.G., Xiong, F., *et al.* (2017) 'Succession and fermentation products of grass carp (Ctenopharyngodon idellus) hindgut microbiota in response to an extreme dietary shift', *Frontiers in microbiology*, 8, p. 1585.

Harder, W. (1975) *Anatomy of Fishes: Figures and plates*. Schweizerbart.

Hawiger, J. and Zienkiewicz, J. (2019) 'Decoding inflammation, its causes, genomic responses, and emerging countermeasures', *Scandinavian journal of immunology*, 90(6), p. e12812.

van der Hee, B. and Wells, J.M. (2021) 'Microbial regulation of host physiology by short-chain fatty acids', *Trends in Microbiology*, 29(8), pp. 700–712.

Hensley, M.R. and Leung, Y.F. (2010) 'A convenient dry feed for raising zebrafish larvae', *Zebrafish*, 7(2), pp. 219–231.

Herbomel, P. and Levraud, J.-P. (2005) 'Imaging early macrophage differentiation, migration, and behaviors in live zebrafish embryos', *Developmental Hematopoiesis*, pp. 199–214.

Herbomel, P., Thisse, B. and Thisse, C. (1999) 'Ontogeny and behaviour of early macrophages in the zebrafish embryo', *Development*, 126(17), pp. 3735–3745.

Hernández, P.P. *et al.* (2018) 'Single-cell transcriptional analysis reveals ILC-like cells in zebrafish', *Science immunology*, 3(29), p. eaau5265.

Hodgkinson, J.W., Grayfer, L. and Belosevic, M. (2015) 'Biology of bony fish macrophages', *Biology*, 4(4), pp. 881–906.

Holmgren, S. and Olsson, C. (2009) 'The neuronal and endocrine regulation of gut function', *Fish Physiology*, 28, pp. 467–512.

Horn, M.H. *et al.* (2006) 'Structure and function of the stomachless digestive system in three related species of New World silverside fishes (Atherinopsidae) representing herbivory, omnivory, and carnivory', *Marine Biology*, 149(5), pp. 1237–1245.

Hoseinifar, S.H., Sun, Y. and Caipang, C.M. (2017) 'Short-chain fatty acids as feed supplements for sustainable aquaculture: An updated view', *Aquaculture Research*, 48(4), pp. 1380–1391.

Howe, K. *et al.* (2013) 'The zebrafish reference genome sequence and its relationship to the human genome', *Nature*, 496(7446), pp. 498–503.

Hu, Y.L., Xiang, L.X. and Shao, J.Z. (2010) 'Identification and characterization of a novel immunoglobulin Z isotype in zebrafish: Implications for a distinct B cell receptor in lower vertebrates', *Molecular Immunology*, 47(4), pp. 738–746. Available at: https://doi.org/10.1016/j.molimm.2009.10.010.

Hwang, W.Y. *et al.* (2013) 'Efficient genome editing in zebrafish using a CRISPR-Cas system', *Nature Biotechnology*, 31(3), pp. 227–229. Available at: https://doi.org/10.1038/nbt.2501.

Imitola, J. *et al.* (2004) 'Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1α/CXC chemokine receptor 4 pathway', *Proceedings of the National Academy of Sciences*, 101(52), pp. 18117–18122.

Jutfelt, F. (2011) 'Barrier function of the gut', *Encyclopedia of fish physiology: from genome to environment*, 2, pp. 1322–1331.

Kapoor, B.G. and Khawna, B. (1993) 'The potential spectrum of the gut in teleost fishes', *Adv. Fish Biol*, 1, pp. 221–226.

Karasov, W.H. and del Rio, C.M. (2020) 'Physiological ecology', in *Physiological Ecology*. Princeton University Press.

Khandekar, G., Kim, S. and Jagadeeswaran, P. (2012) 'Zebrafish thrombocytes: functions and origins', *Advances in hematology*, 2012.

Kim, S. *et al.* (2010) 'Vivo-Morpholino knockdown of αIIb: A novel approach to inhibit thrombocyte function in adult zebrafish', *Blood Cells, Molecules, and Diseases*, 44(3), pp. 169–174.

Kimmel, C.B. *et al.* (1995) 'Stages of embryonic development of the zebrafish', *Developmental dynamics*, 203(3), pp. 253–310.

Kizil, C., Kyritsis, N. and Brand, M. (2015) 'Effects of inflammation on stem cells: together they strive?', *EMBO reports*, 16(4), pp. 416–426.

Kolaczkowska, E. and Kubes, P. (2013) 'Neutrophil recruitment and function in health and inflammation', *Nature reviews immunology*, 13(3), pp. 159–175.

Kramer, D.L. and Bryant, M.J. (1995) 'Intestine length in the fishes of a tropical stream: 2. Relationships to diet—the long and short of a convoluted issue', *Environmental biology of fishes*, 42(2), pp. 129–141.

Kuz'Mina, V. v and Gelman, A.G. (1997) 'Membrane-linked digestion in fish', *Reviews in Fisheries Science*, 5(2), pp. 99–129.

Lam, S.H. *et al.* (2004) 'Development and maturation of the immune system in zebrafish, Danio rerio: A gene expression profiling, in situ hybridization and immunological study', *Developmental and Comparative Immunology*, 28(1), pp. 9–28. Available at: https://doi.org/10.1016/S0145-305X(03)00103-4.

Langenau, D.M. *et al.* (2004) 'In vivo tracking of T cell development, ablation, and engraftment in transgenic zebrafish.', *Proceedings of the National Academy of Sciences of the United States of America*, 101(19), pp. 7369–74. Available at: https://doi.org/10.1073/pnas.0402248101.

Larsen, A. (2014) 'Studies on the microbiota of fishes and the factors influencing their composition'.

Lauzon, H.L. *et al.* (2010) 'Microbiota of Atlantic cod (Gadus morhua L.) rearing systems at pre-and posthatch stages and the effect of different treatments', *Journal of applied microbiology*, 109(5), pp. 1775–1789.

Lawrence, C. (2011) 'Advances in zebrafish husbandry and management', *Methods in cell biology*, 104, pp. 429–451.

Ledent, V. (2002) 'Postembryonic development of the posterior lateral line in zebrafish', *Development*, 129(3), pp. 597-604.

Lewis, K.L., del Cid, N. and Traver, D. (2014) 'Perspectives on antigen presenting cells in zebrafish', *Developmental and Comparative Immunology*, 46(1), pp. 63–73. Available at: https://doi.org/10.1016/j.dci.2014.03.010.

Ley, K. *et al.* (2007) 'Getting to the site of inflammation: the leukocyte adhesion cascade updated', *Nature Reviews Immunology*, 7(9), pp. 678–689.

Lieschke, G.J. *et al.* (2001) 'Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish', *Blood, The Journal of the American Society of Hematology*, 98(10), pp. 3087–3096.

Liu, H. *et al.* (2016) 'The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels', *Scientific reports*, 6(1), pp. 1–12.

Lugo-Villarino, G. *et al.* (2010) 'Identification of dendritic antigen-presenting cells in the zebrafish', *Proceedings of the National Academy of Sciences*, 107(36), pp. 15850–15855.

Ma, J., Rubin, B.K. and Voynow, J.A. (2018) 'Mucins, mucus, and goblet cells', *Chest*, 154(1), pp. 169–176.

Martin, P. *et al.* (2003) 'Wound healing in the PU. 1 null mouse—tissue repair is not dependent on inflammatory cells', *Current biology*, 13(13), pp. 1122–1128.

Medzhitov, R. (2008) 'Origin and physiological roles of inflammation', *Nature*, 454(7203), pp. 428–435.

Merrifield, D.L. and Rodiles, A. (2015) 'The fish microbiome and its interactions with mucosal tissues', in *Mucosal health in aquaculture*. Elsevier, pp. 273–295.

Mountfort, D.O., Campbell, J. and Clements, K.D. (2002) 'Hindgut fermentation in three species of marine herbivorous fish', *Applied and environmental microbiology*, 68(3), pp. 1374–1380.

Mulero, M.C. *et al.* (2019) 'Genome reading by the NF-κB transcription factors', *Nucleic acids research*, 47(19), pp. 9967–9989.

Nalbant, P. *et al.* (1999) 'Functional characterization of a Na+–phosphate cotransporter (NaPi-II) from zebrafish and identification of related transcripts', *The Journal of physiology*, 520(1), pp. 79–89.

Nathan, C. (2006) 'Neutrophils and immunity: challenges and opportunities', *Nature reviews immunology*, 6(3), pp. 173–182.

Nathan, C. and Ding, A. (2010) 'Nonresolving inflammation', *Cell*, 140(6), pp. 871–882.

Nelson, J.S., Grande, T.C. and Wilson, M.V.H. (2016) *Fishes of the World*. John Wiley & Sons.

Nguyen-Chi, M. *et al.* (2015) 'Identification of polarized macrophage subsets in zebrafish', *Elife*, 4, p. e07288.

Nguyen-Chi, M. *et al.* (2017) 'TNF signaling and macrophages govern fin regeneration in zebrafish larvae', *Cell death & disease*, 8(8), pp. e2979–e2979.

Oehlers, S.H. *et al.* (2011) 'A chemical enterocolitis model in zebrafish larvae that is dependent on microbiota and responsive to pharmacological agents', *Developmental Dynamics*, 240(1), pp. 288–298. Available at: https://doi.org/10.1002/dvdy.22519.

Oehlers, S.H. *et al.* (2013) 'Chemically Induced Intestinal Damage Models in Zebrafish Larvae', *Zebrafish*, 10(2), pp. 184–193. Available at: https://doi.org/10.1089/zeb.2012.0824.

Oehlers, S.H.B. *et al.* (2010) 'Expression of zebrafish cxcl8 (interleukin-8) and its receptors during development and in response to immune stimulation', *Developmental and Comparative Immunology*, 34(3), pp. 352–359. Available at: https://doi.org/10.1016/j.dci.2009.11.007.

Petit, J. and Wiegertjes, G.F. (2022) 'Conservation of members of the free fatty acid receptor gene family in common carp.', *Developmental and comparative immunology*, 126, p. 104240. Available at: https://doi.org/10.1016/j.dci.2021.104240.

Petrie-Hanson, L., Hohn, C. and Hanson, L. (2009) 'Characterization of rag 1 mutant zebrafish leukocytes', *BMC Immunology*, 10(1), p. 8. Available at: https://doi.org/10.1186/1471-2172-10-8.

Prykhozhij, S. v and Berman, J.N. (2014) 'The progress and promise of zebrafish as a model to study mast cells', *Developmental & Comparative Immunology*, 46(1), pp. 74–83.

Rawls, J.F., Samuel, B.S. and Gordon, J.I. (2004) 'Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota', *PNAS*, 101(13), pp. 4596–4601. Available at: papers2://publication/uuid/897C5AC0-1CF8-4439-A8CE-1A42D6A7D552.

Reid, H.I. *et al.* (2009) 'Analysis of bacterial populations in the gut of developing cod larvae and identification of Vibrio logei, Vibrio anguillarum and Vibrio splendidus as pathogens of cod larvae', *Aquaculture*, 288(1–2), pp. 36–43.

Renshaw, S.A. *et al.* (2006) 'A transgenic zebrafish model of neutrophilic inflammation', *Blood*, 108(13), pp. 3976–3978.

Ribas, L. and Piferrer, F. (2014) 'The zebrafish (Danio rerio) as a model organism, with emphasis on applications for finfish aquaculture research', *Reviews in Aquaculture*, 6(4), pp. 209–240. Available at: https://doi.org/10.1111/raq.12041.

Ribble, D.O. and Smith, M.H. (1983) 'Relative intestine length and feeding ecology of freshwater fishes', *Growth*, 47(3), p. 292.

Richardson, R. *et al.* (2013) 'Adult Zebrafish as a Model System for Cutaneous Wound-Healing Research', *Journal of Investigative Dermatology*, 133(6), pp. 1655–1665. Available at: https://doi.org/https://doi.org/10.1038/jid.2013.16.

Rieger, A.M., Hall, B.E. and Barreda, D.R. (2010) 'Macrophage activation differentially modulates particle binding, phagocytosis and downstream antimicrobial mechanisms', *Developmental & Comparative Immunology*, 34(11), pp. 1144–1159.

Ringø, E. *et al.* (2016) 'Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story?', *Aquaculture nutrition*, 22(2), pp. 219–282.

Roeselers, G. *et al.* (2011) 'Evidence for a core gut microbiota in the zebrafish', *ISME Journal*, 5(10), pp. 1595–1608. Available at: https://doi.org/10.1038/ismej.2011.38.

Romero, J., Ringø, E. and Merrifield, D.L. (2014) 'The gut microbiota of fish', *Aquaculture nutrition: Gut health, probiotics and prebiotics*, pp. 75–100.

Sahlmann, C. *et al.* (2013) 'Early response of gene expression in the distal intestine of Atlantic salmon (Salmo salar L.) during the development of soybean meal induced enteritis', *Fish & shellfish immunology*, 34(2), pp. 599–609.

Sfacteria, A., Brines, M. and Blank, U. (2015) 'The mast cell plays a central role in the immune system of teleost fish', *Molecular immunology*, 63(1), pp. 3–8.

Shibasaki, Y. *et al.* (2019) 'Identification of primordial organized lymphoid structure in the spleen of teleost fish', *Fish & Shellfish Immunology*, 91, p. 428. Available at: https://doi.org/https://doi.org/10.1016/j.fsi.2019.04.172.

Singleman, C. and Holtzman, N.G. (2014) ' Growth and Maturation in the Zebrafish, Danio Rerio : A Staging Tool for Teaching and Research ', *Zebrafish*, 11(4), pp. 396–406. Available at: https://doi.org/10.1089/zeb.2014.0976.

Smith, T.B., Wahl, D.H. and Mackie, R.I. (1996) 'Volatile fatty acids and anaerobic fermentation in temperate piscivorous and omnivorous freshwater fish', *Journal of fish Biology*, 48(5), pp. 829–841.

Stephens, W. Zac *et al.* (2016) 'The composition of the zebrafish intestinal microbial community varies across development', *ISME Journal*, 10(3), pp. 644–654. Available at: https://doi.org/10.1038/ismej.2015.140.

Stephens, W Zac *et al.* (2016) 'The composition of the zebrafish intestinal microbial community varies across development', *The ISME Journal*, 10(3), pp. 644–654. Available at: https://doi.org/10.1038/ismej.2015.140.

Stevens, C.E. and Hume, I.D. (2004) *Comparative physiology of the vertebrate digestive system*. Cambridge University Press.

Sullam, K.E., Essinger, S.D., Lozupone, C.A., Connor, M.P.O., *et al.* (2012) 'Environmental and ecological factors that shape the gut 2 bacterial communities of fish: a meta-analysis - Supplementary', *Molecular Ecology*, 21(13), pp. 1–16.

Sullam, K.E., Essinger, S.D., Lozupone, C.A., O'CONNOR, M.P., *et al.* (2012) 'Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis', *Molecular ecology*, 21(13), pp. 3363–3378.

Tran, N.T., Wang, G. and Wu, S. (2017) 'A review of intestinal microbes in grass carp Ctenopharyngodon idellus (Valenciennes)', *Aquaculture Research*, 48(7), pp. 3287–3297.

Trede, N.S., Zapata, A. and Zon, L.I. (2001) 'Fishing for lymphoid genes', *Trends in immunology*, 22(6), pp. 302–307.

Urán, P.A. *et al.* (2008) 'Soybean meal induces intestinal inflammation in common carp (Cyprinus carpio L.)', *Fish and Shellfish Immunology*, 25(6), pp. 751–760. Available at: https://doi.org/10.1016/j.fsi.2008.02.013.

Urán, P.A. *et al.* (2009) 'Time-related changes of the intestinal morphology of Atlantic salmon, Salmo salar L., at two different soybean meal inclusion levels', *Journal of fish diseases*, 32(9), pp. 733–744.

Urnov, F.D. *et al.* (2010) 'Genome editing with engineered zinc finger nucleases', *Nature Reviews Genetics*, 11(9), pp. 636–646. Available at: https://doi.org/10.1038/nrg2842.

Wallace, K.N. *et al.* (2005) 'Intestinal growth and differentiation in zebrafish', *Mechanisms of Development*, 122(2), pp. 157–173. Available at: https://doi.org/10.1016/j.mod.2004.10.009.

Wallace, K.N. and Pack, M. (2003) 'Unique and conserved aspects of gut development in zebrafish', *Developmental Biology*, 255(1), pp. 12–29. Available at: https://doi.org/10.1016/S0012-1606(02)00034-9.

Wiegertjes, G.F. *et al.* (2016a) 'Polarization of immune responses in fish: The "macrophages first" point of view', *Molecular Immunology*, 69, pp. 146–156. Available at: https://doi.org/https://doi.org/10.1016/j.molimm.2015.09.026.

Wiegertjes, G.F. *et al.* (2016b) 'Polarization of immune responses in fish: The 'macrophages first'point of view', *Molecular immunology*, 69, pp. 146–156.

Wilson, J.M. and Castro, L.F.C. (2010) 'Morphological diversity of the gastrointestinal tract in fishes', in *Fish physiology*. Elsevier, pp. 1–55.

Wittamer, V. *et al.* (2011) 'Characterization of the mononuclear phagocyte system in zebrafish', *Blood*, 117(26), pp. 7126–7135. Available at: https://doi.org/https://doi.org/10.1182/blood-2010-11-321448.

Witte, M. *et al.* (2014) 'Deficiency in macrophage-stimulating protein results in spontaneous intestinal inflammation and increased susceptibility toward epithelial damage in zebrafish', *Zebrafish*, 11(6), pp. 542–550.

Xie, Y., Meijer, A.H. and Schaaf, M.J.M. (2021) 'Modeling inflammation in zebrafish for the development of anti-inflammatory drugs', *Frontiers in Cell and Developmental Biology*, 8, p. 620984.

Xu, J., Du, L. and Wen, Z. (2012) 'Myelopoiesis during Zebrafish Early Development', *Journal of Genetics and Genomics*, 39(9), pp. 435–442. Available at: https://doi.org/https://doi.org/10.1016/j.jgg.2012.06.005.

Yoder, J.A. *et al.* (2002) 'Zebrafish as an immunological model system.', *Microbes and infection*, 4(14), pp. 1469–78. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12475637.

Yoder, J.A. *et al.* (2004) 'Resolution of the novel immune-type receptor gene cluster in zebrafish', *Proceedings of the National Academy of Sciences*, 101(44), pp. 15706–15711.

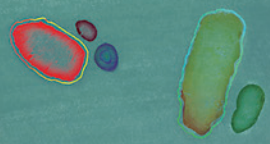
Zapata, A. *et al.* (2006) 'Ontogeny of the immune system of fish', *Fish & shellfish immunology*, 20(2), pp. 126–136.

Zihler, F. (1981) 'Gross morphology and configuration of digestive tracts of Cichlidae (Teleostei, Perciformes): phylogenetic and functional, significance', *Netherlands Journal of Zoology*, 32(4), pp. 544–571.

Zou, J. and Secombes, C.J. (2016) 'The function of fish cytokines', *Biology*, 5(2), p. 23.

Chapter 2

Exposure to antibiotics affects saponin immersion-induced immune stimulation and shift in microbial composition in zebrafish larvae



Adrià López Nadal, David Peggs, Geert F. Wiegertjes and Sylvia Brugman

Frontiers in Microbiology, 29 October 2018
https://doi.org/10.3389/fmicb.2018.02588

Abstract

In the last decades, pollution of the environment by large scale use of antibiotics in agriculture and human medicine have led to increased antimicrobial resistance in both the environment and the host animal microbiome. Disturbances in the host microbiome can result in impaired immunity and reduced resilience of aquaculture species. Here, we investigated whether environmentally measured levels of the commonly used antibiotics ciprofloxacin and oxytetracycline influences the host microbiome and susceptibility toward saponin-induced immune stimulation in larval zebrafish. Firstly, neutrophil and macrophage reporter zebrafish larvae were exposed to different concentrations of soy saponin by immersion. A dose-dependent increase in neutrophil presence in the intestinal area was observed together with increased expression of immune genes *il1b, tnfb, il22* and *mmp9*. To investigate the effect of antibiotics, larval zebrafish were immersed in ciprofloxacin or oxytetracycline in the presence or absence of a low dose of saponin. *In vivo* imaging revealed that antibiotic treatment did not reduce the number of neutrophils that were recruited to the intestinal area upon saponin exposure, although it did tend to lower pro-inflammatory cytokine levels. Microbial sequencing of whole larvae revealed that exposure to a low dose of saponin already shifted the microbial composition. The combination of oxytetracycline and saponin significantly increased α-diversity compared to the controls. In conclusion, the current study provides evidence that the combination of low levels of antibiotics with low levels of anti-nutritional factors (saponin) can induce inflammatory phenotypes and can modify the microbiota, which might lead to altered disease susceptibility.

Introduction

With a growing world population, reaching an estimated 9 billion people in the year 2050, the need for food to feed the world is a pressing matter. Aquaculture is one of the fastest growing production sectors globally, and fish consumption increased from 9.9 kg/capita in the 1960s on average to a staggering 19.7 kg in 2013 and it is estimated to grow further. Aquaculture is now surpassing captured fisheries and amounts to 90 million tons of farmed fish worldwide (FAO report, SOFIA, 2016). For sustainable fish production to meet global demand now and in the future, performance and sustainability of fish feeds should be improved both from an economic and ecological stand point.

The development of high-quality sustainable aquaculture feed is a challenge due to the varying availability of raw materials that ensure sufficient protein levels and the presence of unhealthy anti-nutritional factors. For example, in the past, costly fish meal was replaced by cheaper plant-based feeds such as soybean meal (Sales, 2009). However, soybean meal may contain large amounts of anti-nutritional compounds, such as soy saponin. Soy saponin has been consistently shown to induce enteritis and alter the microbiome composition in farmed fish (Chikwati *et al.*, 2012; Costas *et al.*, 2014; Krogdahl *et al.*, 2015). More recently, a multitude of feed sources have become available such as peas, faba bean and rapeseed meals, as well as highly refined concentrates. As the feed industry adapts to account for new ingredient sources, appropriate *in vivo* models are required to test feeds for their effects on fish health prior to large scale production.

Next to the importance of feed for fish health, the fish environment (water, sediment, and plants) contains many microbes that can both be beneficial as well as detrimental to their health (reviewed in Bentzon-Tilia *et al.*, 2016; and in de Bruijn *et al.*, 2018). Beneficial bacteria can help digest feed as well as reduce the level of toxic metabolites (such as ammonia or nitrate) ensuring good water quality (reviewed in de Bruijn *et al.*, 2018). However, the large amounts of antibiotics that have been used as growth promoters in animal husbandry and human medicine are posing a threat to our health and those of our aquaculture fish (Ding and He, 2010; Klein *et al.*, 2018). Large scale antibiotic resistance and the rise of opportunistic infections calls for multidisciplinary research efforts to increase the resilience of all species including aquaculture fish (reviewed in Watts *et al.*, 2017).

In a recent review of the European scenario it was shown that the levels of antibiotic in the water can reach as high as several micrograms per liter (Carvalho and Santos, 2016). These antibiotics, besides inducing antimicrobial resistance (Gullberg *et al.*, 2011; Pindling *et al.*, 2012), also have an impact on the fish microbial communities of gut, skin and gills, which in turn influences fish disease susceptibility (Navarrete *et al.*, 2008; Brugman *et al.*, 2009; Pindling *et al.*, 2012; Tacchi *et al.*, 2015; Zhou *et al.*, 2018a; Zhou *et al.*, 2018b). For example, previously, we showed that adult zebrafish exposed to a high dose (mg/L range)

of the antibiotic vancomycin showed an overgrowth of *Cetobacterium somerae* (a fish commensal) and displayed reduced severity of chemically induced enterocolitis, whereas fish exposed to a high dose (mg/L range) of colistin sulphate showed overgrowth of *Aeromonas sp.* and were not protected from enterocolitis (Brugman *et al.*, 2009). Furthermore, Zhou and coworkers exposed adult zebrafish to low dose (ng/L range) antibiotics sulfamethoxazole or oxytetracycline for a 6-week period and reported an increased metabolic rate and higher *Aeromonas hydrophila*-induced mortality. Gut function of these zebrafish was impaired as evidenced by a decrease in intestinal goblet cell numbers, alkaline phosphatase and acid phosphatase activity. Furthermore an increased expression of pro-inflammatory cytokines *tnfa* and *il1* was observed (Zhou *et al.*, 2018b).

Given the potential for antibiotic treatments to alter host responses to antigens such as anti-nutritional factors in the feed, there is a need to understand whether reported low (ng/L – µg/L) environmental concentrations of antibiotics might change the microbial composition, which in turn might influence disease susceptibility. In this study, we set-up an immersion-based saponin immune stimulation model using zebrafish larvae. Subsequently, we addressed whether environmentally encountered levels (µg/L range) of the antibiotics ciprofloxacin or oxytetracycline influence the saponin-induced immune stimulation and the fish microbiome.

Materials and methods

Ethics statement

The present study was approved by the Dutch Committee on Animal Welfare (2017.W-0034) and the Animal Welfare Body (IvD) of the Wageningen University (Netherlands). Furthermore, we adhere to our standard biosecurity and institutional safety procedures at Wageningen University and Research.

Animals

Adult Tg(mpeg1:mCherry/mpx:eGFPi^{114}) (Renshaw *et al.*, 2006; Bernut *et al.*, 2014) zebrafish (kindly provided by Prof. Meijer, Leiden University), expressing mCherry under the macrophage-specific mpeg1 promotor and GFP under the neutrophil-specific mpx promotor were housed in Zebtec family tanks (Tecniplast, Buguggiate, Italy) under continuous flow-through at 28°C (14/10-hour light/dark cycle) at Carus facilities (WUR, Wageningen, Netherlands). Zebrafish were fed with a mixture of Artemia 230.000 npg (Ocean Nutrition Europe, Essen, Belgium) and Tetramin Flakes (Tetra, Melle, Germany) twice per day. Embryos were obtained by natural spawning and raised with E3 water (0.10 mM NaCl in demineralized water, pH 7.6) in petri dishes at 28°C (12/12-hour light/dark cycle)

(Westerfield, 2007). Dead or fungus-infected embryos were identified by microscopy and discarded in tricaine/E3 solution [8.4% (v/v) 24 mM Tricaine (Sigma-Aldrich, DL, United States) stock solution in E3]. Larval ages are expressed in days post-fertilization (dpf). From 5 dpf onward larvae were fed with live daily cultured *Tetrahymena pyriformis*.

Dose-response experiment saponin exposure

Double Tg(mpeg1:mCherry/mpx:eGFPi[114]) zebrafish larvae were randomly distributed in 6 well plates (n = 20 fish/well) and exposed to different concentrations [0, 0.5, 0.7 and 1.0 mg/ml] of saponin [ultrapure Soy Saponin 95%, kindly provided by Trond Kortner NMBU Oslo Norway, origin: Organic Technologies, Coshocton, OH (4)] dissolved in the E3 (10 ml solution/well) from 6–9 dpf. Mortality was registered and all media were refreshed daily. At 24 h (7 dpf) and 72 h (9 dpf) after the start of the immersion, zebrafish (n = 6–11/group) were anaesthetized embedded and imaged using fluorescent microscopy (as described below). Per time point several larvae were euthanized for further analysis with an overdose MS-222 (8.4 ml of 24 mM Tricaine (Sigma-Aldrich, DL, United States) in 100 ml E3). Pools of 5 larvae were used for RNA extraction (3 pools per group at 24 h, 7–9 pools per group at 72 h) and gene expression was measured on cDNA by Real Time PCR (as described below). Two independent experiments were performed and data were combined.

Experimental design and sampling strategy antibiotics and saponin exposure

A graphical representation of the experimental design and analysis performed in each time-point is displayed in **Figure 1**. To assess the effect of antibiotics, 4 dpf Tg(mpeg1:mCherry/mpx:eGFPi[114]) fish were randomly distributed in five 6 well-plates (n = 20 fish/well) and 3 treatment conditions were established: (1) control (E3), (2) ciprofloxacin 5 µg/L (Sigma-Aldrich, DL, United States) or (3) oxytetracycline hydrochloride 5 µg/L (Sigma-Aldrich, DL, United States) (10 ml solution/well). The dose of antibiotics was based on several reviews and experimental papers summarizing environmental concentrations of antibiotics in water environments (Ding and He, 2010; Carvalho and Santos, 2016; Watts *et al.*, 2017; Zhou *et al.*, 2018b; Patrolecco *et al.*, 2018) to be at a low dose (ng-µg/L range) and not at high dose (mg/L range). At 6 dpf, 4 pools of 5 larvae were sampled to assess changes in gene expression at baseline. Moreover, at 6 dpf DNA was isolated from 3 pools of 5 larvae to investigate microbiome composition at baseline. *In vivo* imaging was performed on n = 10 larvae/group to visualize innate immune cells. Subsequently, after sampling, at 6 dpf ultrapure soy saponin was applied to half of the remaining larvae at a concentration 0.5 mg/ml (to induce mild immune stimulation) so each treatment group was split into two, resulting in 6 treatment groups: (1) control, (2) ciprofloxacin (5 µg/L), (3) oxytetracycline hydrochloride (5 µg/L), (4) saponin (0.5 mg/ml), (5) ciprofloxacin + saponin (5 µg/L +

0.5 mg/ml), and (6) oxytetracycline hydrochloride + saponin (5 µg/L + 0.5 mg/ml). All treatment media were refreshed daily. At 9 dpf *in vivo* imaging was performed on $n = 10$ larvae/group to visualize innate immune cells. Gene expression was performed on 4 pools of 5 larvae to investigate immune gene expression and from 3 pools of 5 larvae DNA was isolated for microbiological analysis.

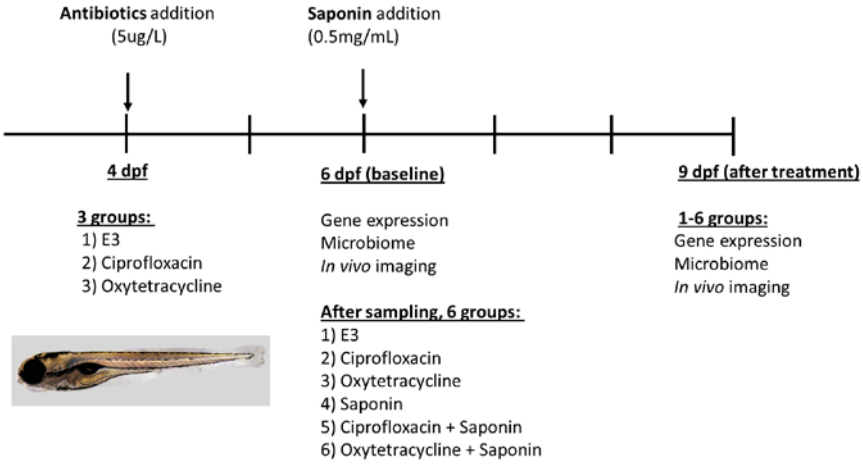


Figure 1: Experimental design and sampling strategy for the antibiotics/saponin experiments.

Fluorescent *in vivo* imaging

Tg(mpeg1:mCherry/mpx:eGFPi[114]) zebrafish larvae were anaesthetized with tricaine/E3 solution (4.2 ml of 24 mM Tricaine (Sigma-Aldrich, DL, United States) in 100 ml E3) and embedded in 1% low melting point agarose (Thermo Fisher Scientific, MA, United States). Larvae were imaged as whole mounts with a Leica M205 FA Fluorescence Stereo Microscope. After image acquisition, pictures were analyzed with ImageJ® software (United States National Institutes of Health, Bethesda, United States). The intestinal regions were manually selected per fish on the basis of the bright light picture and subsequently copied to the green and red channel pictures (**Supplementary Figure 1**). Within this intestinal region individual cells were counted for each fish. Furthermore, corrected total cell fluorescence (CTCF) was measured in ImageJ® on total fish larvae by using the following formula: Integrated density–(area of total fish x mean fluorescence of the background reading).

Relative gene expression

In order to assess changes in gene expression, total RNA was isolated from pools of larvae (*n* = 5/pool) with the RNeasy® Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was assessed with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA, United States). The quality of RNA was assessed by analysis of the 260/280 (1.9–2.0) and 260/230 (2.0–2.2) ratio on the nanodrop. cDNA was synthetized including a DNase treatment [DNase I (1 U/µl)], followed by synthesis using Superscript™ III First Strand Synthesis Systems (Invitrogen, CA, United States). Finally, Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) was performed with the use of ABsolute™ qPCR SYBR® Green Mix (Thermo Fisher Scientific, MA, United States) using the Thermal cycler Rotor-Gene 6000™ (Corbett Research, Cambridge, United Kingdom). The PCR program used was the following: 95°C 3 min. 40 × (95°C 10 s, 60°C 10 s, 72°C 30 s) followed by a melting curve 95°C 30 s, 65°C 5 s increase to 95°C in 0.5°C steps. Data were normalized to elf1a and the Pfaffl quantification method with efficiency correction (Pfaffl, 2001) was applied as described in (Forlenza *et al.*, 2012). The primers used are listed in **Table 1**.

Table 1 Forward (FW) and reverse (RV) sequences of the primers employed for the Real Time qPCR.

Gene	FW primer	RV primer
elfa1	5'-CTGGAGGCCAGCTCAAACAT-3'	5'-ATCAAGAAGAGTAGTAGTACCG-3'
il1b	5'-TGCGGGCAATATGAAGTCA-3'	5'-TTCGCCATGAGCATGTCC-3'
cxcl8a	5'-TGTTTTCCTGGCATTTCTGACC-3'	5'-TTTACAGTGTGGGCTTGGAGGG-3'
ccl38.5	5'-GTCTGGTGCTCTTCGCTTTC-3'	5'-TGCAGAGAAGATGCGTCGTA-3'
il22	5'-GGAGGGTCTGCACAGAG-3'	5'-GTCTCCCCGATTGCTT-3'
tnfb	5'-AAACAACAAATCACCACACC-3'	5'-ACACAAAGTAAAGACCATCC-3'
mmp9	5'-ACGGCATTGCTGACAT-3'	5'-TAGCGGGTTTGAATGG-3'
il10	5'-AGGGCTTTCCTTTAAGACTG-3'	5'-ATATCCCGCTTGAGTTCC-3'

16S rRNA gene profiling

In order to study the microbiome composition total DNA was isolated from three pools of 5 larvae per treatment condition and time-point (6 and 9dpf). Samples were kept in 2 ml Eppendorf® tubes with 100 µl lysis buffer (100 nM NaCl, 10 nM Tris pH~8, 15 nM EDTA, 0.5% w/v SDS) and 7 µl of Proteinase K (19 mg/ml) (QIAGEN, Hilden, Germany). Samples were incubated at 56°C until dissolved. Subsequently, 35 µl of a saturated 6 M NaCl solution was added, leaving the samples on a shaker for 15 min. After centrifugation at 21000 × *g* for 15 min the DNA-containing supernatants were transferred to a new tubes and 270 µl

of ice-cold 100% ethanol was added. Samples were incubated at –20°C for 10 min and after 5 min. centrifugation at 21000 × *g* 15°C, the pellet was washed and dissolved in 50 µl of RNase-free water. A DNA clean-up step was performed using DNA Clean & Concentrator™ kit (Zymo Research, CA, United States) following manufacturer's instructions. DNA concentration was measured using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA, United States). Samples below 100 ng per sample, 260/280 ratio ≤ 1.80 and 260/280 ratio ≤ 1.50 were excluded from further analysis.

Illumina 16S rRNA gene amplicon libraries were generated and sequenced at BaseClear (Leiden, Netherlands). In short, barcoded amplicons from the V3–V4 region of 16S rRNA genes were generated using a 2-step PCR. 10–25 ng genomic (g)DNA was used as template for the first PCR with a total volume of 50 µl using the 341F (5'-CCTACGGGNGGCWGCAG-3') and the 785R (5'-GACTACHVGGGTATCTAATCC-3') primers appended with Illumina adaptor sequences. Control PCR reactions were performed alongside each separate amplification without addition of template. PCR products were purified and the size of the PCR products were checked on Fragment analyzer (Advanced Analytical) and quantified by fluorometric analysis. Purified PCR products were used for the 2nd PCR in combination with sample-specific barcoded primers (Nextera XT index kit, Illumina). Subsequently, PCR products were purified, checked on a Fragment analyzer (Advanced Analytical) and quantified, followed by multiplexing, clustering, and sequencing on an Illumina MiSeq with the paired-end (2×) 300-bp protocol and indexing. The sequencing run was analyzed with the Illumina CASAVA pipeline (v1.8.3) with demultiplexing based on sample-specific barcodes. The raw sequencing data produced was processed removing the sequence reads of too low quality (only "passing filter" reads were selected) and discarding reads containing adaptor sequences or PhiX control with an in-house filtering protocol. A total number of ~588.000 reads were distributed in ~22.000 reads per sample on average. In addition, reads containing (partial) adapters were clipped (up to a minimum read length of 50-bp.). A quality assessment on the remaining reads was performed using the FASTQC quality control tool version 0.10.0. The Illumina paired reads were merged into single reads (so-called pseudo-reads) through sequence overlap with SNAP version 1.0.23 (Naccache *et al*., 2014), after removal of the forward and reverse primers (Edgar, 2010). Chimeric pseudo-reads were removed and the remaining reads were aligned to the RDP 16S gene databases (Cole *et al*., 2014). Based on the alignment scores of the pseudo-reads, the taxonomic depth of the lineage is based on the identity threshold of the rank; Species 99%, Genus 97%, Family 95%, Order 90%, Class 85%, and Phylum 80%. A total number of ~105.000 high-quality, paired-end, unique reads were clustered into 578 OTUs. These OTUs were further filtered excluding the ones contributing ≤ 0.01% of the dataset resulting in 239 OTUs. An overview of the control quality measurements for the samples is displayed in **Table 2**.

Table 2 Control quality measurements for all the samples: pseudo-reads, classified reads, coverage percentage and number of observed OTUs per sample.

Samples	Pseudo-reads	Classified reads	Coverage percentage	Observed OTUs n°/sample
1) Control 6dpf	22302	21427	97.1	99
2) Control 6dpf	23513	22716	98.7	76
4) Control 6dpf	23296	22335	98.2	83
6) Ciprofloxacin 6dpf	20581	19661	98.2	96
7) Ciprofloxacin 6dpf	27550	26536	98.2	76
8) Ciprofloxacin 6dpf	27275	26246	98.0	122
9) Oxytetracyclin 6dpf	17427	16873	97.7	52
10) Oxytetracyclin 6dpf	21250	20580	97.7	65
11) Oxytetracyclin 6dpf	19794	19120	98.1	127
13) Control 9dpf	21635	20902	97.4	62
15) Control 9dpf	26136	25222	97.9	64
16) Control 9dpf	16466	15979	97.7	68
17) Saponin 9dpf	24279	23397	96.3	149
18) Saponin 9dpf	21079	20401	97.8	133
19) Saponin 9dpf	18037	17327	97.9	123
21) Ciprofloxacin 9dpf	21892	21183	97.9	84
23) Ciprofloxacin 9dpf	19922	19282	97.6	68
24) Ciprofloxacin 9dpf	21037	20319	97.6	96
26) Ciprofloxacin + Saponin 9dpf	19005	18269	97.7	125
27) Ciprofloxacin + Saponin 9dpf	20790	20044	98.0	103
28) Ciprofloxacin + Saponin 9dpf	20382	19681	98.3	115
29) Oxytetracyclin 9dpf	19808	19185	97.4	114
31) Oxytetracyclin 9dpf	17192	16646	97.6	104
32) Oxytetracyclin 9dpf	19497	18839	97.9	93
33) Oxytetracyclin + Saponin 9dpf	24147	23125	96.9	161
34) Oxytetracyclin + Saponin 9dpf	21400	20566	98.2	174
36) Oxytetracyclin + Saponin 9dpf	19352	18108	98.1	154

Statistics

The data collected from the fluorescent imaging of the saponin dose response was analyzed using Prism version 5.03 (GraphPad©); linear regression and one-way ANOVA with *post hoc* test after confirmation of normal distribution of the data (Kolmogorov-Smirnov test). The α-diversity graphs, as well as the relative gene expression and the

innate immune cells counts were generated in Prism version 5.03 (GraphPad©). The former plots were firstly tested with D'Agostino and Pearson omnibus normality algorithms and further analyzed with either one/two-way(s) Analysis of Variance (ANOVA) and Tukey's Post-test or Kruskall-Wallis test and Dunn's Multiple Comparison Post-test for normal and non-normal distributed data, respectively.

The dataset containing 239 abundance-standardized OTUs was employed to further establish relationships among bacterial communities. Data was rarefied using MicrobiomeAnalyst© (Dhariwal *et al*., 2017) and α-diversity indexes including Observed OTUs, Shannon index, Simpson index, Chao1 and Fisher index were calculated accordingly. In order to assess β-diversity, Principal Coordinate Analysis (PCoA) plots were derived from unweighted UniFrac and Bray-Curtis dissimilarity distances by permutational multivariate analysis of variance (PERMANOVA) using MicrobiomeAnalyst©. Furthermore, Redundancy Analysis (RDA) plots were assessed by using Canoco© 5.0 (Canoco version 5.0, Braak, C.J.F. ter; Smilauer, P Microcomputer Power) in order to correlate microbial communities with the treatments. These analyses were based on Bray-Curtis dissimilarity distances and assessed using permutational multivariate analysis of variance (PERMANOVA).

Results

Dose-dependent increase of neutrophil recruitment to the gut area after three days of saponin exposure

Exposing zebrafish larvae to highly purified saponin (95% pure) from 6 to 9 dpf decreased the percentage of survival in a dose-dependent fashion (**Figure 2A**). Zebrafish mortality increased significantly at 1 mg/ml soy saponin compared to controls. Increased neutrophil recruitment to the intestinal area (region indicated in **Supplementary Figure 1**) increased in a dose-dependent manner at 72 but not at 24 h after exposure (**Figures 2B,C**). Linear regression on the intestinal neutrophil count revealed a significant dose-response to saponin immersion (**Supplementary Figure 2**). However, the corrected total cell fluorescence (CTCF, total corrected fluorescent signal in the green channel) in the entire fish is not increased (**Figure 2C**), suggesting that saponin induced intestinal-region specific effects. Interestingly, since some larvae (as the one depicted in **Figure 2B**) showed stronger fluorescence signal in the kidney area, corrected total cell fluorescence was also assessed in the kidney region. However, both at 24 and 72 h we did not observe a significant increase in kidney fluorescence with increasing saponin dose (data not shown). In contrast to the neutrophils, the macrophages were not affected by saponin at 0.5, 0.7 or 1.0 mg/ml doses at both 24 and 72 h (**Figures 2B,C**).

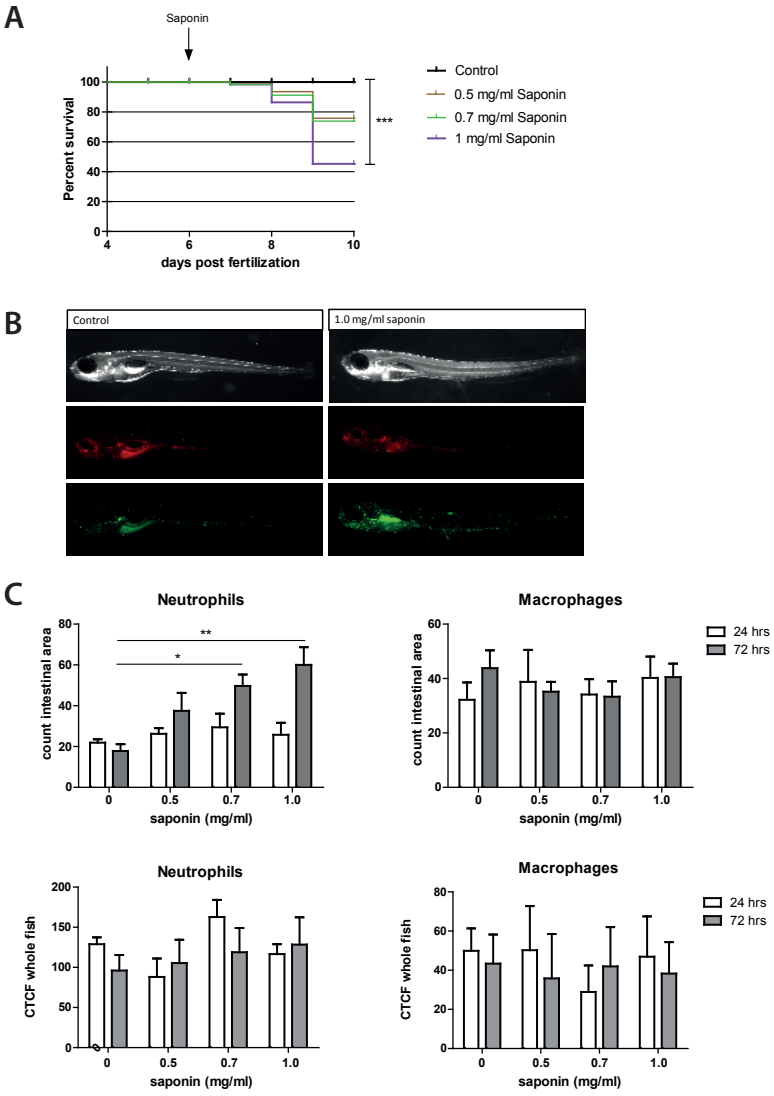


Figure 2: Effect of saponin immersion on zebrafish larvae. **(A)** Percent survival of zebrafish exposed to control (E3), 0.5 mg/ml saponin, 0.7 mg/ml saponin and 1 mg/ml saponin from 6-9 dpf (n = 40 fish/treatment) (Log-rank Mantel-Cox Test for Chi-square, $^{***}p < 0.0005$). **(B)** Representative pictures of the saponin-treated Tg(mpeg1:mCherry/mpx:eGFPi[114]) fish displaying green neutrophils and red macrophages. **(C)** Quantification of neutrophils and macrophages in the intestinal area (n = 6–11 fish/group) (one way ANOVA Kruskal-Wallis test with Dunn's Multiple comparison Post-Test, mean ± SEM, $^{*}p < 0.05$ $^{**}p < 0.01$). Top: counted cells in intestinal area. Bottom: Corrected Total Cell Fluorescence (CTCF, measure for total fluorescent pixels in the whole fish). Two independent experiments were performed and data are combined.

Saponin dose-dependently induced pro- and anti-inflammatory cytokine expression

As can be observed from **Figure 3**, expression of pro-inflammatory cytokine *il1b* increased significantly after immersion in 1.0 mg/ml saponin for 72 h compared to controls (**Figure 3A**). The expression of *tnfb* increased significantly after 72 h when larvae were exposed to a dose of 0.7 or 1.0 mg/ml of saponin (**Figure 3E**). Increased expression of *mmp9* (involved in breakdown of extracellular matrix, indicative of tissue damage) was seen after exposure to 0.7 mg/ml or 1.0 mg/ml saponin immersion after 72 h (**Figure 3F**). *Il22*, a regulatory cytokine of the il10 family, showed increased expression at 72 h after immersion in 1.0 mg/ml saponin (**Figure 3D**). While *il10* expression data showed a significant value for the Kruskall-Wallis test ($p = 0.02$), *post hoc* testing using Dunn's multiple comparison did not show differences between groups (**Figure 3G**). The expression of both *cxcl8a* and *ccl38.5* did not change upon saponin exposure (**Figures 3B** and **5C**).

Zebrafish exposed to oxytetracycline from 4 to 6 dpf showed slightly lower *il1b* expression, however, neutrophil recruitment was not affected

In order to address whether early exposure to antibiotics ciprofloxacin or oxytetracycline already affects zebrafish larvae at baseline, we exposed the larvae to either 5 ug/L ciprofloxacin or 5 ug/L oxytetracycline from 4 to 6 dpf. Neutrophil and macrophage recruitment to the intestinal area as measured by the number of these innate cells was not altered (**Supplementary Figure 3**). Furthermore, gene expression analysis revealed that oxytetracycline but not ciprofloxacin induced a small but significant decrease in *il1b* expression (**Supplementary Figure 3**).

Ciprofloxacin or oxytetracycline did not reduce saponin-induced neutrophil recruitment to the intestinal area upon co-treatment

To understand whether antibiotics protected from or enhanced the saponin-induced immune stimulation, we exposed the fish to either oxytetracycline or ciprofloxacin (4–9 dpf) in the presence or absence of a low dose (0.5 mg/ml) of saponin immersion (6–9 dpf). We specifically chose this dose of 0.5 mg/ml to induce mild (sub-phenotypical) immune activation, so to mimic low amounts of anti-nutritional factors. We recorded mortality to assess cytotoxicity derived from saponin and ciprofloxacin/oxytetracycline exposure (**Figure 4A**). All saponin treated groups showed a lower, but not significant, survival compared to controls or antibiotics alone.

As can be observed from **Figures 4B** and **4C**, the combination of ciprofloxacin and low-dose saponin significantly increased neutrophil recruitment to the intestinal area. Exposure to only saponin or the combination of oxytetracyclin and saponin only showed

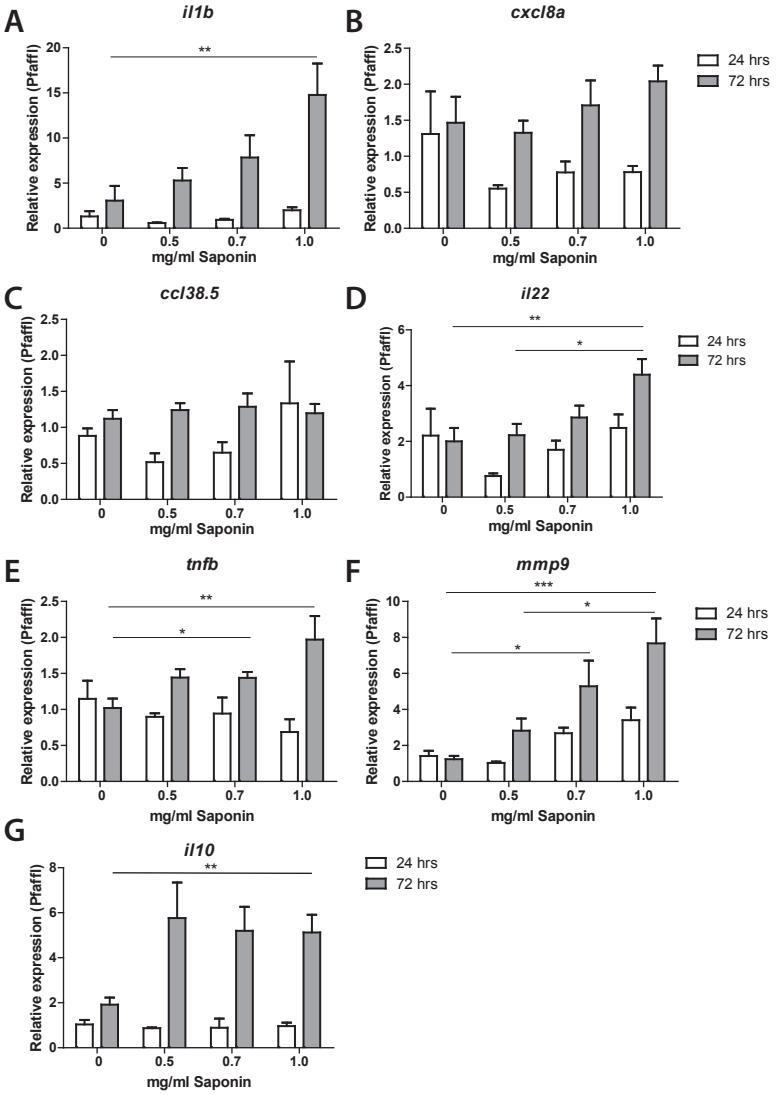


Figure 3: Relative gene expression of saponin-treated zebrafish. Zebrafish were immersed in different doses of saponin (0, 0.5, 0.7 and 1 mg/ml, from 6 to 9 dpf). At 24 h (7 dpf) and 72 h (9 dpf) zebrafish larvae were euthanized and 5 whole zebrafish were pooled for each sample. After RNA extraction and cDNA synthesis, qPCR was performed for the following cytokines: **(A)** il-1β, **(B)** cxcl8a, **(C)** ccl38.5, **(D)** il22, **(E)** tnfb, **(F)** mmp9 and **(G)** il10. Three pools of five larvae were used per group at 24 h, and 7–9 pools of five larvae per group at 72 h. Data were tested for normality with Kolmogorov-Smirnov. Non-parametric analysis was performed by one way ANOVA Kruskal-Wallis test with Dunn's Multiple comparison post-test for il1b and mmp9. Parametric analysis was performed by one way ANOVA with Tukey post hoc for il22, and tnfb. Results are displayed as mean ± SEM. Two independent experiments were performed and the data were combined.

a trend toward increased neutrophil presence in the intestinal area ($p < 0.10$). Antibiotic treatment alone or in combination with saponin did not show significant changes in gene expression. However, *il1b*, *cxcl8*, and *il22* genes expression all tended to be lower in antibiotic co-treatment, with the exception of *mmp9* that tended to be higher in groups receiving saponin (**Figures 5A, 5B, 5D** and **5F**). The expression of *ccl38.5*, *tnfb*, and *il10* was not different between treatment groups (**Figures 5C, 5E** and **5G**).

Combination of oxytetracycline and saponin significantly increased microbiota diversity

Assessment of the microbiota composition at phylum level showed that Proteobacteria was the most abundant phylum observed (**Figure 6A**). In all groups receiving saponin, the relative abundance of Bacteroidetes and Actinobacteria seemed increased, however, these changes were not significantly different from the 9 dpf control. Interestingly, this trend in Bacteroidetes and Actinobacteria was also observed in the fish that received oxytetracycline only. The increase in diversity richness was further confirmed by the observed OTUs (**Figure 6B**) and the α-diversity indexes (**Figures 6C–E**). The combination of saponin and oxytetracycline displayed a significant increase in α-diversity (Shannon, Chao and Fisher) compared to control at 9 dpf (**Figures 6C–E**). In order to assess β-diversity we performed Principal Coordinate Analysis (PCoA) (**Figures 6F,G**). At 6 dpf, clustering of each treatment (R2: 0.23, $p<0.53$) did not reveal a significant relationship whereas at 9 dpf (R2: 0.86, $p<0.001$) both saponin and antibiotic treatment were the main determinant for the microbial communities variation.

Redundancy Analysis revealed that saponin promoted a microbial shift which was further enhanced by oxytetracycline

To get more insight into the microbiome shift upon saponin addition a Redundancy Analysis (RDA) was performed at 6 dpf (basal level, **Figure 7A**) and at 9 dpf (end of the treatment, **Figure 7B**). At basal level (6 dpf) the samples clustered by treatment depending on the top 25 most discriminating OTUs. However, those differences were not significant and the X and Y axis can just explain 24.05% of the variation observed. On the other hand, at 9 dpf, after saponin addition, the shift was substantial. The treatments clustered separately among saponin-treated groups and non-saponin-treated groups. Differences were significant ($p = 0.002$) and the X and Y axis accounted for 47.21% of the variation observed. The top 25 most discriminating (not *per se* most abundant) OTUs are displayed in **Figure 7C** (6 dpf) and **Figure 7D** (9 dpf) and correlated with the treatment groups. Importantly, the angle between the genus and the imaginary line from the treatment to the ($x = 0, y = 0$) coordinate displays the correlation among genus and treatment. Therefore, the genus Escherichia and Shigella were correlated with control group while *Curvibacter*,

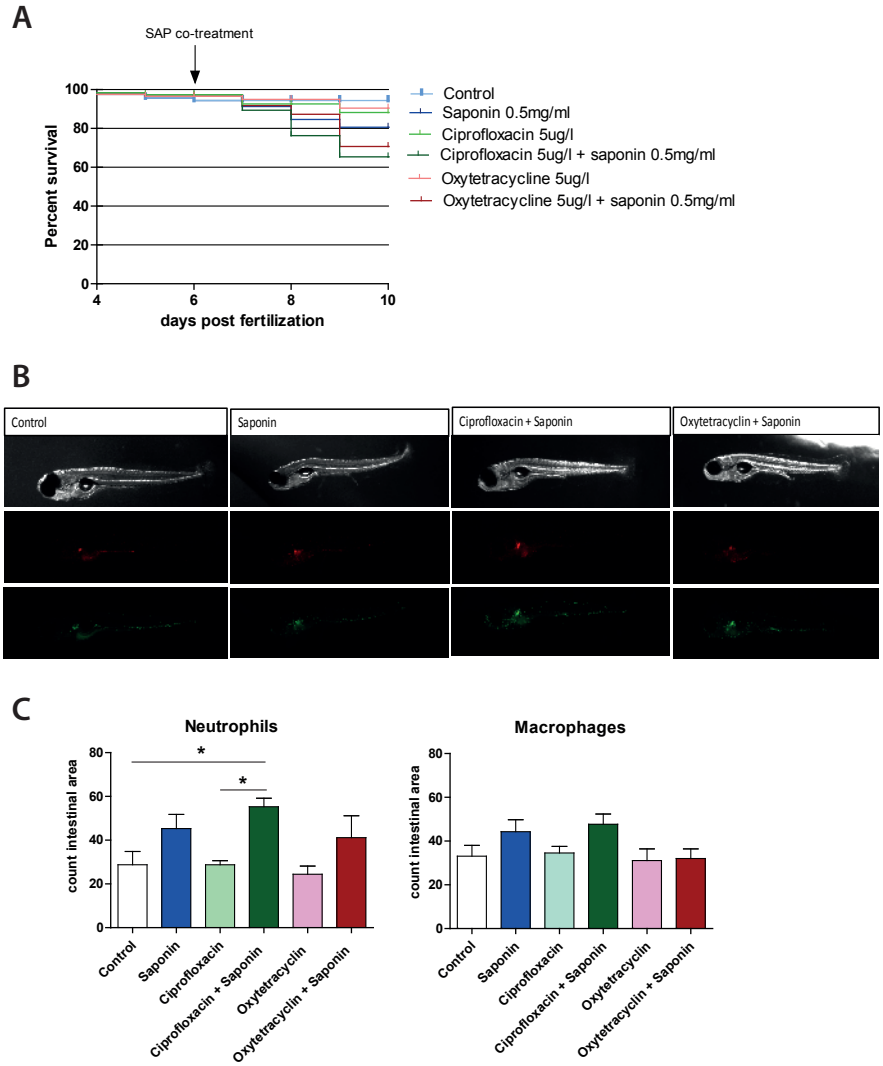


Figure 4: Effect of antibiotic exposure on saponin-immune-stimulation. **(A)** Percent survival of zebrafish exposed to control (E3), ciprofloxacin (4–9 dpf) (5 ug/L) or oxytetracycline (4–9 dpf) (5 ug/ml) +/- saponin (0.5 mg/ml) from 6–9 dpf (n = 100 fish/treatment) (Log-rank Mantel-Cox Test for Chi-square). **(B)** Representative pictures of the antibiotic/saponin-treated Tg(mpeg1:mCherry/mpx:eGFPi[114]) fish displaying green neutrophils and red macrophages. **(C)** Quantification of neutrophils and macrophages in the intestinal area (n = 10 fish/group) (one way ANOVA Kruskal-Wallis test with Dunn's Multiple comparison Post-Test, mean ± SEM, *p < 0.05). Two independent experiments were performed and one representative experiment is shown.

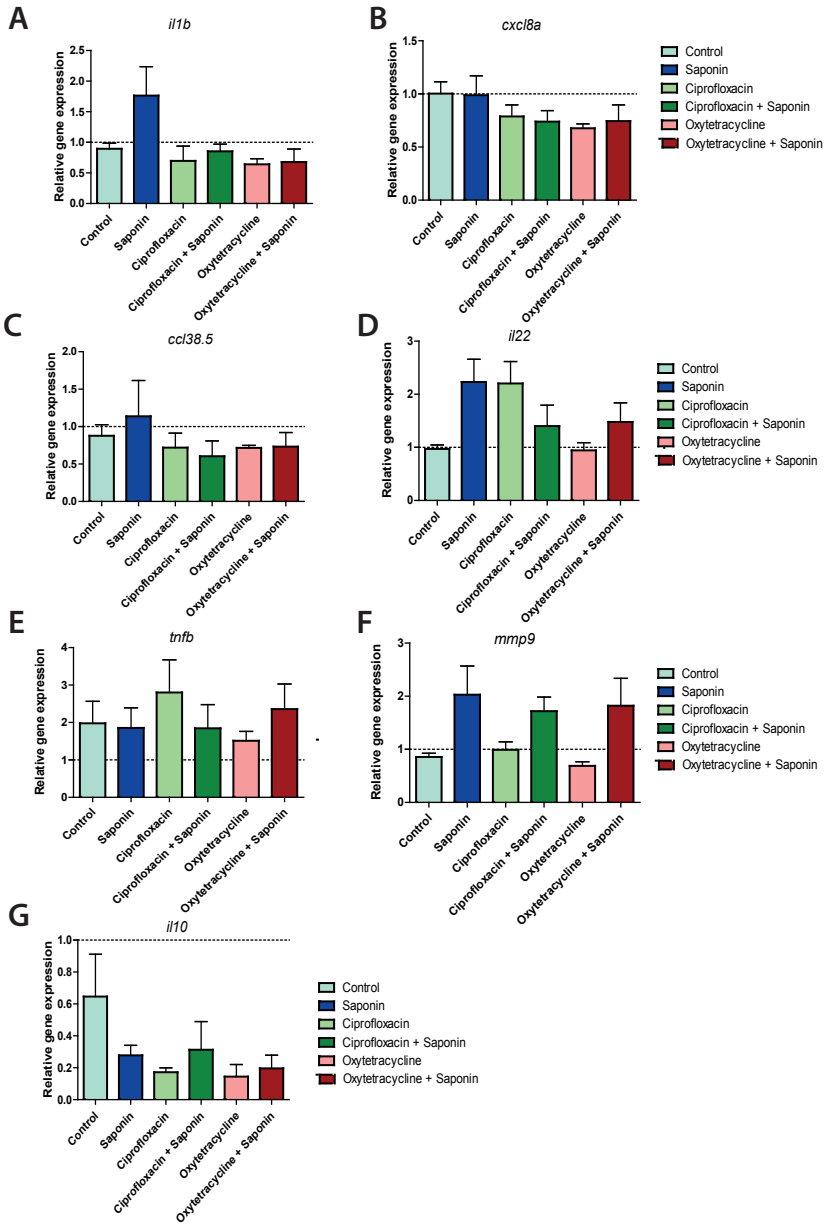


Figure 5: Relative gene expression of antibiotic/saponin-treated zebrafish. At 72 h after exposure (9 dpf) zebrafish larvae were euthanized and 5 whole zebrafish were pooled for each sample. After RNA extraction and cDNA synthesis, qPCR was performed for the following cytokines: **(A)** il1b, **(B)** cxcl8a, **(C)** ccl38.5, **(D)** il22, **(E)** tnfb, **(F)** mmp9 and **(G)** il10. n = 4 pools of 5 larvae/treatment, one way ANOVA Kruskal-Wallis test with Dunn's Multiple comparison Post-Test, mean ± SEM.

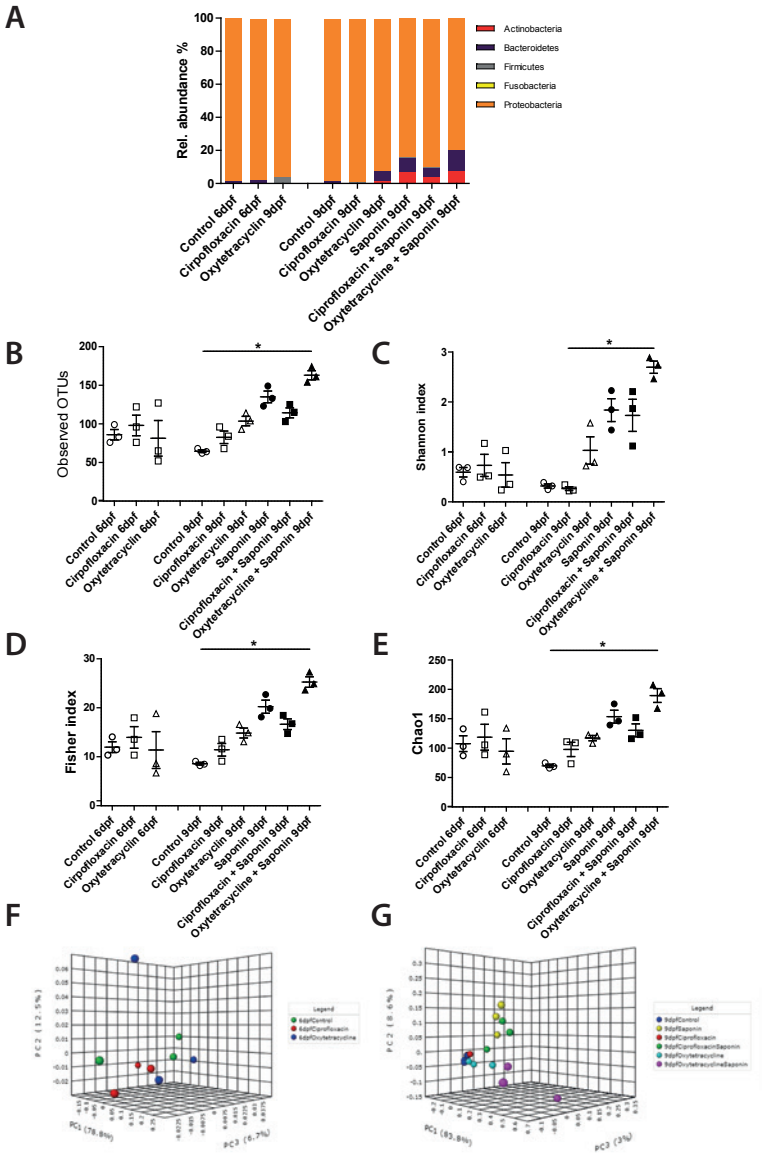


Figure 6: Saponin altered the microbiome and co-exposure with oxytetracycline increased microbiota diversity. **(A)** Relative abundance in microbiota at phylum level for the treatment groups. Several richness and α-diversity indexes were analysed: **(B)** Observed OTUs (richness), **(C)** Shannon's diversity index, **(D)** Fisher index, **(E)** Chao1 index (n = 3 pools of 5 larvae/treatment) (one way ANOVA Kruskal-Wallis test with Dunn's Multiple comparison Post-Test, mean ± SEM, *$p < 0.05$,). β-diversity PCoA plots are displayed for 6dpf (baseline) **(F)** and 9 dpf (end of the treatments exposure) **(G)** (Statistics: PERMANOVA).

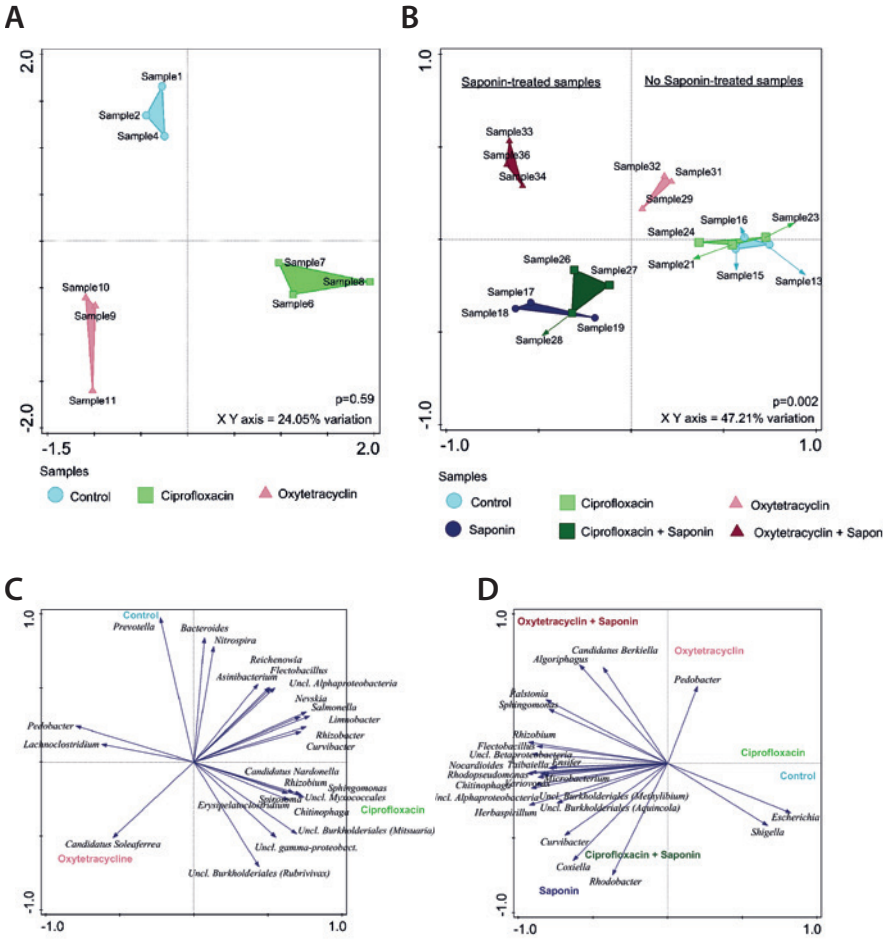
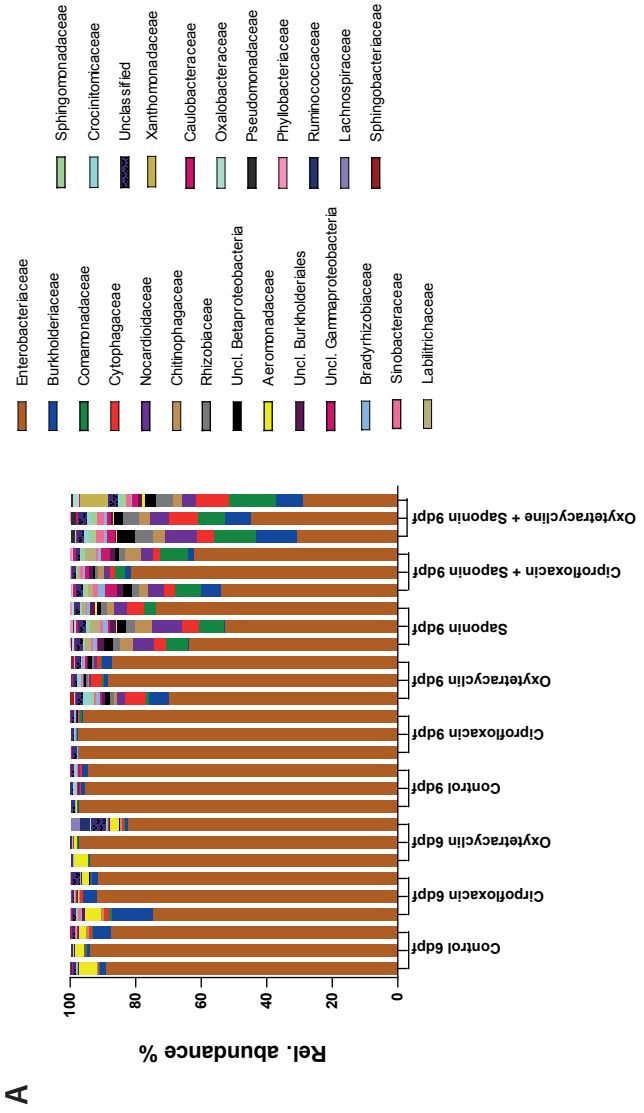


Figure 7: Saponin promoted a microbiome shift which was further enhanced by oxytetracycline in zebrafish larvae. Redundancy Analysis (RDA) was performed at 6dpf **(A)** and at 9 dpf **(B)** (n = 3 pools of 5 larvae/treatment). The distances among the samples approximates the average dissimilarity of the genera composition between the two samples being compared as measured by Euclidean distances. The top 25 most discriminating (not per se most abundant) OTUs are displayed at **(C)** 6 dpf and **(D)** 9 dpf for all the treatments.

Coxiella and *Rhodobacter* were associated with a saponin and saponin + ciprofloxacin-treated group. On the other hand, *Pedobacter* was correlated with oxytetracycline-treated fish while *Candidatus berkiella* and *Algoriphagus* were associated with oxytetracycline + saponin-treated fish. Interestingly, the oxytetracycline-treated groups differed from the ciprofloxacin and the control groups (with and without saponin), indicating that the microbiome shift was saponin but also antibiotic treatment dependent.

The diversity of several genera of bacteria increased at the expense of Escherichia after saponin and oxytetracycline exposure

To assess the weight of individual genera the overall average ≥ 0.05% abundant genus was assessed and the top 6 abundance are depicted in Figure 8 (top 7 – 10 can be found in **Supplementary Figure S4**). Strikingly, *Escherichia* was the most abundant bacterium in every sample. However, a significant reduction of Escherichia was observe in the oxytetracycline + saponin-treated fish compared to the controls (**Figure 8B**). Changes were observed in *Limnobacter* (**Figure 8C**), *Variovorax* (**Figure 8D**), *Flectobacillus* (**Figure 8E**), *Nocardioles* (**Figure 8F**) and *Lacibacter* (**Figure 8G**). All tended to be more abundant in the saponin treated groups, while *Limnobacter* and *Flectobacillus* also tended to be higher in the oxytetracyclin alone group. *Aeromonas* (**Supplementary Figure S4**) was found at 6 dpf but not at 9 dpf suggesting that this genus is related to earlier life stages in our zebrafish larvae.


A
Rel. abundance %
100
80
60
40
20
0
Control 6dpf
Ciprofloxacin 6dpf
Oxytetracylin 6dpf
Control 9dpf
Ciprofloxacin 9dpf
Oxytetracylin 9dpf
Saponin 9dpf
Ciprofloxacin + Saponin 9dpf
Oxytetracycline + Saponin 9dpf
Enterobacteriaceae
Burkholderiaceae
Comamonadaceae
Cytophagaceae
Nocardioidaceae
Chitinophagaceae
Rhizobiaceae
Uncl. Betaproteobacteria
Aeromonadaceae
Uncl. Burkholderiales
Uncl. Gammaproteobacteria
Bradyrhizobiaceae
Sinobacteraceae
Labilithrichaceae
Sphingomonadaceae
Crocinitomicaceae
Unclassified
Xanthomonadaceae
Caulobacteraceae
Oxalobacteraceae
Pseudomonadaceae
Phyllobacteriaceae
Ruminococcaceae
Lachnospiraceae
Sphingobacteriaceae

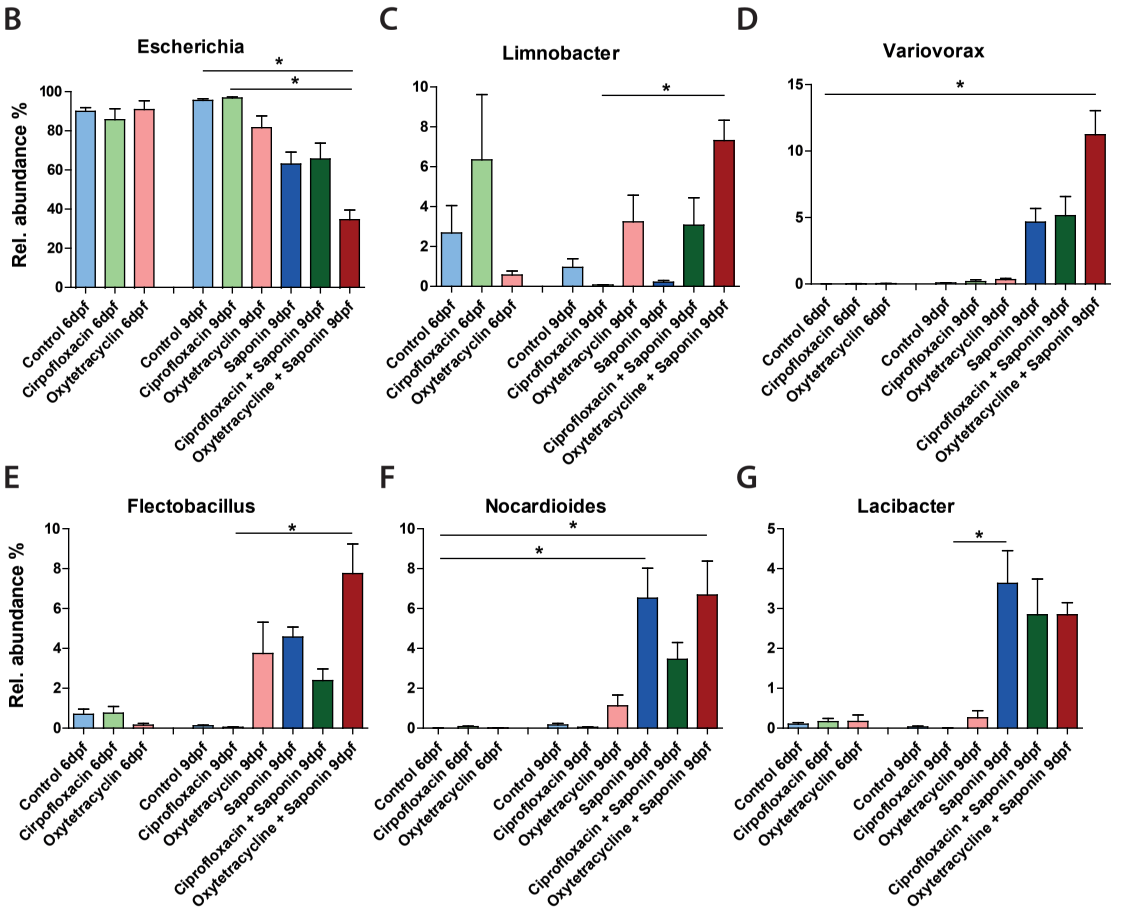


Figure 8: Diversity of several bacterial genera increased at the expense of *Escherichia* after saponin and oxytetracycline exposure. **(A)** The overall average ≥ 0.05% abundant genera are displayed (3 pools of 5 larvae/treatment). The top 6 most abundant genera are displayed: **(B)** *Escherichia*, **(C)** *Limnobacter* **(D)** *Variovorax* **(E)** *Flectobacillus* **(F)** *Nocardioides*, **(G)** *Lacibacter* (mean ± SEM, $^{*}p < 0.05$).

Discussion

In this study we investigated whether environmental levels of antibiotics in the water could influence zebrafish larval immune responses toward sub-phenotypical levels of anti-nutritional factors. We found that immersion in saponin induced immune stimulation in a dose dependent manner (as measured by neutrophil influx to the intestinal region and pro-inflammatory cytokine responses in the whole animal). Combined exposure to ciprofloxacin and saponin increased neutrophil influx to the gut area significantly compared to controls and exposure to either saponin or ciprofloxacin alone. Moreover, immersion in saponin combined with oxytetracycline significantly increased the diversity of the microbiota: the abundance of Bacteroidetes and Actinobacteria increased at the expense of Proteobacteria. Beta-diversity analysis revealed that the treatments (antibiotics and saponin) were microbial shift determining factors.

Saponins contain one carbohydrate chain linked to a fat soluble region and they are able to disrupt biological membranes due to their amphipathic nature. Several studies show that saponins are the main anti-nutritional factor in soybean meal causing intestinal inflammation in fish species (Chikwati *et al.*, 2012; Krogdahl *et al.*, 2015). As already shown by Hedrera and coworkers (Hedrera *et al.*, 2013) saponin is able to induce an immune response in zebrafish at 3.3 g/kg inclusion level in feed. Here, it is important to note that levels of saponins within soybean meal can differ greatly. In this study, as in the study by Hedrera highly pure sources of saponin (95 and 90%, respectively) have been used which ameliorate the reproducibility of the results. An important difference between our study and the study by Hedrera is that in this study the saponin was supplied to the water and not incorporated into the feed. Therefore, our data yield information on the response of the whole fish, and not necessarily on intestinal specific effects. We did observe that the number of neutrophils present in the intestinal area does increase dose-dependently upon saponin immersion. A dose-dependent increase in whole fish neutrophil fluorescence (as measured by corrected total cell fluorescence, CTCF) was not observed. Likewise, the CTCF originating from neutrophils in the (head)kidney area in larvae treated with saponin was also not increased upon higher saponin dose. The fact that increased neutrophil presence is observed in our study in the gut area might be partly explained by larvae being fed Tetrahymena throughout the study from 5 dpf. Therefore, the surrounding water containing saponin is ingested together with the feed, and saponin will reach the larval intestines. Although, the aquaculture fish industry has almost fully replaced soybean meal and saponin in the feed, this saponin-induced immune stimulation model can be used as a screening method to assess other compounds for their ability to cause comparable immune stimulation, or their ability to protect from inflammation.

In the present study, a dominance of Proteobacteria in our control larvae was observed, which is in line with much of the literature (Roeselers *et al.*, 2011; Sullam *et al.*,

2012; Stephens *et al.,* 2016). However, the dominant Proteobacteria observed in our larvae was *E. coli*, which is not often found in other studies. Exposure to saponin in the rearing water, shifter the entire microbiome and tended to increase its diversity (as indicated by the observed OTUs and α-diversity). We hypothesize that supplying an additional substrate (saponin) in a very short period, from 6–9 dpf, next to the live feed (tetrahymena) might have just favored other less abundant species from the *Bacteroidetes* and *Actinobacteria* phyla to grow out at the expense of the dominant Proteobacteria. This might be different when older zebrafish with a more diversified and stable microbiota would have been used. An increase of *Bacteroidetes* was also reported in 30 dpf zebrafish fed a high fat diet, resulting in an altered microbiome compared to controls (Arias-Jayo *et al.,* 2018). However, in the study of Arias-Jayo and colleagues fish were fed with a commercially available pellet feed during 25 days 3 times per day and therefore we cannot compare it with the settings of our study.

Interestingly, fish that were only exposed to oxytetracycline tended to show increased microbial diversity. This is not in line with current literature, in which most studies investigating antibiotics observe reduced diversity (Navarrete *et al.*, 2008; Ding and He, 2010, Pindling *et al.*, 2012; Zhou *et al.*, 2018a; Zhou *et al.*, 2018b). The fact that saponin (and soybean meal) increases bacterial diversity in fish is observed by others (Bakke-McKellep *et al.*, 2007; Merrifield *et al.*, 2009). Furthermore, in our study we have used very young larvae (4–9 dpf) in which the microbial community is still developing and that at this stage the microbiota of zebrafish larvae greatly resembles the environmental microbiota (Stephens *et al.,* 2016). Investigating effects of antibiotics and saponin exposure to older fish, with a more diversified and stable microbiota might give different results.

Oxytetracycline by itself also tended to increase diversity and had some effects on baseline gene expression; significantly reducing *il1b* expression (**Supplementary Figure 3**). Furthermore, ciprofloxacin and oxytetracycline affect microbes differently; ciprofloxacin mainly inhibits DNA synthesis and replication of aerobic Gram-negative bacteria, while oxytetracycline inhibits protein synthesis of both anaerobic Gram-positive as well as Gram-negative bacteria (Perrin-Guyomard *et al.*, 2005; Ding and He, 2010). Understanding the effect of these antibiotics not only on the presence but also transcriptional activity of microbes associated to a fish host might be very interesting to identify why oxytetracycline has other effects on the host compared to ciprofloxacin.

Our study is in line with a previous study performed in Atlantic Salmon where oxytetracycline was added at 3 g/kg to soybean meal diet. The oxytetracycline did not affect disease severity, however it did influence the microbial community (Bakke-McKellep *et al.*, 2007). Considering the fact that we used low levels of oxytetracycline and ciprofloxacin, far lower than the levels that can be found at aquaculture sites during treatment (Ding and He, 2010; Plhalova *et al.*, 2014; Carvalho and Santos, 2016), it would be very interesting to

follow especially the oxytetracycline-exposed fish to older age and assess their disease susceptibility. Zhou and coworkers (Zhou *et al.*, 2018a) fed adult zebrafish oxytetracycline-containing feed for 6 weeks and observed that these fish displayed higher mortality upon *Aeromonas* challenge as well as displayed lower activity of alkaline phosphatase and acid phosphatase needed for intestinal homeostasis (Bates *et al.*, 2007).

Conclusion

In conclusion, in this study we have shown that saponin immersion dose-dependently induces immune stimulation, as evidenced by increased pro-inflammatory cytokine expression and neutrophil recruitment to the intestinal area. Low levels of antibiotics present in surface water can influence saponin-induced changes in the microbiome (increased α-diversity in oxytetracycline + saponin) and increased neutrophil recruitment (ciprofloxacin + saponin). Therefore, this study highlights the importance of background levels of environmental pollutants such as antibiotics in the assessment of feed effects on fish health, which may be missed in controlled laboratory settings.

Authors contribution

AL performed the experiments and drafted the manuscript. DP advised on the experiments and wrote the manuscript. GW wrote the manuscript and provided the funding. SB performed the experiments, wrote the manuscript and provided the funding.

Funding

This study was funded by NWO-TTW Applied and Engineering Sciences (project number 15566).

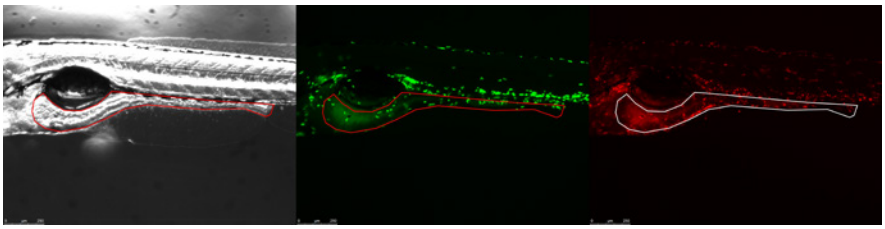
Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

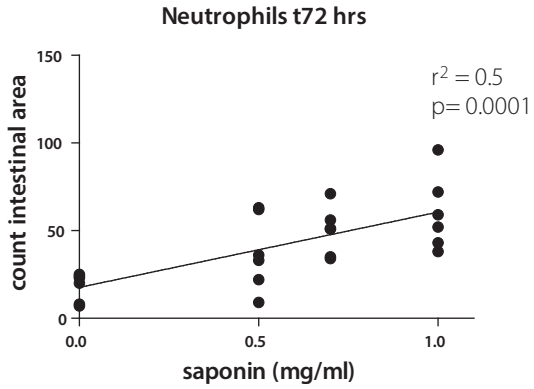
Acknowledgments

The authors would like to thank Professor Trond Kortner of NMBU, Oslo Norway, for providing us with the 95% pure saponin. Furthermore, the authors would like to thank Raka Choudhury and Marcela Fernandez Gutierrez at the Host-Microbe Interactomics group at Wageningen University and Research for their valuable advice on the microbiota analysis.

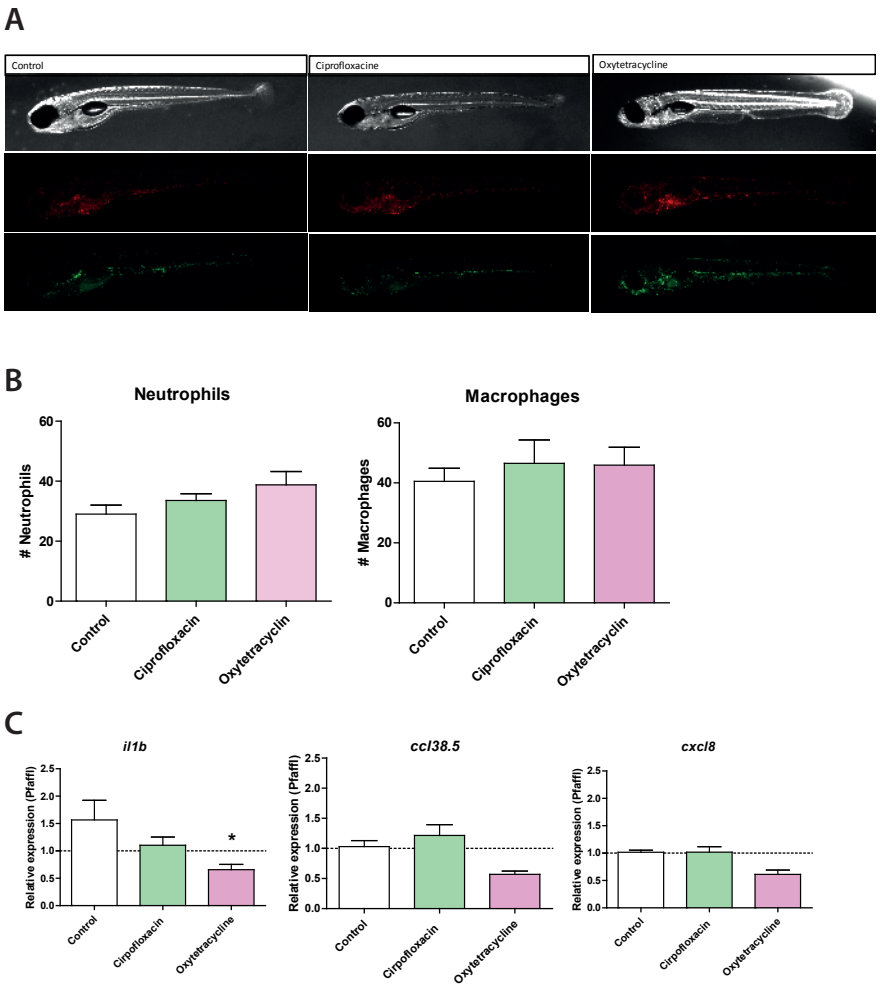
Supplementary Material



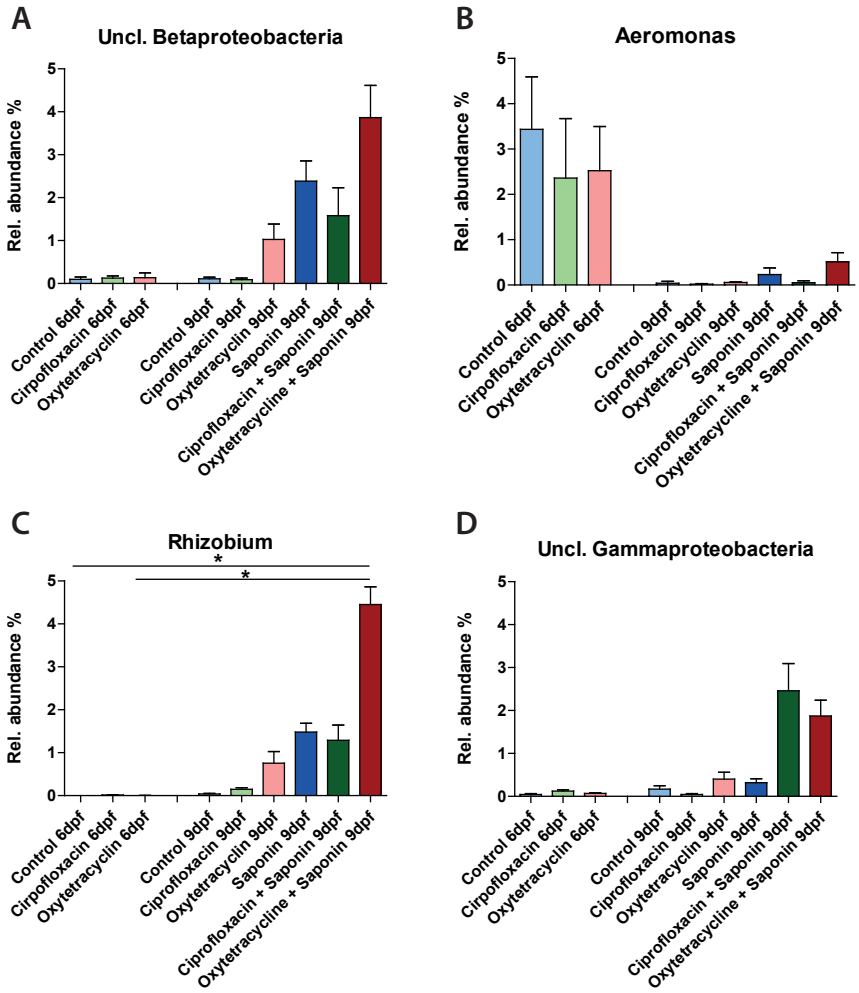
Supplementary Figure 1: Representative picture of the intestine region selected in the bright field and copied to the green and red channel pictures for neutrophil and macrophage count.



Supplementary Figure 2: Linear regression analysis of the neutrophil count in the intestinal area of the saponin dose-response experiment.



Supplementary Figure 3: **(A)** Representative pictures of neutrophil and macrophage imaging at baseline (6 dpf) for control (E3), Ciprofloxacin and Oxytetracycline treated larvae (exposure from 4–6 dpf), **(B)** Quantification of neutrophil and macrophage number in the intestinal area (n = 10 larvae/treatment). **(C)** Relative gene expression on pools of whole zebrafish larvae (n = 4 pools of 5 larvae/treatment) for the following cytokines: il1b, ccl38.5, cxcl8, (one way ANOVA Kruskal-Wallis test with Dunn's Multiple comparison Post-Test, mean ± SEM.



Supplementary Figure 4: The top 7-10 most abundant genera (mean ± SEM, *p < 0.05) are displayed: **(A)** Unclassified *Betaproteobacteria* **(B)** *Aeromonas* **(C)** *Rhizobium* **(D)** Unclassified *Gammaproteobacteria*.

References

Arias-Jayo, N., Abecia, L., Alonso-Sáez, L., Ramirez-Garcia, A., Rodriguez, A., and Pardo, M. A. (2018). High-fat diet consumption induces microbiota dysbiosis and intestinal inflammation in zebrafish. *Microb. Ecol.* 76, 1089–1101. doi: 10.1007/s00248-018-1198-9

Bakke-McKellep, A. M., Penn, M. H., and Salas, P. M. (2007). Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar* L.). *Br. J. Nutr.* 97, 699–713. doi: 10.1017/S0007114507381397

Bates, J. M., Akerlund, J., Mittge, E., and Guillemin, K. (2007). Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe* 2, 371–382. doi: 10.1016/j.chom.2007.10.010

Bentzon-Tilia, M., Sonnenschein, E. C., and Gram, L. (2016). Monitoring and managing microbes in aquaculture - towards a sustainable industry. *Microb. Biotechnol.* 9, 576–584. doi: 10.1111/1751-7915.12392

Bernut, A., Herrmann, J. L., and Kissa, K. (2014). *Mycobacterium abscessus* cording prevents phagocytosis and promotes abscess formation. *Proc. Natl. Acad. Sci. U.S.A.* 111, E943–E952. doi: 10.1073/pnas.1321390111

Brugman, S., Liu, K. Y., and Lindenbergh-Kortleve, D. (2009). Oxazolone-induced enterocolitis in zebrafish depends on the composition of the intestinal microbiota. *Gastroenterology* 137, 1757–1767. doi: 10.1053/j.gastro.2009.07.069

Carvalho, I. T., and Santos, L. (2016). Antibiotics in the aquatic environments: a review of the European scenario. *Environ. Int.* 94, 736–757. doi: 10.1016/j.envint.2016.06.025

Chikwati, E. M., Venold, F. F., Penn, M. H., Rohloff, J., Refstie, S., and Guttvik, A. (2012). Interaction of soyasaponins with plant ingredients in diets for Atlantic salmon, *Salmo salar* L. *Br. J. Nutr.* 107, 1570–1590. doi: 10.1017/S0007114511004892

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., and Sun, Y. (2014). Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* 42, D633–D642. doi: 10.1093/nar/gkt1244

Costas, B., Couto, A., Azeredo, R., Machado, M., Krogdahl, A., and Oliva-Teles, A. (2014). Gilthead seabream (*Sparus aurata*) immune responses are modulated after feeding with purified antinutrients. *Fish Shellfish Immunol.* 41, 70–79. doi: 10.1016/j.fsi.2014.05.032

de Bruijn, I., Liu, Y., Wiegertjes, G. F., and Raaijmakers, J. M. (2018). Exploring fish microbial communities to mitigate emerging diseases in aquaculture. *FEMS Microbiol. Ecol.* 94:fix161. doi: 10.1093/femsec/fix161

Dhariwal, A., Chong, J., Habib, S., King, I. L., Agellon, L. B., and Xia, J. (2017). Microbiome analyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* 45, W180–W188. doi: 10.1093/nar/gkx295

Ding, C., and He, J. (2010). Effect of antibiotics in the environment on microbial populations. *Appl. Microbiol. Biotechnol.* 87, 925–941. doi: 10.1007/s00253-010-2649-5

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. doi: 10.1093/bioinformatics/btq461

Forlenza, M., Kaiser, T., Savelkoul, H. F., and Wiegertjes, G. F. (2012). The use of real-time quantitative PCR for the analysis of cytokine mRNA levels. *Methods Mol. Biol.* 820, 7–23. doi: 10.1007/978-1-61779-439-1_2

Gullberg, E., Cao, S., Berg, O. G., Ilback, C., Sandegren, L., and Hughes, D. (2011). Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog.* 7:e1002158. doi: 10.1371/journal.ppat.1002158

Hedrera, M. I., Galdames, J. A., Jimenez-Reyes, M. F., Reyes, A. E., Avendano-Herrera, R., and Romero, J. (2013). Soybean meal induces intestinal inflammation in zebrafish larvae. *PLoS One* 8:e69983. doi: 10.1371/journal.pone.0069983

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., and Levin, S. A. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. U.S.A.* 115, E3463–E3470. doi: 10.1073/pnas.1717295115

Krogdahl, A., Gajardo, K., Kortner, T. M., Penn, M., Gu, M., and Berge, G. M. (2015). Soya saponins induce enteritis in Atlantic salmon (*Salmo salar* L.). *J. Agric. Food Chem.* 63, 3887–3902. doi: 10.1021/jf506242t

Merrifield, D. L., Dimitroglou, A., Bradley, G., Baker, R. T., and Davies, S. J. (2009). Soybean meal alters autochthonous microbial populations, microvilli morphology and compromises intestinal enterocyte integrity of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J. Fish Dis.* 32, 755–766. doi: 10.1111/j.1365-2761.2009.01052.x

Naccache, S. N., Federman, S., Veeraraghavan, N., Zaharia, M., Lee, D., and Samayoa, E. (2014). A cloud-compatible bioinformatics pipeline for ultrarapid pathogen identification from next-generation sequencing of clinical samples. *Genome Res.* 24, 1180–1192. doi: 10.1101/gr.171934.113

Navarrete, P., Mardones, P., Opazo, R., Espejo, R., and Romero, J. (2008). Oxytetracycline treatment reduces bacterial diversity of intestinal microbiota of Atlantic salmon. *J. Aquat. Anim. Health* 20, 177–183. doi: 10.1577/H07-043.1

Patrolecco, L., Rauseo, J., and Ademollo, N. P. (2018). Persistence of the antibiotic sulfamethoxazole in river water alone or in the co-presence of ciprofloxacin. *Sci. Total Environ.* 640–641, 1438–1446. doi: 10.1016/j.scitotenv.2018.06.025

Perrin-Guyomard, A., Poul, J. M., Corpet, D. E., and Sanders, P. (2005). Impact of residual and therapeutic doses of ciprofloxacin in the human-flora-associated mice model. *Regul. Toxicol. Pharmacol.* 42, 151–160. doi: 10.1016/j.yrtph.2005.03.001

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29:e45. doi: 10.1093/nar/29.9.e45

Pindling, S., Azulai, D., Zheng, B., Dahan, D., and Perron, G. G. (2018). Dysbiosis and early mortality in zebrafish larvae exposed to subclinical concentrations of streptomycin. *FEMS Microbiol. Lett.* 365:fny188. doi: 10.1093/femsle/fny188

Plhalova, L., Zivna, D., Bartoskova, M., and Blahova, J. (2014). The effects of subchronic exposure to ciprofloxacin on zebrafish (*Danio rerio*). *Neuro Endocrinol. Lett.* 35(Suppl. 2), 64–70.

Renshaw, S., Loynes, A., and Trushell, C. A. (2006). A transgenic zebrafish model of neutrophilic inflammation. *Blood* 108, 3976–3978. doi: 10.1182/blood-2006-05-024075

Roeselers, G., Mittge, E. K., Stephens, W. Z., Parichy, D. M., Cavanaugh, C. M., and Guillemin, K. (2011). Evidence for a core gut microbiota in the zebrafish. *ISME J.* 5, 1595–1608. doi: 10.1038/ismej.2011.38

Sales, J. (2009). The effect of fish meal replacement by soyabean products on fish growth: a meta-analysis. *Br. J. Nutr.* 102, 1709–1722. doi: 10.1017/S0007114509991279

Stephens, W. Z., Burns, A. R., Stagaman, K., Wong, S., Rawls, J. F., and Guillemin, K. (2016). The composition of the zebrafish intestinal microbial community varies across development. *ISME J.* 10, 644–654. doi: 10.1038/ismej.2015.140

Sullam, K. E., Essinger, S. D., Lozupone, C. A., O'Connor, M. P., Rosen, G. L., and Knight, R. (2012). Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Mol. Ecol.* 21, 3363–3378. doi: 10.1111/j.1365-294X.2012.05552.x

Tacchi, L., Lowrey, L., Musharrafieh, R., Crossey, K., Larragoite, E. T., and Salinas, I. (2015). Effects of transportation stress and addition of salt to transport water on the skin mucosal homeostasis of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 435, 120–127. doi: 10.1016/j.aquaculture.2014.09.027

Watts, J. E. M., Schreier, H. J., Lanska, L., and Hale, M. S. (2017). The rising tide of antimicrobial resistance in aquaculture: sources, sinks and solutions. *Mar Drugs* 15:158. doi: 10.3390/md15060158

Westerfield, M. (2007). *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 5th Edn. Eugene: University of Oregon Press.

Zhou, L., Limbu, S. M., Qiao, F., Du, Z. Y., and Zhang, M. (2018a). Influence of long-term feeding antibiotics on the gut health of zebrafish. *Zebrafish* 15, 340–348. doi: 10.1089/zeb.2017.1526

Zhou, L., Limbu, S. M., Shen, M., Zhai, W., Qiao, F., and He, A. (2018b). Environmental concentrations of antibiotics impair zebrafish gut health. *Environ. Pollut.* 235, 245–254. doi: 10.1016/j.envpol.2017.12.073

Chapter 3

Environmental microbes determine macrophage response towards saponin-induced inflammation in zebrafish larvae

Adrià López Nadal, Evelien Kidess, Jos Boekhorst,
Geert F. Wiegertjes and Sylvia Brugman

Manuscript submitted

Abstract

The microbial consortium within an organism is crucial for its development and immune status. Alteration of the host microbiome by antibiotics or antinutritional factors may contribute to increased disease susceptibility. Here, we investigated whether oxytetracycline (OxyT) treated adult zebrafish harboured microbiota composition able to alter larvae recipient microbiota composition and modulate their immune response towards a saponin challenge. Zebrafish larvae exposed to OxyT-treated adult gut content (3-6dpf) showed altered microbiota composition compared to controls. Subsequently, to investigate the susceptibility towards inflammation, the differently content-exposed larvae were challenged with the anti-nutritional factor saponin (6-8 dpf). By using transgenic zebrafish larvae we monitored the presence of neutrophils and macrophages *in vivo* in the whole fish as well as around the gut area. Interestingly, saponin-treated OxyT-content exposed larvae showed less macrophages in the overall fish as well as around the gut area than saponin-treated control-exposed larvae. Fewer macrophages associated with a decreased expression of *il22* in saponin-treated OxyT-content exposed larvae compared to controls. Overall, the microbial composition of the zebrafish larvae and their macrophage response to saponin-induced inflammation depended on the environmental microbes

Introduction

The environment is teeming with microbes that are in intimate contact with their hosts. While mammals are shielded from the environment by developing in a womb for the first months of life, fish are immediately exposed to their surroundings from the moment of fertilization of the eggs. Zebrafish hatch within two days after fertilization when reared at 28.5 °C (Westerfield, 2007). Environmental microbes already colonize the eggs and from around 3-4 days-post-fertilization (dpf) when the mouth opens colonize the gut tube (reviewed in de Bruijn *et al.*, 2018). Until 5 dpf zebrafish derive their nutrition from the yolk sac after which feeding commences (Wallace *et al.*, 2005).

Microbial colonization during early life induces developmental programs in the host. Both metabolic and immune processes are among the most conserved host pathways that are influenced by colonizing microbes (Rawls *et al.*, 2004). For example, colonization of germ-free zebrafish larvae revealed conserved responses in nutrient metabolism, epithelial cell turn-over and innate immunity. In a later study, 66 homologues genes responded to microbial colonization in both mice and zebrafish and 54 of these genes changed in the same direction (Rawls *et al.*, 2006), indicating conserved vertebrate host responses towards microbial colonization. Colonizing microbes are crucial for a proper development of the host. As a matter of fact, germ-free zebrafish did not survive more than 20 dpf (Rawls *et al.*, 2004). Additionally, microbes are needed for a proper function of the immune system, germ-free mice have underdeveloped lymphoid organs, decreased levels of anti-microbial peptides and intestinal lymphocytes as well as reduced levels of IgA, the main antibody at mucosal sites, which all reverses upon colonization (Hapfelmeier *et al.*, 2010; reviewed in Chinen & Rudensky, 2012).

Interestingly, immune responses induced by colonizing microbes in turn can shape the microbial community. For example, colonizing germ free mice with a mixed faecal suspension of conventional mice led to outgrowth of (pathobiont) species, such as *Helicobacter*, *Sphingomonas* and *Mucispirillum* from 0-4 days after conventionalization. This coincided with a robust host transcriptomics response, altered goblet cell immunohisto-chemical profiles as well as increased production of antimicrobial peptides, after which the abundance of the pathobiont species sharply declined (el Aidy *et al.*, 2014). So not only do colonizing microbes induce host responses, but these host responses also alter the microbial composition. This phase of 'transient inflammation' might even be necessary to 'educate' the host immune response and repress the pathobionts that induced the response in the first place.

Certain bacteria can induce differentiation in several types of immune cells, as has been shown in mice. Segmented filamentous bacteria for example are excellent inducers of Th17 cells (Ivanov *et al.*, 2009) and *Clostridial* species within the clusters IV, XIVa and XVIII

can influence regulatory T cell development in mice (Furusawa *et al.*, 2013). This also means that the colonizing microbes have an influence on immune set-point of the host. An altered microbial community might result in different abundances of subtypes of immune cells. Since fish are exposed to the environment from the moment of fertilization of the eggs and environmental microbes might have different effects on the zebrafish host immune system, this study aims to investigate whether exposure to different environmental microbes during early development affects the immune response (neutrophil and macrophage presence) in zebrafish larvae and/or changes their susceptibility towards saponin-induced intestinal inflammation.

Materials and Methods

Animals

Adult transgenic (mpeg1:mCherry / mpx:eGFP^{i114}) were housed and fed as previously described (López Nadal *et al.*, 2018). Embryos were obtained by natural spawning and bleached at 1 dpf using 0.004% NaCl in E2 medium (Bleaching Solution made from 10-13% NaOCl stock (Aldrich 425044); E2 medium: 0.10 mM NaCl in demineralized water, pH 7.3) followed by washing in E2 and treatment with pronase (10 ul of 30 mg/mL Pronase stock solution in 50 ml E2 medium (Pronase: Roche 165921; store stock at –20 °C)) and additional washing. After bleaching eggs were transferred to sterile culture flasks (T75, n=20 eggs/flask containing 25 ml of E2 medium, 8 flasks). Flasks were kept at 28°C (14/10-hour light/dark cycle) (Westerfield, 2007). The experimental design including the treatments employed and the sample size is summarized in **Figure 1.**

Oxytetracycline exposure

Adult transgenic (mpeg1:mCherry / mpx:eGFP^{i114}) were exposed to normal system water or Oxytetracycline (OxyT) (5 mg/L in 2L, Sigma-Aldrich, DL, United States) in system water for 24 hours. After 24 hrs fish were transferred to clean tank water and 6 hours later euthanized through MS-222 overdose (300 mg/L end concentration; stock concentration: 4 mg/ml MS-222- Finquel/MS-222, Argent Laboratories in Tris buffered solution) to harvest intestinal content. Intestinal content of three control adult zebrafish were pooled and diluted in 1 ml sterile PBS. Similarly intestinal content of three OxyT-exposed fish were pooled and diluted in 1 ml of sterile PBS.

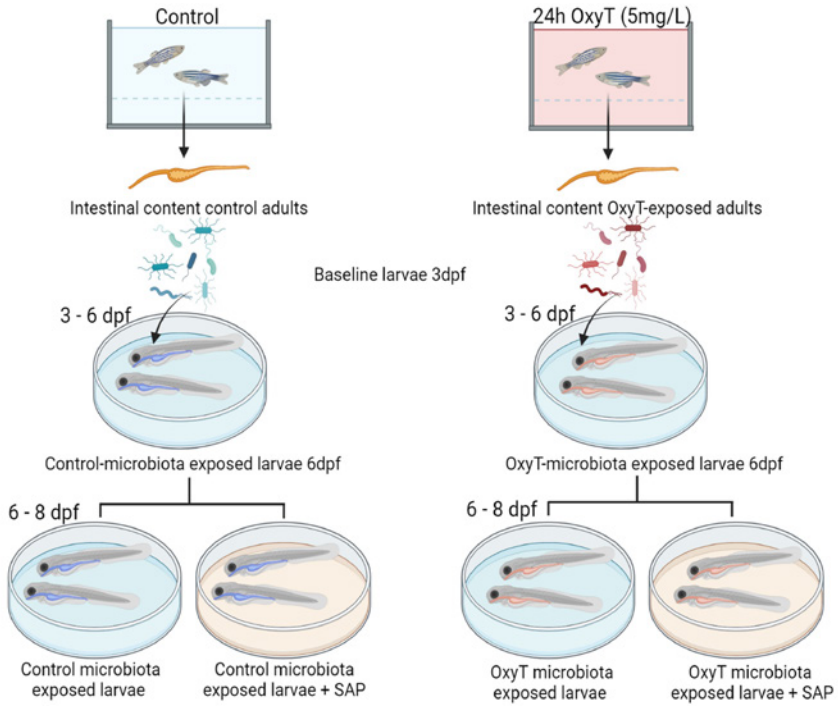


Figure 1: Experimental design and treatments employed for the study.

Exposure of larvae to intestinal content

Fifty microliters of pooled intestinal content were added per flask (25 ml E2) containing 3 dpf larvae (n=20 larvae per flask). Six flasks received intestinal content of untreated adult zebrafish, eight flasks received intestinal content of OxyT-treated adult zebrafish. At 3 dpf, two pools of n=5 larvae that were untreated were euthanized with tricaine (300mg/L) and saved in sterile PBS at -20°C for DNA isolation and 16S sequencing.

Baseline sampling control versus oxytetracycline-exposed larvae

At 6 dpf n=15 control (3 pools of 5 larvae, group G2 in **Figure 1**) and n=15 OxyT-content exposed larvae (3 pools of 5 larvae, group G3 in **Figure 1**) were euthanized (300 mg/L buffered tricaine) and kept in sterile PBS at -20°C for DNA isolation and 16S sequencing. N= 15 Control (3 pools of 5 larvae) and n = 25 OxyT-content exposed larvae (5 pools of 5 larvae) were euthanized and transferred to tubes containing RNAlater and stored at –20°C for RNA extraction.

Induction of inflammation

At 6 dpf, half of the control and half of the OxyT content exposed larvae were exposed to 0.5 mg/ml saponin (ultrapure Soy Saponin 95%, kindly provided by Trond Kortner NMBU Oslo Norway, origin: Organic Technologies, Coshocton, OH (Krogdahl *et al.*, 2015)), the other half were exposed to E2 (both saponin and control solutions were made fresh and E2 in the flasks was completely replaced with either the saponin in E2 or E2 solution). This resulted in two flasks of control content exposed larvae + E2 (G5 in **Figure 1**), two flasks of OxyT-content exposed larvae + E2(G7 in **Figure 1**), four flasks of control content exposed larvae + saponin (G6 in **Figure 1**), six flasks of OxyT-content exposed larvae + saponin (G8 in **Figure 1**). Larvae were exposed to either control or saponin from 6-8 dpf and the solution was refreshed daily. Dead larvae were recorded each day to obtain a survival curve for each group.

Fluorescent *in vivo* imaging

At 6 and 8 dpf part of the larvae were anaesthetized with 168 ug/ml (end concentration) buffered MS-222 and embedded in 1% Ultrapure low melting point agarose (Thermo Fisher Scientific, MA, United States). Larvae were imaged using the LeicaFA205 fluorescent microscope for the GFP and mCherry signal using the same exposure time and gain per channel for each larva (n=9 control 6 dpf, n=10 OxyT 6 dpf, n=8 control + E2 8 dpf, n=10 control + SAP 8 dpf, n=8 OxyT + E2 8 dpf, n=8 OxyT + SAP 8 dpf). After imaging larvae were removed from the agarose gel and euthanized in an overdose MS-222 as described above. Fluorescent images were analysed by Fiji Image J (United States National Institutes of Health, Bethesda, United States) images were processed by calculating the % area of fluorescence and subtracting the background for each picture.

RNA isolation, cDNA synthesis and qPCR

At 6 and 8 dpf part of the larvae were euthanized using an overdose of buffered MS-222. Pools of 5 larvae per sample (n) were stored in RNAlater at -20°C until further processing (n=3 control 6 dpf, n=6 OxyT6 dpf, n=4 control 8 dpf, n=3 control + E2, n=3 control + saponin, n=6 OxyT+ E2, n=4 OxyT+ saponin). RNA isolation was performed using the Qiagen MicroRNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 500 ng RNA using the Superscript III First strand synthesis kit (Invitrogen, cat no. 11752-050/111752-250). QPCR was performed using GoTaq qPCR Master Mix (Promega, WI, United States) according to the manufacturer's instructions using the primers depicted in **Table 1**.

Table 1 Primer used for the Real Time qPCR

Gene	FW primer	Reverse primer
elfa1	5'-CTGGAGGCCAGCTCAAACAT-3'	5'-ATCAAGAAGAGTAGTAGTACCG-3'
mmp9	5'-ACGGCATTGCTGACAT-3'	5'-TAGCGGGTTTGAATGG-3'
il22	5'-GGAGGGTCTGCACAGAG-3'	5'-GTCTCCCCGATTGCTT-3'
il10	5'-AGGGCTTTCCTTTAAGACTG-3'	5'-ATATCCCGCTTGAGTTCC-3'
cxcl8a	5'-TGTTTTCCTGGCATTTCTGACC-3'	5'-TTTACAGTGTGGGCTTGGAGGG-3'
cxcl8b.1	5'-CCACTGAATTGTCCTTTCATCA-3'	5'-TGATGAAAGGACAATTCAGTGG-3'
tnfa	5'-AACAAGATGGAAGTGTGCTGAGA-3'	5'-GGTCCTGGTCATCTCTCCAGT-3'
tnfb	5'-AAACAACAAATCACCACACC-3'	5'-ACACAAAGTAAAGACCATCC-3'
ccl38.5	5'-GTCTGGTGCTCTTCGCTTTC-3'	5'-TGCAGAGAAGATGCGTCGTA-3'
cxcl11aa (cxcl11.1)	5'-CCATCAGGAAACCGAACAA-3'	5'-CACCAAGACACACACATCTC-3'
cxcl11af (cxcl11.6)	5'-TGTCGTCTTAAAGGCTATTGG-3'	5'-TCGGTGCTGATGTTGATG-3'
bdef1	5'-AGCCCCAGAGCATATTTAT-3'	5'-TGGTCCGAAGTAAAGTTCA-3'
bdef2	5'-GGCGCTTCTGTTTTGA-3'	5'-TTTCCCCCAATATTAACAAT-3'
bdef3	5'-GCCAAGCCAATGATACA-3'	5'-GACCGCTATTATGC-3'

DNA isolation and 16S sequencing

Pools of larvae of 3, 6 and 8 dpf were transferred to 100 µl lysis buffer containing 100mMNaCl, 10mM Tris pH 8, 15 mM EDTA 0.5% SDS). Proteinase K (7 µl of 19 mg/ml stock, QIAGEN, Hilden, Germany) was added to the samples in lysis buffer and samples were incubated at 56°C until the larvae were fully dissolved. After briefly spinning the tube to remove any condense on the lid, 35 µl of 6M NaCl was added, and samples were incubated at room temperature under continuous shaking (700 rpm). Samples were subsequently centrifuged for 15 min at 16,800g (maximum speed) and the supernatant was transferred to a clean 1.5 ml Eppendorf tube. Next, 270 µl 100% (cold) ethanol was added and samples were kept in –80 C for one hour. Samples were centrifuged for 5 minutes at full speed, and the pellet washed with 70% ethanol. After centrifugation, the supernatant was removed, and pellets were air dried before adding 50 µl RNase/DNase free water. DNA concentrations were measured and send for sequencing at BaseClear (Leiden, the Netherlands). Next Generation Sequencing (NGS) Illumina technology with the following forward (FW) and reversed (RV) primers were used to sequence a 300 bp region of the V3-V4 hypervariable region of the 16S rRNA gene: FW (TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGCCTACG-GGNGGCWGCAG) and RV (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVG-GGTATC T AATCC).

Bioinformatics analysis 16S sequencing data

16S data was analysed with Qiime2 (Bolyen *et al.*, 2019), using DADA2 (Callahan *et al.*, 2016) for the generation of amplicon sequence variants (ASV) and the Silva database (Pruesse *et al.*, 2007) (v138) for taxonomic classification. Alpha diversity indexes (Shannon and Faith's distance) were calculated as implemented in Qiime2. The pairwise distances within and between groups were calculated from the Bray-Curtis Distances in Canoco v5.15v (Braak and Smilauer, 2012). Redundancy analyses (RDA) and principal component analyses (PCA) were performed with Canoco v5.15 (Braak and Smilauer, 2012) using analysis type "constrained" or "unconstrained", respectively. Response variables were log-transformed with the formula log(10000*relative_abundance+1). RDA p-values were determined through permutation testing (500 permutations). Boxplots were generated using Prism v.9.0.0 (GraphPad Software, San Diego, California USA). Additional data handling and format conversions were done in Python (https://www.python.org/).

Statistical analysis

Data was checked for normal distribution by a Shapiro-Wilk test or Kolmogorov-Smirnov depending on sample size. Un-paired t-test was used when comparing two treatments with normally distributed data. Man-Whitney t-test was applied when comparing two treatments with non-normally distributed data. For datasets containing more than two treatments with normally distributed data one-way ANOVA test with Tukey's multiple comparison post-hoc test was applied. In case of non-normally distributed data, one-way ANOVA Kruskal-Wallis test with Dunn's multiple comparison post-hoc test was applied.

Results

Exposure to intestinal content of oxytetracycline-treated adults changes the microbiota of larval zebrafish

Zebrafish larvae of 3 dpf were exposed to either intestinal content from OxyT-exposed adult zebrafish or control (tank water)-exposed adult zebrafish until 6dpf. From 6 dpf to 8 dpf both groups were split in controls (E2) and saponin-exposed (0.5mg/ml). To explore the differences in microbiota a PCA and an RDA of all the samples were performed (**Supplementary Figure 1**). The PCA separated the baseline samples, non-exposed to any adult content (3dpf) from the rests of the samples exposed to adult gut content (horizontal axis). Moreover, there is a separation between the 6dpf and the 8dpf samples and only the non-challenged 8dpf exposed to control adult gut content still cluster with the 6dpf samples (vertical axis). The RDA confirmed that the microbial composition between the groups is significantly different ($p = 0.002$).

First, it was investigated how OxyT-treated adult gut microbiota affected the colonization and development of the bacterial communities in the zebrafish larvae in the unchallenged groups (without saponin) (at 6 dpf and 8 dpf). The diversity of the microbiota was increased in zebrafish larvae that received an adult gut content treated with OxyT at 8dpf (and not at 6 dpf) compared to controls (α-diversity, Shannon **Figure 2A**). Besides OxyT-treated adult gut content promoted more phylogenetic diverse microbiota overtime (6 vs 8dp): the taxons are further away in the phylogenetic three from each other (α-diversity, Phylogenetic Diversity, **Figure 2A**). How the bacterial communities differ from one group to another was explored by comparing pairwise distances within and between the groups (β-diversity, **Figure 2B**). Exposure to OxyT-treated adult content significantly increased the variation in the microbial communities overtime (OxyT-exp larvae 6vs 8dpf, left **Figure 2B**). When comparing the differences in microbiota between groups it is observed that: i) the Bray-Curtis distance between 6 and 8 dpf OxyT-content exposed larvae is significantly decreased when compared to controls and ii) the distance between control and OxyT-content exposed larvae is significantly decreased at 8 dpf compared to 6 dpf. Overall, there was a homogenizing effect of the microbial variation in OxyT-content exposed larvae overtime.

As can be observed from the RDA (**Figure 1C**) exposing larvae of 3 dpf to different intestinal content (control or OxyT-exposed adult content) resulted in different microbial communities at 6 dpf (p=0.002**, Figure 1C**) that at least remain different up until 8 dpf (p=0.002, **Figure 1D**). When evaluating the abundance of the top discriminative genera, OxyT-content exposed larvae display more *Escherichia-Shigella* and less *Pelomonas* and *Undibacteria* at 6 dpf, while OxyT-content exposed larvae at 8 dpf display more *Cryseobacteria*, *Rhizobium*, *Aeromonas*, *Pelomonas* and *Pseudomonas* than control-content exposed larvae (**Figure 1E**). The fluctuations of the microbial species in terms of relative abundance are depicted in a heatmap for all the non-saponin challenged groups (**Supplementary Figure 2**). Most of the discriminatory genera are increased in abundance in the OxyT-content exposed larvae at 8 dpf compared to all the other groups, with the exception of *Undibacterium*. Interestingly, oxytetracycline-content exposed larvae appear to display more *Escherichia-Shigella* already at 6 dpf, while the control content-exposed only reach these levels at 8 dpf. In conclusion, exposing 3 dpf larvae to luminal content of control or OxyT-exposed adults gives rise to distinct microbiota profiles until at least 8 dpf.

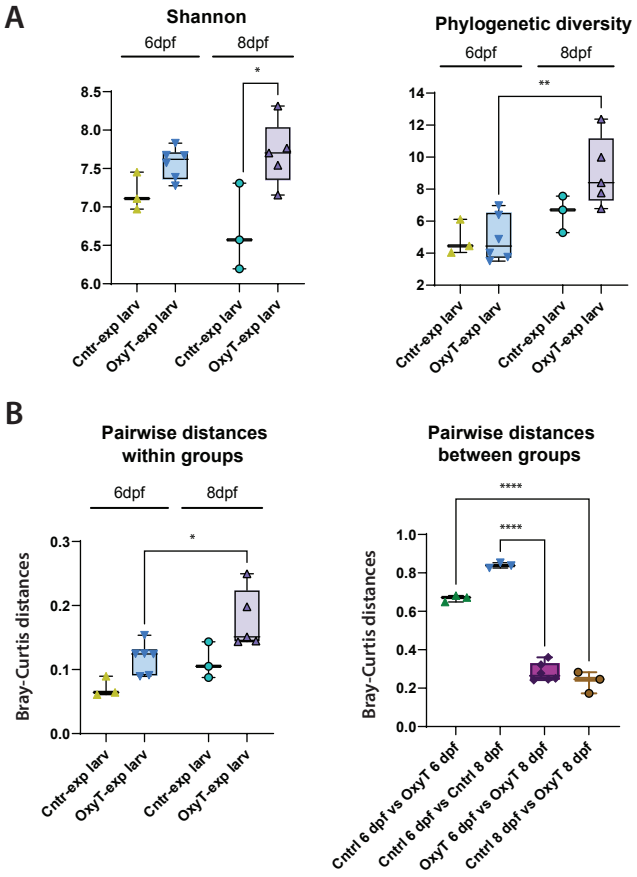


Figure 2: **(A)** Alpha-diversity indexes: Shannon and Phylogenetic diversity for the 6 and 8 dpf control- and OxyT-content exposed larvae. **(B)** Beta-diversity measure of the pairwise distances within and between groups (6 and 8 dpf control- and OxyT-content exposed larvae). *p≤0.05, **p≤0.01, ****p≤0.0001 Kruskal-Wallis test after testing for non-normally distributed data by Shapiro-Wilk test. Whiskers: min. to max. all points with median. **(C)** RDA of the baseline (3 dpf) and the 6 dpf control- and OxyT-content exposed larvae (p=0.002, x axis explains 46.5% of the variation and y axis explains 17.9% of the variation observed). Each symbol corresponds to an individual sample and ellipses are 2D approximations at the 66% confidence level. The top 20 most discriminative genera are depicted in arrows pointing towards the phenotype they associate with. **(D)** RDA of the baseline (3 dpf) and the 6 dpf and 8dpf control- and OxyT-content exposed larvae (p=0.002, x axis explains 24.9% of the variation and y axis explains 20.9% of the variation observed). Each symbol corresponds to an individual sample and ellipses are 2D approximations at the 66% confidence level. The top 20 most discriminative genera are depicted in arrows pointing towards the phenotype they associate with. **(E)** Top most discriminative genera of both RDAs, threshold values: RDA score ≥ 0.4 and average relative abundance ≥ 1%.

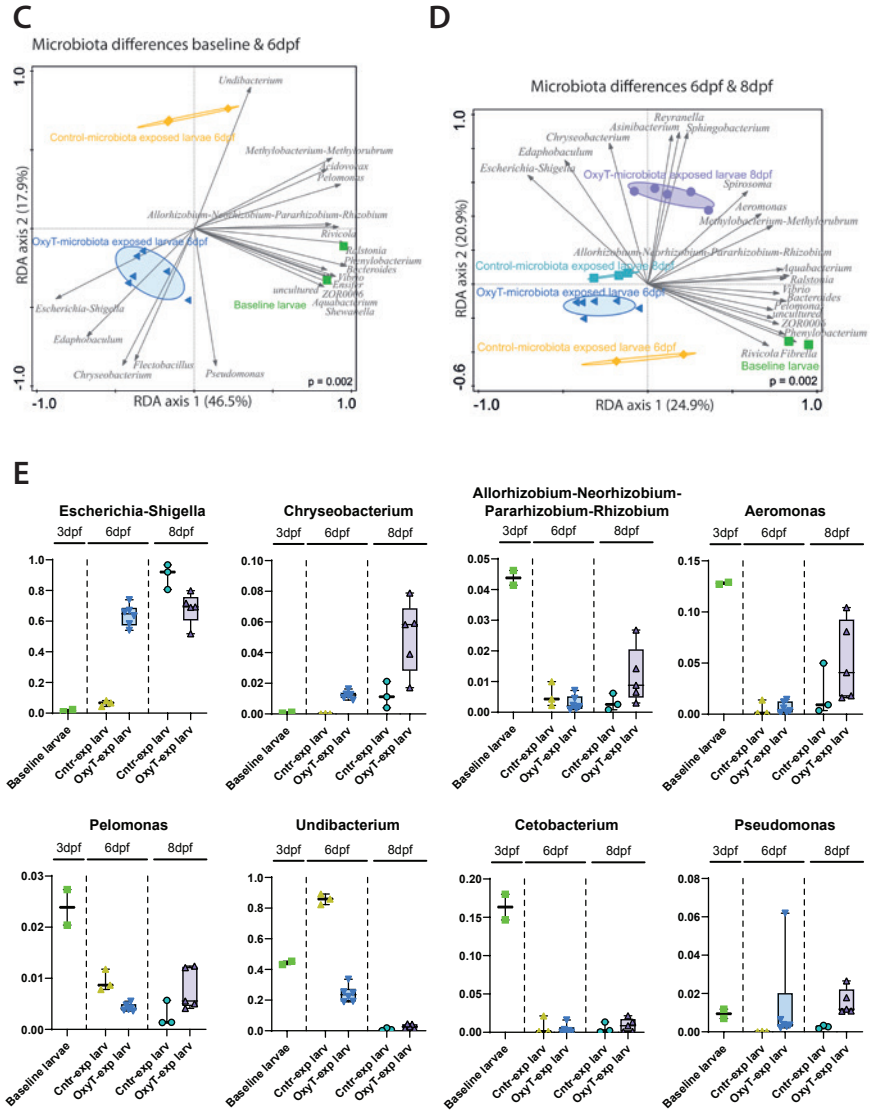


Figure 2: Continued.

Oxytetracycline content-exposed larvae show no macrophage response after 3 days of saponin exposure

Next, we investigated whether changing the microbial community in zebrafish larvae might have an influence on the response towards an inflammation-inducing compound (saponin). Previous research performed in our laboratory showed that 0.5 mg/ml saponin exposure during 3 days induces a mild inflammatory response in the majority of the zebrafish larvae. This response is characterized by neutrophil and (to a lesser extent) macrophage recruitment and pro-inflammatory cytokine expression (López Nadal *et al.*, 2018).

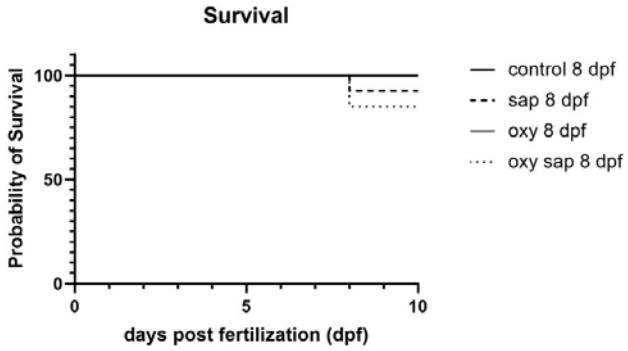


Figure 3: Survival of larvae exposed to control or 0.5 mg/ml saponin.

As can be observed from the survival graph, saponin affected survival in both OxyT as well as control-content exposed groups, although the mortality tended to be higher in the OxyT-content exposed group (7,5% in control + sap versus 15% in OxyT + SAP). Both saponin groups show significantly lower survival compared to their respective control groups (Mantel-Cox test, $p<0.01$).

In order to investigate the innate immune responses (neutrophil and macrophage presence) in the zebrafish larvae exposed to different microbial communities, zebrafish were anaesthetized and embedded in low-melting point agarose and imaged using a fluorescent microscope at 6 dpf (before) and 8 dpf (after saponin or control exposure). Representative pictures are shown in **Figure 4A**. At 6 dpf, there were no differences in the presence of neutrophils and macrophages in the overall fish nor in the gut area (**Figure 4A, 4B**). Both saponin-exposed groups showed an increase in neutrophil presence in the overall fish and the gut area. Interestingly, the zebrafish larvae that were exposed to OxyT-exposed intestinal content did not show increased macrophage presence both in the whole fish and gut area in response to saponin treatment. The control-exposed larvae showed increased macrophage presence in response to saponin exposure.

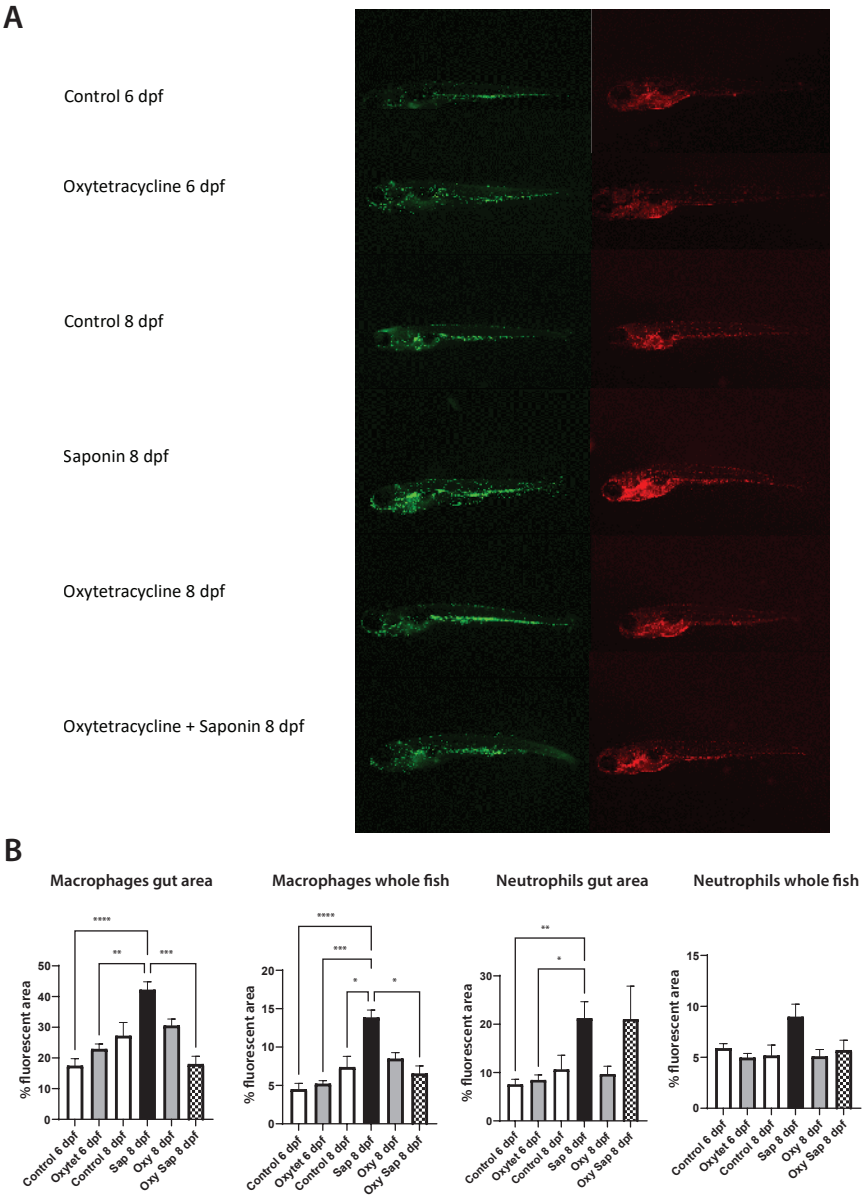


Figure 4: Neutrophil (GFP) and macrophage (mCherry) imaging in zebrafish larvae at baseline (6 dpf) and after exposure to saponin or control (E2) (8 dpf). **(A)** representative fluorescent imaging of GFP+ neutrophils and mCherry+ macrophages. **(B)** Quantification of % area GFP and mCherry fluorescence corrected for background. Statistics: neutrophil graphs: one-way ANOVA Kruskal-Wallis test with Dunn's multiple comparison post-hoc test; macrophage graphs: one-way ANOVA test with Tukey's multiple comparison post-hoc test.

Oxytetracycline or control intestinal content exposed zebrafish larvae show a differential gene-expression response towards saponin

Both at 6 dpf (before) and at 8 dpf (after saponin exposure) gene expression was analysed in pools of larvae. Upon exposure to saponin zebrafish larvae (both control and OxyT content-exposed) display increased expression of *mmp9*; a hallmark of saponin-induced damage (**Figure 5;** (López Nadal *et al.*, 2018)). This matrix metallopeptidase is involved in the degradation of extracellular matrix and activation of pro-inflammatory cytokines and chemokines such as *il1b* and *cxcl8*. The concentration of saponin used in this experiment is shown to induce mild inflammatory responses and indeed in this experiment the gene expression of *cxcl8b1, il1β* and *tnfb* (and *il10*) after saponin is only slightly higher (but not significantly). Since differences in macrophage presence were observed after saponin exposure between those zebrafish larvae that received control or OxyT-exposed content, gene expression of known macrophage recruiting chemokines were analysed. *Ccl38*

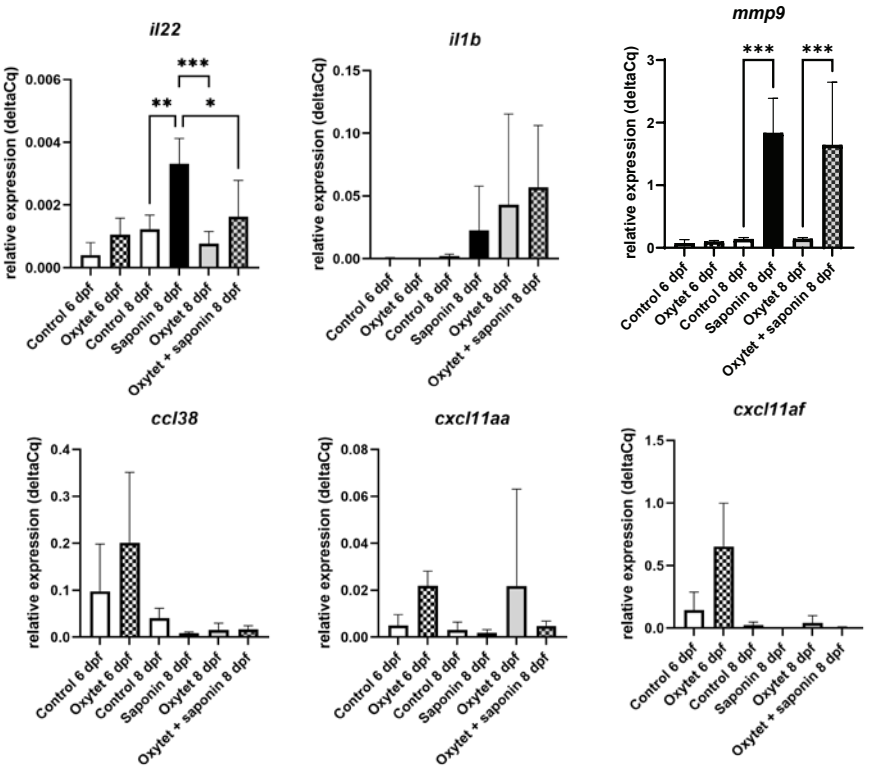


Figure 5: Gene expression in pools of total larvae exposed to control (E2) or oxytetracycline-exposed intestinal content of adults (6 dpf), with or without saponin-induced inflammation (8 dpf). Statistics: one-way ANOVA Kruskal-Wallis test with Dunn's multiple comparison post-hoc test.

(ccl38.5), *cxcl11aa* (*cxcl11.1*) and *cxcl11af* (*cxcl11.6*), recruit macrophages via the macrophage chemokine receptors ccr2 (*ccl38a5*) or cxcr3 (*cxcl11.1* and *cxcl11.6*) respectively. These macrophage-recruiting chemokines were however not differentially expressed between both OxyT and control treated groups, with or without saponin. Of the neutrophil-recruiting chemokines (*il1β*, *cxcl8a* and *cxcl8b1)* only *cxcl8b1* tended to be increased (although not significantly) in the OxyT + saponin 8 dpf group. Interestingly, the only cytokine measured that associated with the difference in macrophage presence upon saponin exposure was *il22*.

Saponin exposure from 6-8 dpf in larvae harbouring different microbiota, affects that microbiota in a differential way

We previously showed that a prior exposure to oxytetracyclin in zebrafish larvae aggravates the microbial disruption promoted by a subsequent saponin treatment (López Nadal *et al*., 2018). Here, we assessed whether the saponin-induced dysbiosis was influenced by the exposure of the treated content of the adult zebrafish (**Figure 6**). Saponin significantly increased the number of different species harboured in OxyT-content exposed larvae compared to controls (α-diversity, Shannon index, **Figure 6A**). However these species were not significantly distant in the phylogenetic tree compared to the other groups (α-diversity , Phylogenetic Diversity, **Figure 6A**).

Again, the differences in bacterial communities within and between groups were assessed by distances (β-diversity, left **Figure 6B**). Saponin-treated OxyT-content exposed larvae presented the most dissimilar microbiota composition compared to the other groups. The dissimilarity of the microbiota composition between groups is high in all the comparisons that include the saponin-treated OxyT-content exposed larvae group and low in the ones that this latter group is not present (right, **Figure 6B**). Overall, the saponin-treatment created a more dissimilar microbiota composition in the larvae that were exposed to OxyT-treated adult content in contrast to the homogenization of the bacterial composition in OxyT-content exposed larvae.

As can be seen in the RDA, the saponin treatment and the adult gut content exposure resulted in significantly dissimilar microbiota composition of the zebrafish larvae ($p = 0.002$, **Figure 6C**). The saponin treatment promoted an altered microbiota composition in control-content exposed larvae that resembled the non-treated OxyT-content exposed larvae microbiota. Saponin promoted the most dissimilar microbiota composition in OxyT-content exposed larvae when comparing the groups. Saponin treated OxyT-content exposed larvae showed increased abundance in *Aeromonas* and *Pseudomonas* and a reduced abundance in *Escherichia-Shigella* and *Chryseobacteria* (**Figure 6D**). Saponin treated control-content exposed showed an increased abundance in *Chryseobacteria* compared to the other groups (**Figure 6D**). The control-content exposed larvae showed

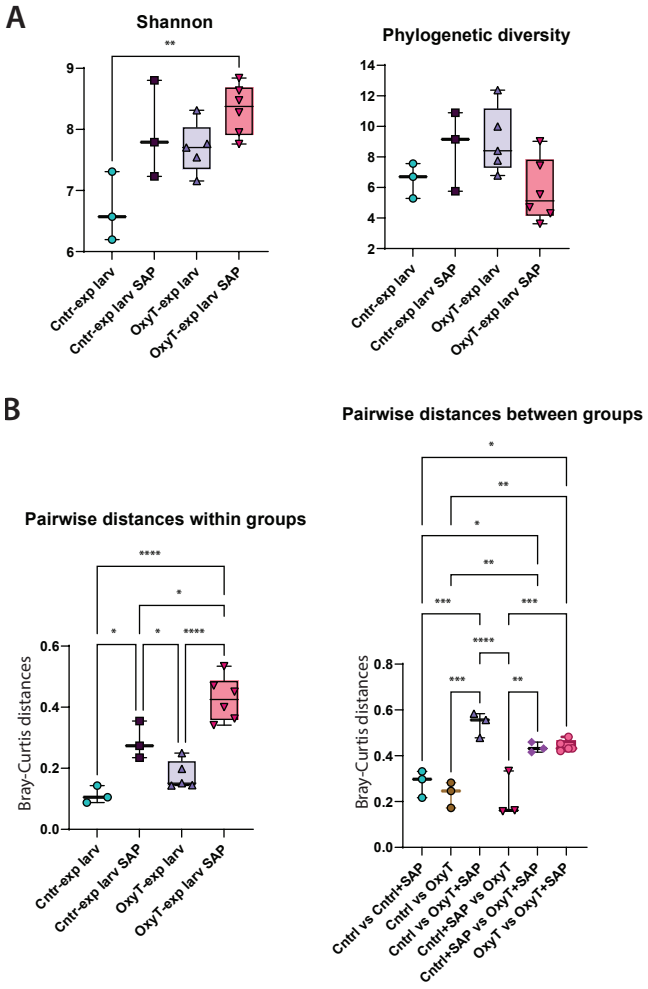


Figure 6: **(A)** Alpha-diversity indexes: Shannon and Phylogenetic diversity for the 8 dpf control- and OxyT-content exposed larvae challenged with saponin. **(B)** Beta-diversity measure of the pairwise distances within and between groups 8dpf control- and OxyT-content exposed larvae challenged with saponin. *p≤0.05, **p≤0.01,*** p≤0.005, **** p≤0.0001 Kruskal-Wallis test after testing for non-normally distributed data by Shapiro-Wilk test. Whiskers: min. to max. all points with median. **(C)** RDA of the 8dpf control- and OxyT-content exposed larvae challenged with saponin (p=0.002, x axis explains 15.1% of the variation and y axis explains 11.7% of the variation observed. Each symbol corresponds to an individual sample and ellipses are 2D approximations at the 66% confidence level. The top 20 most discriminative genera are depicted in arrows pointing towards the phenotype they associate with. **(D)** Top most discriminative genera of both RDAs, threshold values: RDA score ≥ 0.4 and average relative abundance ≥ 1%.

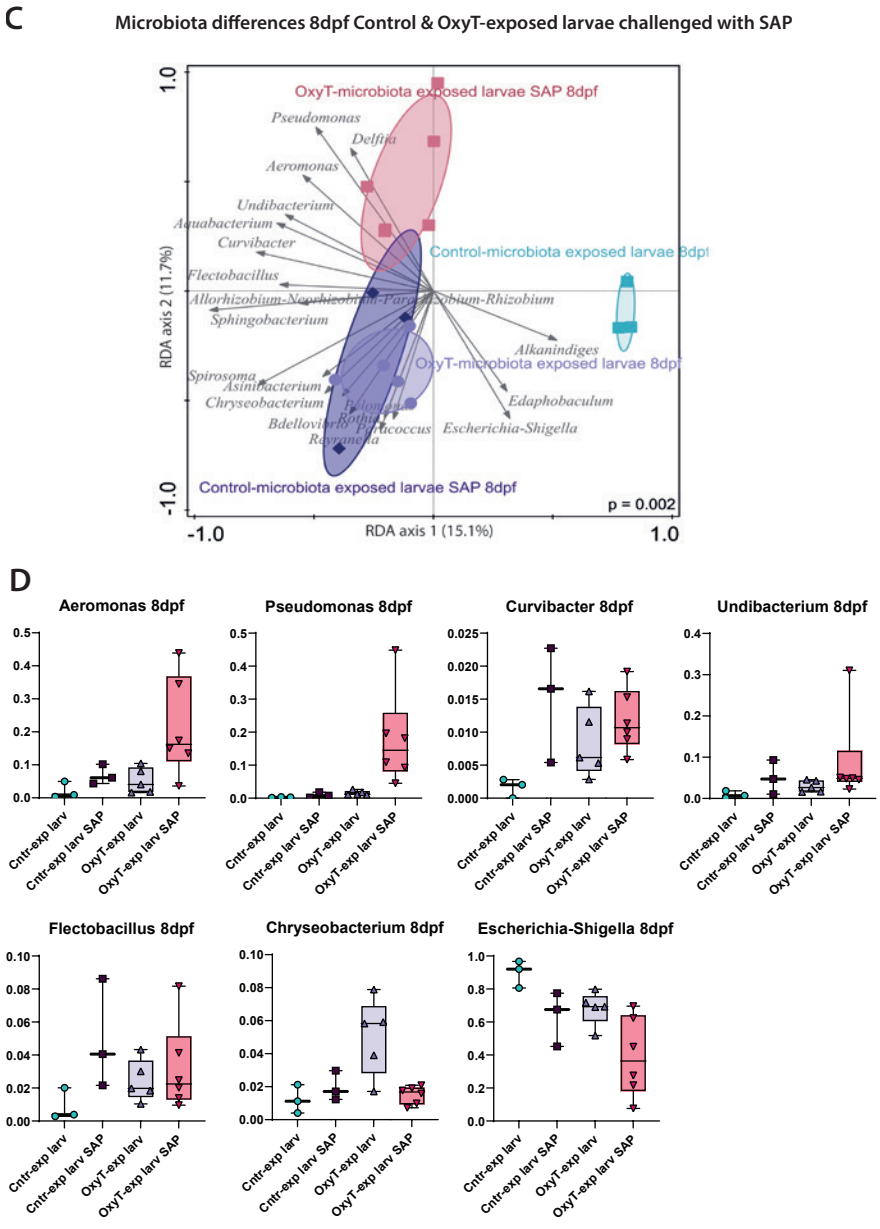


Figure 6: Continued.

a reduced abundance in *Sphingobacterium*, *Pseudomonas*, *Spirosoma*, *Curvibacter*, *Aeromonas*, *Deftia*, *Aquabacterim* and *Edaphobaculum* and an increased abundance in *Escherichia-Shigella* compared to the other groups (**Supplementary Figure 3**). Overall, the saponin-treatment in OxyT-content exposed larvae promoted a more dissimilar microbiota composition characterized by a decreased *Escherichia-Shigella* abundance and an increased *Aeromonas* and *Pseudomonas* abundance.

Discussion

In this study zebrafish larvae were exposed to luminal contents of oxytetracycline or control exposed adult zebrafish to investigate whether: i) microbial colonization in zebrafish larvae is affected by exposure to certain microbial consortia and whether ii) the dissimilar microbial communities influence the immune responses towards an anti-nutritional factor (saponin). Overall microbiota composition of the zebrafish larvae (hatched from bleached eggs) depended on the microbial mix of the adult gut content that were exposed. Differences in microbiota compositions between control-content exposed larvae and OxyT-content exposed larvae subsisted at least until 8 dpf. Differently colonized larvae exposed to saponin (6-8dpf) resulted in distinct immune profiles. Mortality in saponin-treated OxyT-content exposed larvae was twice as high compared to saponin-treated control-content exposed larvae although not statistically significant. Interestingly, saponin-treated OxyT-content exposed larvae showed less macrophages (overall fish and gut area) compared to saponin-treated control-content exposed larvae. Furthermore, *il22* gene expression levels were lower in OxyT-content exposed larvae in response to saponin, compared to the controls.

This research clearly indicates that differences in microbial composition already early in life can affect responses towards inflammation-causing substances such as the anti-nutritional factor saponin. Previous research performed in our laboratory showed that changing the microbiota in adult zebrafish before induction of oxazolone colitis also affected disease severity and susceptibility (Brugman *et al.*, 2009). In that study, exposure to vancomycin reduced the abundance of *Escherichia* and increased the abundance of commensal *Cetobacterium*, resulting in less neutrophil recruitment and reduced enterocolitis scores. Interestingly, while in adult zebrafish an increase in *Escherichia* and decrease in *Cetobacterium* is often associated with dysbiosis and disease (Bhute *et al.*, 2020; Ofek *et al.*, 2021; Wang *et al.*, 2021), healthy zebrafish larvae harbour mostly *Escherichia-Shigella* early in life (López Nadal *et al.*, 2018; Ikeda-Ohtsubo *et al.*, 2020). *Cetobacterium* are present in the zebrafish larvae guts in a relative abundance <1% that becomes around 14% in the adult zebrafish gut (Stephens *et al.*, 2016), demonstrating that the developmental

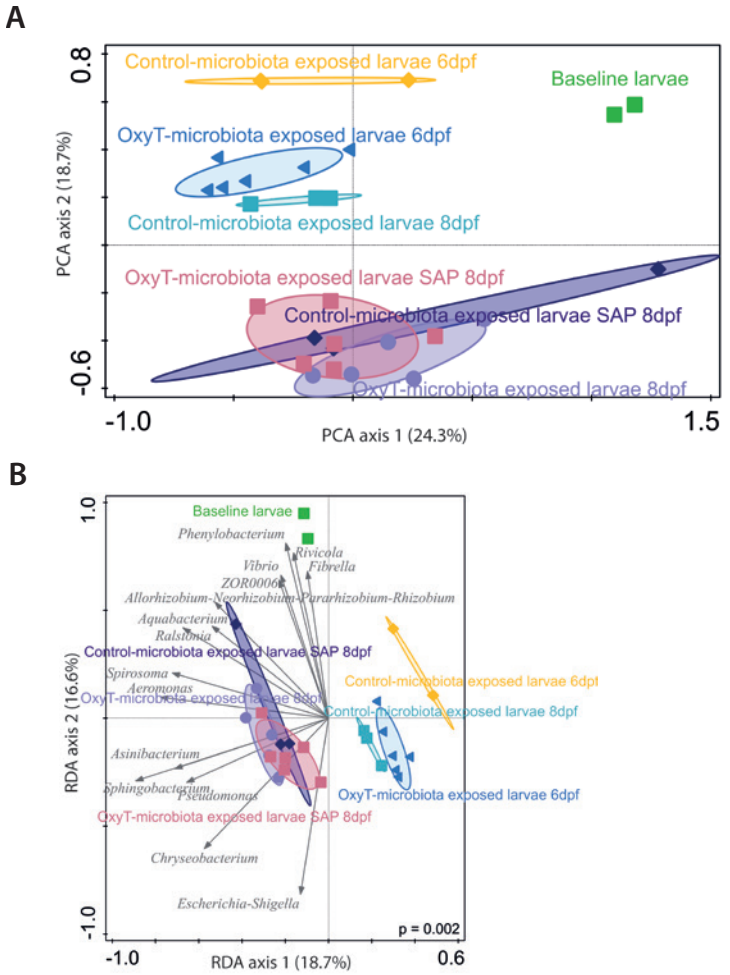
stage of the zebrafish as well as the immune maturation are crucial to the specific microbial community composition (López Nadal *et al*., 2020).

We previously showed that a prior exposure to oxytetracycline (5ug/L, 4-6 dpf) declined survival to a saponin challenge (0.5mg/ml, 6-9 dpf) and disrupted the microbiota composition decreasing the abundance of *Escherichia-Shigella* and increasing the abundance of *Limnobacter* and *Flectobacillus* among other genera (López Nadal *et al*., 2018). In that previous study, the zebrafish larvae were directly treated with the antibiotic while in this present study we examined if OxyT-treated gut microbial communities from adult zebrafish would impact the microbial composition and the susceptibility to saponin-induced inflammation in the larvae. Interestingly, saponin-treated OxyT-content exposed larvae also presented reduced survival and a decrease in relative abundance of *Escherichia-Shigella* and an increase in other genera (among which *Flectobacillus*), suggesting that the resulting gut microbiota composition of the adult zebrafish after the oxytetracyclin exposure has the capacity to colonize and disrupt zebrafish larvae microbiota in a similar manner than the direct exposure to the oxytetracycline. Future research might address whether the microbiota composition of the larvae is affected by any parental microbial contribution in standard rearing conditions.

Larvae exposed to oxytetracycline treated adult gut content have an altered macrophage response towards saponin. While the control-content exposed larvae recruit both neutrophils and macrophages to the gut area, the OxyT-content exposed larvae do not seem to recruit macrophages. Analysis of macrophage recruiting chemokines (*cxcl11* and *ccl38.5*), however, did not give an explanation as to why less macrophages were recruited. It might be that in order to pick up differences in chemokine responses, gene expression should have been measured earlier during the saponin exposure. Since we also did not observe significant differences in *cxcl8a* and *cxcl8b1* expression upon saponin while clear neutrophil recruitment is visible, this might indicate that peak levels of recruiting chemokines might proceed this 8 dpf timepoint. Interestingly, expression of *il22* associated with macrophages recruitment. Expression levels of *il22* and recruitment of macrophages were both decreased in OxyT-content-exposed larvae after saponin. In mammals, macrophages are reported to express the IL22 receptor and induce IL22-producing group 3 innate lymphoid cells (ILC3) (Treerat, Nature 2017; Bain Immunol Rev. 2014). Furthermore, IL22-/- mice showed a decreased number of recruited alveolar macrophages during the chronic stages of Mycobacterium infection (Treerat *et al*., 2017). Whether the reduced *il22* expression levels result in less macrophages in our OxyT-content-exposed zebrafish or whether the reduced *il22* expression is a result of less recruited (activated) macrophages remains to be elucidated. Future research in our lab will further investigate the cross-talk between immune cells, bacteria and the IL22 signalling axis in health and disease in zebrafish.

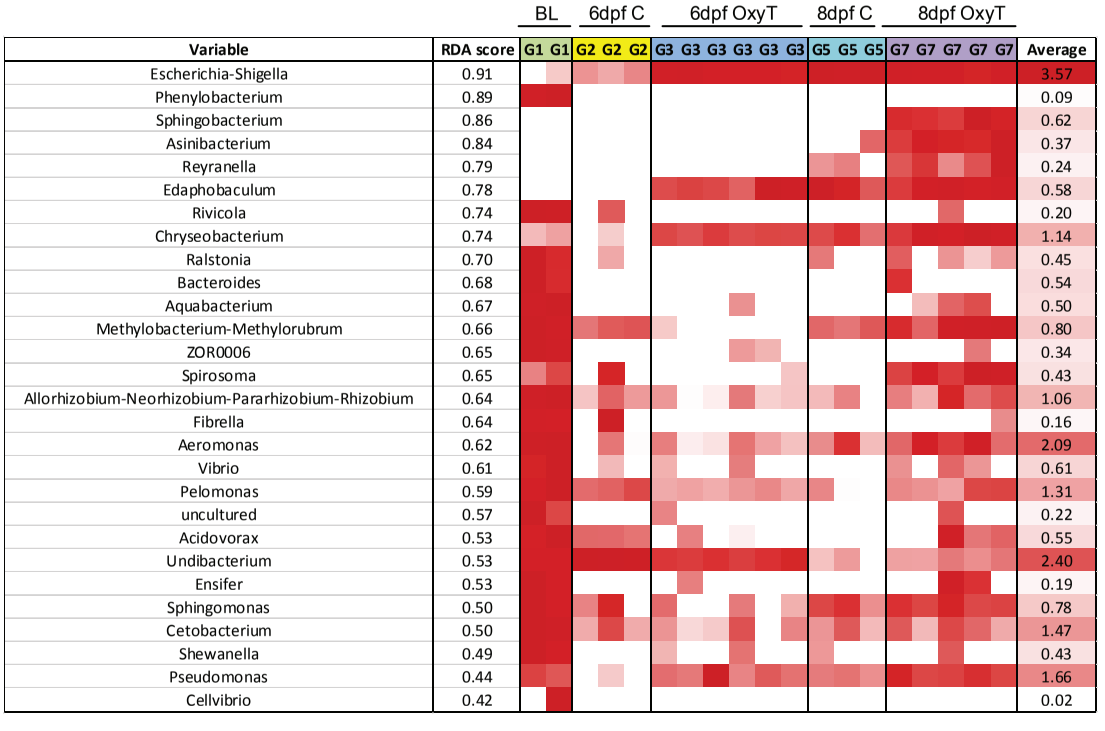
Ample research in mammals and zebrafish has shown that the microbiota composition is an important determinant of immune set-point. Different immune cell populations will be recruited to the intestines for example in response to colonizing segmented filamentous bacteria (SFB) in mice (Th17) (Ivanov *et al.*, 2009). Currently, more and more research in zebrafish is performed to understand which immune cells and mediators interact with the microbiota and how microbes affect zebrafish health. In conclusion, here we show that fish harbouring altered microbiota can respond differently to saponin-induced inflammation. Antibiotic perturbations in aquatic environments may have large effects on fish health by modifying their immune system and susceptibility to diseases and inflammatory-associated events.

Supplementary figures

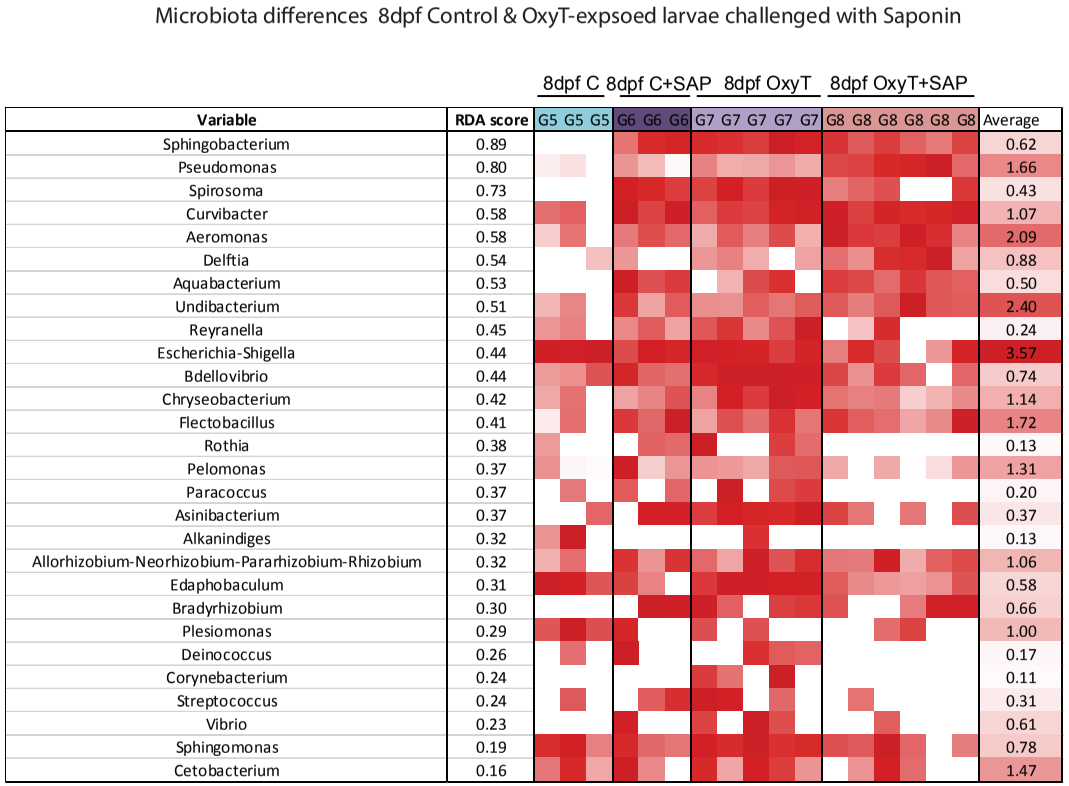


Supplementary Figure 1: **(A)** PCA of all samples analysed in the experiment. Samples clustered by x axis: baseline compared to content exposed larvae (24.3% of the variation explained) and y axis: saponin treatment and OxyT-content exposed at 8dpf compared to the other groups (18.7% of the variation explained). **(B)** RDA of all sampled used in the study with the top 15 genera correlated with the treatments. Each symbol corresponds to an individual sample and ellipses are 2D approximations at the 66% confidence level. The top 20 most discriminative genera are depicted in arrows pointing towards the phenotype they associate with.

Microbiota differences baseline, 6dpf and 8dpf Control & OxyT-expsoed larvae



Supplementary Figure 2: Heatmap of the relative abundance of the 28 most discriminating genera in the RDA **(Figure 2D)**. Colour indicates abundance, normalized by row (genus), with the lowest value in white and the highest value in red, log-transformed as "log(relative abundance*10000+1)".



Supplementary Figure 3: Heatmap of the relative abundance of the 28 most discriminating genera in the RDA (Figure 3D). Colour indicates abundance, normalized by row (genus), with the lowest value in white and the highest value in red, log-transformed as "log(relative abundance*10000+1)".

References

Bhute, S. S., Escobedo, B., Haider, M., Mekonen, Y., Ferrer, D., Hillyard, S. D., Friel, A. D., van Breukelen, F., & Hedlund, B. P. (2020). The gut microbiome and its potential role in paradoxical anaerobism in pupfishes of the Mojave Desert. *Animal Microbiome*, *2*(1), 1–9.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology*, *37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Brugman, S., Liu, K. Y., Lindenbergh-Kortleve, D., Samsom, J. N., Furuta, G. T., Renshaw, S. A., Willemsen, R., & Nieuwenhuis, E. E. S. (2009). Oxazolone-Induced Enterocolitis in Zebrafish Depends on the Composition of the Intestinal Microbiota. *Gastroenterology*, *137*(5), 1757-1767.e1. https://doi.org/10.1053/j.gastro.2009.07.069

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Chinen, T., & Rudensky, A. Y. (2012). The effects of commensal microbiota on immune cell subsets and inflammatory responses. *Immunological Reviews*, *245*(1), 45–55.

el Aidy, S., Derrien, M., Aardema, R., Hooiveld, G., Richards, S. E., Dane, A., Dekker, J., Vreeken, R., Levenez, F., & Doré, J. (2014). Transient inflammatory-like state and microbial dysbiosis are pivotal in establishment of mucosal homeostasis during colonisation of germ-free mice. *Beneficial Microbes*, *5*(1), 67–77.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T. A., Nakato, G., Takahashi, D., Nakanishi, Y., Uetake, C., Kato, K., & Kato, T. (2013). Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. *Nature*, *504*(7480), 446–450.

Hapfelmeier, S., Lawson, M. A. E., Slack, E., Kirundi, J. K., Stoel, M., Heikenwalder, M., Cahenzli, J., Velykoredko, Y., Balmer, M. L., & Endt, K. (2010). Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses. *Science*, *328*(5986), 1705–1709.

Ikeda-Ohtsubo, W., López Nadal, A., Zaccaria, E., Iha, M., Kitazawa, H., Kleerebezem, M., & Brugman, S. (2020). Intestinal microbiota and immune modulation in zebrafish by fucoidan from Okinawa mozuku (Cladosiphon okamuranus). *Frontiers in Nutrition*, *7*, 67.

Ivanov, I. I., Atarashi, K., Manel, N., Brodie, E. L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K. C., Santee, C. A., & Lynch, S. v. (2009). Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*, *139*(3), 485–498.

Krogdahl, Å., Gajardo, K., Kortner, T. M., Penn, M., Gu, M., Berge, G. M., & Bakke, A. M. (2015). Soya Saponins Induce Enteritis in Atlantic Salmon (Salmo salar L.). *Journal of Agricultural and Food Chemistry*, *63*(15), 3887–3902. https://doi.org/10.1021/jf506242t

López Nadal, A., Ikeda-Ohtsubo, W., Sipkema, D., Peggs, D., McGurk, C., Forlenza, M., Wiegertjes, G. F., & Brugman, S. (2020). Feed, Microbiota, and Gut Immunity: Using the Zebrafish Model to Understand Fish Health . In *Frontiers in Immunology* (Vol. 11). https://www.frontiersin.org/article/10.3389/fimmu.2020.00114

López Nadal, A., Peggs, D., Wiegertjes, G. F., & Brugman, S. (2018). Exposure to Antibiotics Affects Saponin Immersion-Induced Immune Stimulation and Shift in Microbial Composition in Zebrafish Larvae. *Frontiers in Microbiology*, *9*(October), 1–16. https://doi.org/10.3389/fmicb.2018.02588

Ofek, T., Lalzar, M., Laviad-Shitrit, S., Izhaki, I., & Halpern, M. (2021). Comparative Study of Intestinal Microbiota Composition of Six Edible Fish Species. *Frontiers in Microbiology*, *12*.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., & Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research*, *35*(21), 7188–7196. https://doi.org/10.1093/nar/gkm864

Rawls, J. F., Mahowald, M. A., Ley, R. E., & Gordon, J. I. (2006). Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell*, *127*(2), 423–433. https://doi.org/10.1016/j.cell.2006.08.043

Rawls, J. F., Samuel, B. S., & Gordon, J. I. (2004). Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *PNAS*, *101*(13), 4596–4601. papers2://publication/uuid/897C5AC0-1CF8-4439-A8CE-1A42D6A7D552

Stephens, W. Z., Burns, A. R., Stagaman, K., Wong, S., Rawls, J. F., Guillemin, K., & Bohannan, B. J. M. (2016). The composition of the zebrafish intestinal microbial community varies across development. *ISME Journal*, *10*(3), 644–654. https://doi.org/10.1038/ismej.2015.140

Treerat, P., Prince, O., Cruz-Lagunas, A., Muñoz-Torrico, M., Salazar-Lezama, M. A., Selman, M., Fallert-Junecko, B., Reinhardt, T. A., Alcorn, J. F., & Kaushal, D. (2017). Novel role for IL-22 in protection during chronic Mycobacterium tuberculosis HN878 infection. *Mucosal Immunology*, *10*(4), 1069–1081.

Wang, A., Zhang, Z., Ding, Q., Yang, Y., Bindelle, J., Ran, C., & Zhou, Z. (2021). Intestinal Cetobacterium and acetate modify glucose homeostasis via parasympathetic activation in zebrafish. *Gut Microbes*, *13*(1), 1–15.

Westerfield, M. (2007). *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*. University of Oregon.

Chapter 4

Zebrafish display cxcl8a-dependent selective thermal preference during inflammation

Adrià López Nadal, Fanny Beekman, Detmer Sipkema,
Geert F. Wiegertjes, Sylvia Brugman

Manuscript in preparation

Abstract

Zebrafish are ectotherms that rely on thermal sensing of environmental temperatures to regulate their inner temperature. In the context of bacterial and viral infections, it has been reported that fish swim to warmer waters to increase their body temperature, which increases their resistance to such infections, a process named behavioural fever. In the present study we aimed to investigate whether thermal preference also plays a role during inflammation. To this end, we adapted a live-tracking device to be able to observe and quantify zebrafish larvae locomotion and thermal preference upon saponin-induced inflammation. Inflamed zebrafish reduced their distance moved and velocity, and spent more time in the compartment with higher temperature compared to controls. Besides, saponin-exposed fish showed an increased number of neutrophils and macrophages in the gut area as well as an increased expression of pro-inflammatory cytokines (*il1b, mmp9, il10, cxcl8a, ccl38.5, tnfa* and *tnfb*). When saponin-exposed fish were co-treated with dexamethasone as an immunosuppressive glucocorticoid, thermal preference, neutrophil counts and expression of *cxcl8a* all reversed to control values. Finally, we engineered *cxcl8a-/-* fish by CRISPR-Cas9 technology and showed that naive knockout fish displayed reduced locomotion. Moreover, *cxcl8a-/-* fish did not swim to the higher temperature compartment upon saponin challenge, i.e. did not show thermal preference upon inflammation. The present work reveals for the first time a (zebra)fish-specific thermal preference behaviour upon inflammation rather than infection. Moreover, we showed that such behaviour is dependent on *cxcl8a* expression. Such findings may contribute to the understanding of the resolution of inflammatory events in fish and the contribution of cxcl8a expression during fever-like behaviour.

Introduction

Zebrafish became a consolidated model to study complex biological processes in vertebrates during the last decades (David Jonah Grunwald, 2002) due to its many advantages, such as well-annotated genome that eased the generation of mutant zebrafish lines by the CRISPR-Cas technique and the availability of several transgenic zebrafish (reviewed in (David Jonah Grunwald, 2002; Yoder *et al.*, 2002; Traver *et al.*, 2003; López Nadal *et al.*, 2020). Zebrafish are poikilotherms and ectotherms, their internal temperature varies considerably, being unable to regulate inner thermal homeostasis (Spence *et al.*, 2008). Zebrafish are native to South Asia and found in ponds and slow-flowing water streams with temperatures ranging from 10°C to 40°C (Engeszer *et al.*, 2007; Arunachalam *et al.*, 2013). While naturally-occurring environmental temperatures can vary tremendously, there was a collective effort from the scientific community to establish standard conditions for laboratory-reared zebrafish. As a matter of fact, research facilities kept zebrafish husbandry under stable rearing temperatures for more than 100 generations (Aleström *et al.*, 2020).

Already in 1976, it was described that to limit pathogen infection fish swim to warmer waters to increase their body temperature and to boost the immune system, a process named behavioural fever (Reynolds, Casterlin and Covert, 1976). In a similar manner, after dsRNA-induced behavioural fever the expression of several genes associated to anti-viral functions increased in the brain of zebrafish and ultimately increased fish survival (Boltaña *et al.*, 2013). A more recent study showed that viruses may co-evolve with its host to modulate behavioural fever in their favour. For example, herpesvirus led to an altered common carp thermal preference via expression of a decoy TNF-α receptor that delayed behavioural fever in fish, promoting virus replication and survival. Interestingly, TNF-α appeared to be crucial for the onset of behavioural fever and acted as a pyrogenic cytokine (Rakus *et al.*, 2017). Inflammation appears often as a response to the infectious agent that causes the behavioural fever and such inflammation is characterized at an early stage by release of mediators (such as pro-inflammatory cytokines and chemokines) by tissue resident immune and non-immune cells to increase blood flow, vasodilation and vascular permeability promoting swelling. Then, innate immune cells such as neutrophils (Henry *et al.*, 2013) and macrophages (Gray *et al.*, 2011) are recruited by chemoattractant cytokines and promote resolution of the inflammatory processes (as reviewed in Campos-Sánchez and Esteban, 2021). However, to the best of our knowledge, whether an infectious agent (virus or bacterium) is necessary for the onset of behavioural fever or whether fish also experiment similar changes in thermal preference merely upon inflammation it is an unexplored matter.

Soy saponin it is an amphipathic anti-nutritional compound within soybean meal (SBM) that interacts with cell membranes promoting pore formation, vesiculation and

membrane domain disruption. We used soy saponin in solution to promote an inflammatory state in zebrafish larvae characterized by an increase in neutrophil presence in the gut area as well as increased expression of pro-inflammatory cytokines, such as *il1b, cxcl8a, tnfa* and *mmp9* (López Nadal *et al.*, 2018). However, a fish behaviour associated to non-infectious inflammation has not been reported so far in fish. By using a novel prototype of an infra-red live-tracking camera visualizing plates with temperature gradients by controlled by two separate temperature control units we were able to study the locomotion parameters and the thermal preference of zebrafish larvae upon non-infectious inflammation. Saponin-inflammation was characterized by quantification of the expression of several pro-an anti-inflammatory cytokines together with *in vivo* imaging of transgenic larvae and the quantification of neutrophils and macrophages. Finally, we engineered Cxcl8a knock-out zebrafish by Crisp-Cas9 technology to study the role of this chemoattractant cytokine on thermal preference upon saponin-induced inflammation.

Materials and methods

Ethics Statement

The present study was approved by the Dutch Committee on Animal Welfare (2017.W-0034.014) and the Animal Welfare Body (IvD) of the Wageningen University (Netherlands). Furthermore, we adhere to the standard biosecurity and institutional safety procedures at Wageningen University and Research (The Netherlands).

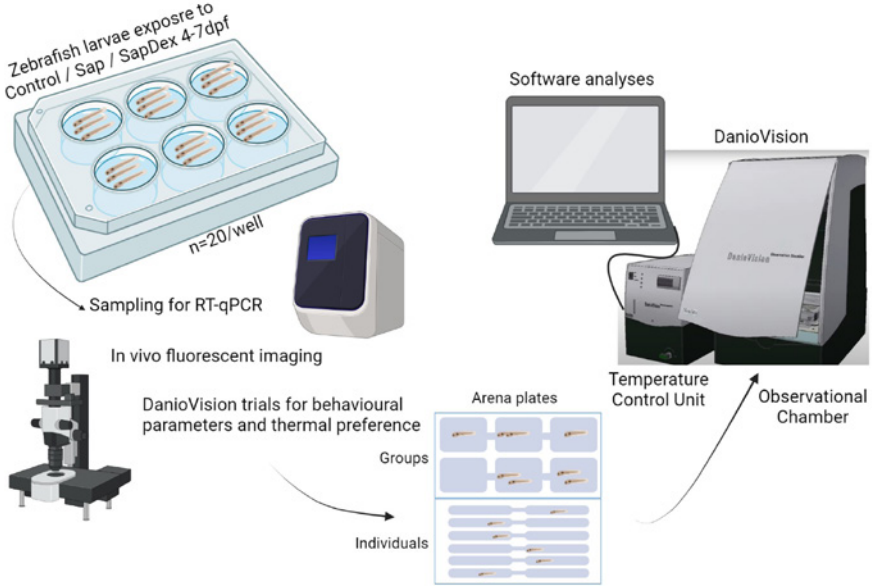
Animals

Adult Tg (mpeg1:mCherry / mpx:eGFPi[114]) and wildtype (AB) zebrafish were housed and fed as previously described (López Nadal *et al.*, 2018). Embryos were obtained by natural spawning and raised with E2 media (0.10 mM NaCl in demineralized water, pH 7.3) in petri dishes at 28°C (12/12-hour light/dark cycle) (Westerfield, 2007). From 1 to 3 days post-fertilization (dpf), the embryos were checked and infected embryos were discarded using a MS-222 (tricaine mesylate: tricaine/E3 (8.4% v/v 24 mM tricaine)) (Sigma-Aldrich, DL, United States of America) solution.

Experimental design

Larvae were randomly distributed in 6 well plates (n = 20 fish/well) and exposed to either control, saponin [0.5 mg/ml] or saponin combined with dexamethasone [0.5 mg/ml SAP + 1.1μM DEX] 10 ml solution/well from 4–7 dpf]. Ultrapure Soy Saponin 95%, was kindly provided by Trond Kortner NMBU Oslo Norway, origin: Organic Technologies, Coshocton, OH (Krogdahl *et al.*, 2015). Dexamethasone was commercially bought (Merk Life Science

NV, Amsterdam, The Netherlands). After the treatments read-out parameters described below were quantified. The techniques used and the experimental design are summarized in the **Graphical Abstract:**



DanioVision and EthoVision – Setup and parameters (data generation and analysis)

Fish behaviour, locomotion and thermal preference were recorded by using the adapted DanioVision™ apparatus (Noldus, Wageningen, The Netherlands). The device consists of an observational chamber (OC) (**Figure 1**) with an infrared-sensitive camera and two separate temperature control units (TCUs). The infrared camera tracked the zebrafish larvae during the trials. The TCUs heated or cooled water in two independent recirculating systems to the desired temperature and send the flowing water to the base of the click-in custom-made experimental plate within the OC. Water passing-through underneath the plates heated or cooled the E2 media in the arenas of the experimental plates. Temperature was also measured in the E2 media at the left and right extremes of the experimental plates to assure that the desired temperatures were reached. DanioVision™ was connected to a computer with EthoVision® XT software (version 14.2, Noldus, Wageningen, Netherlands). The software recorded the movement and location of zebrafish larvae during trials and produced the raw data for several readouts, including distance moved (cm), velocity (cm/s) and cumulative duration (s) in pre-delimited zones within the arenas of experimentation. EthoVision® generated high-quality images of the track visualization

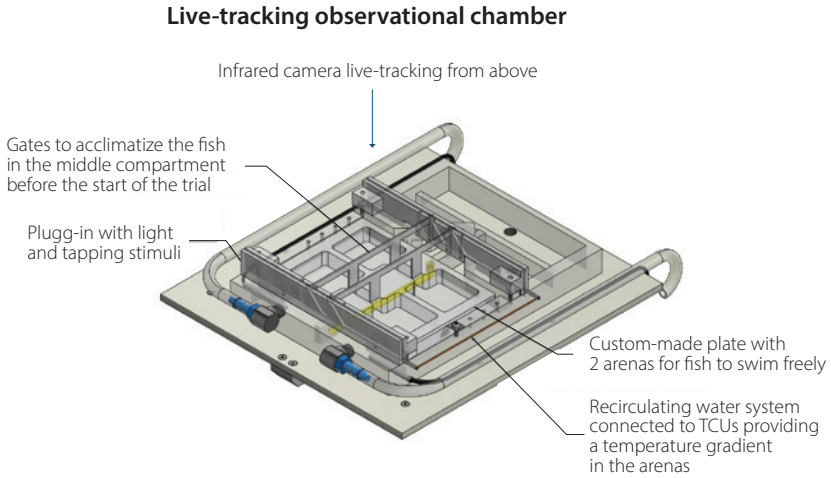


Figure 1: Observational chamber within the live-tracking device to study zebrafish larvae locomotion and thermal preference.

of the swimming route and heatmaps of the location of the zebrafish larvae using a colour scale from blue (less time spent) to red (more time spent). Custom-made plates of 6 independent arenas were used to examine individual fish swimming behaviour and 2 arena plates were used to study fish group swimming behaviours (n=4 fish per arena). Prior to the start of the trials, plates were filled with E2 media and clicked-in the set-up in the OC, temperature was set accordingly, gates were placed manually in the corridors of the zones and fish were placed in the middle zone to allow fish acclimatization to the new environment for 10 minutes (**Supplementary Figure 1)**. Right before the start of the recording, there was a tapping stimulus (intensity level 4 out of 8) and a white light stimulus (15% intensity) to evoke movement of the fish (needed for initial fish detection).

Gene expression

In order to assess changes in gene expression, total RNA was isolated from pools of larvae (n = 5/pool) with the RNeasy® Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration and quality (260/280 ratio values between 1.9–2.0 and 260/230 ratio values between 2.0–2.2) were assessed by using the NanoDrop 1000 Spectrophotometer (ThermoFisher Scientific, MA, United States). cDNA was synthetized including a DNase treatment [DNase I (1 U/µl)], followed by synthesis using Superscript™ III First Strand Synthesis Systems (Invitrogen, CA, United States). Finally, Real-Time quantitative Polymerase Chain Reaction (RT-qPCR) was performed with the use of ABsolute™ qPCR SYBR® Green Mix (ThermoFisher Scientific, MA, United States) and data

was normalized as previously described in (López Nadal *et al.*, 2018). All primers used for the RT-qPCR were selected to characterize the inflammation and are listed in **Table 1**.

Table 1 Forwards (FW) and reversed (RV) primers employed for the real-time quantitative PCR (RT-qPCR).

Gene name	FW primer (5'-3')	RV primer (5'-3')
elf1a	CTGGAGGCCAGCTCAAACAT	ATCAAGAAGAGTAGTAGTACCG
il1b	TGCGGGCAATATGAAGTCA	TTCGCCATGAGCATGTCC
mmp9	ACGGCATTGCTGACAT	TAGCGGGTTTGAATGG
il10	AGGGCTTTCCTTTAAGACTG	ATATCCCGCTTGAGTTCC
cxcl8a	TGTTTTCCTGGCATTTCTGACC	TTTACAGTGTGGGCTTGGAGGG
ccl38.5	GTCTGGTGCTCTTCGCTTTC	TGCAGAGAAGATGCGTCGTA
tnfa	AACAAGATGGAAGTGTGCTGAGA	GGTCCTGGTCATCTCTCCAGT
tnfb	AATCACCACACCTTCAGCTTCC	ACACCGCCAACCCATTTCA

Fluorescent *in vivo* imaging

Tg (mpeg1:mCherry / mpx:eGFPi[114]) larvae were anaesthetized in a buffered solution containing tricaine (4.2 ml of 24 mM Tricaine (Sigma-Aldrich, DL, United States) in 100 ml E2) , embedded in 1% low-melting point agarose (ThermoFisher Scientific, MA, United States) and *in vivo* imaged with Leica M205 FA Fluorescence Stereo Microscope (Leica, Amsterdam, The Netherlands) as previously described in (López Nadal *et al.*, 2018). Pictures were analyzed with ImageJ® software (United States National Institutes of Health, Bethesda, United States): fluorescent imaged pixels were converted to black and white and quantified as individual cell counts as well as a percentage of the area of the cell of interest compared to the imaged zebrafish larvae. Boxplots were generated using Prism v.9.0.0 (GraphPad Software, San Diego, California USA).

CRISPR-Cas9 *cxcl8a* knock-out fish

To generate cxcl8a mutant fish, Cispr-cas9 mediated gene-editing was performed using a guide RNA targeting the first exon of zebrafish *cxcl8a* (ENSDARG00000104795; GRCz11) sgRNA: AGGAATGAGCTTGAGAGGTC. Full sequence of ordered oligo [CCGCTAGC-TAATCGACTCACTATA-AGGAATGAGCTTGAGAGGTC–GTTTTAGAGCTAGAA ATAGCAAG] and common reverse primer [AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTT ATTTTAACTTGCTATTTCTAGCTCTAAAAC]. One cell stage embryos were injected with 1 nl containing 150 ng/ul sgRNA, 2 ng/ul cas9 enzyme (IDT AltR s.p. Cas9 Nuclease V3 #1081058), and 0.3 ul phenol red. Analysis of the offspring of these F0 yielded heterozygous fish with different mutations in the germline. Cxcl8a+/- F1 offspring (n=5 with different mutations)

were selected and outcrossed to AB wildtype fish to yield F2 cxcl8a+/- zebrafish. The founder and offspring with a germline transmission of a mutation in *cxcl8a* leading to a premature stop was selected.

Statistics

Data was checked for normal distribution by a Shapiro-Wilk test. Un-paired t-test was used when comparing two treatments with normally distributed data. Man-Whitney t-test was applied when comparing two treatments with non-normally distributed data. For datasets containing more than two treatments with normally distributed data one-way ANOVA test with Tukey's multiple comparison post-hoc test was applied. In case of non-normally distributed data, one-way ANOVA Kruskal-Wallis test with Dunn's multiple comparison post-hoc test was applied.

Results

Zebrafish larvae present exploratory behaviour and prefer lower temperatures than 28.5°C

Wild-type (WT) and Tg (mpeg1:mCherry / mpx:eGFPi^{114}) zebrafish larvae presented an exploratory behavior at 28.5°C (**Figure 2A**). Fish explored the borders of the arena plates where they spent slightly more time, as showed in the heatmap (**Figure 2B**). Fish did not show any special preference when comparing the time spent in the three different compartments (left, right middle) and swam freely in the 2 arenas custom-made plate for the duration of the experiment (1.5 hours) (**Figure 2C**). Fish presented similar locomotion parameters throughout the length of light period time we employed for our experiments (9-17h) (**Supplementary Figure 2**) and no significant differences in distance moved or velocity were found, indicating that the timing of the experiments did not contribute to the measurements obtained in the trials.

Next, we investigated the preferred temperature of 5 dpf WT and Tg (mpeg1:mCherry / mpx:eGFPi^{114}) zebrafish larvae in a temperature range from 24°C to 34°C. Both WT and Tg fish swam around all arenas in the custom-made plate (as seen in the track visualization in **Figure 3A**) showing an exploratory behaviour. All fish, WT and Tg, individually and in group spent more time in the coldest water at 24°C (left compartment) compared to the 29°C (middle) and 34°C (right) compartment (**Figure 3B** and **Figure 3C**).

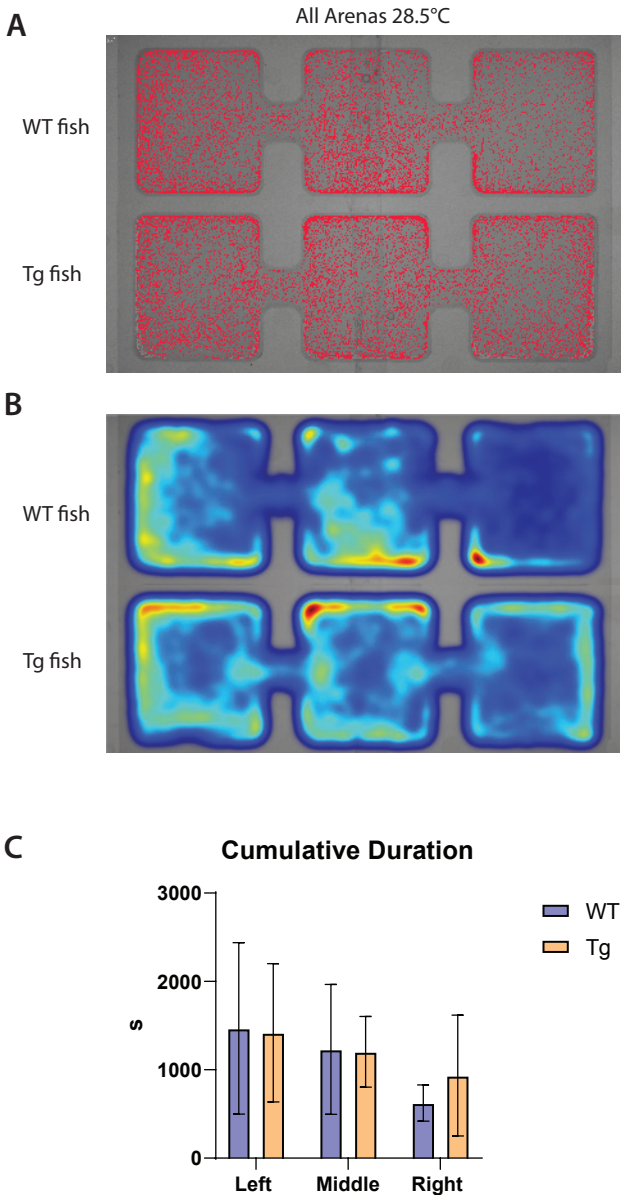


Figure 2: **(A)** Track visualization of 5 dpf fish (n=4 per arena; arena up: WT fish, arena down: Tg fish; trial 1.5h). **(B)** Heatmap of 5dpf fish location (n=4 per arena; arena up: WT fish, arena down: Tg fish; trial 1.5h), color scale blue (less time spent) to red (more time spent). **(C)** Cumulative duration (in seconds) of 5 dpf fish WT and Tg (mpx:GFPi114;mpeg1:mCherry) in the left, middle and right compartment, no significant differences were found.

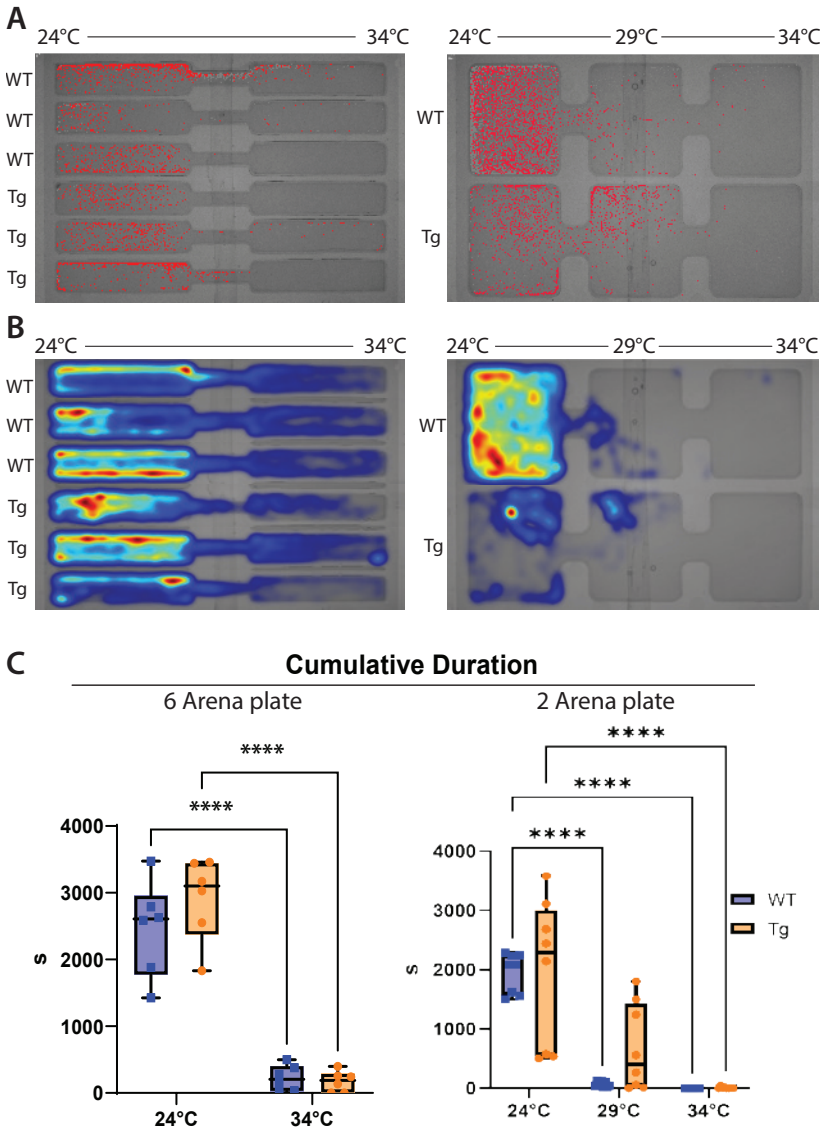


Figure 3: **(A)** Left: Fish track visualization (n=6, 1 per arena; 3 arenas top: WT fish, 3 arenas bottom: Tg fish; trial 1h). Right: Fish track visualization (n=4 per arena; arena top: WT fish, arena bottom: Tg fish; trial 1h). **(B)** Left: Fish location heatmap (n=6, 1 per arena; 3 arenas top: WT fish, 3 arenas bottom: Tg fish; trial 1h). Right: Fish location heatmap (n=4 per arena; arena top: WT fish, arena bottom: Tg fish; 10 minutes acclimatization + trial 1.5h), color scale blue (less time spent) to red (more time spent). **(C)** Left: Individual fish cumulative duration (seconds) of WT and Tg fish at the 24°C zone and the 34°C zone. Right: Group cumulative duration (seconds) of WT and Tg fish at the 24°C zone, the 29°C zone and the 34°C zone. 2 Way ANOVA with Tukey's multiple comparison test, ****p<0.0001.

Saponin reduces locomotion parameters and increases fish preference for warmer waters

In our previous work, it was shown that exposure to 0.5 mg/ml saponin (3-6 dpf/6-9 dpf) leads to a mild inflammatory response (Lopez Nadal *et al.*, 2018). Here, it is shown that Tg fish exposed to 0.5mg/ml saponin from 4-7dpf showed a decreased distance moved and decreased velocity compared to control fish (**Figure 4A**). Although saponin-exposed fish displayed reduced locomotion, they explored all the zones within the arenas as seen from the coloured track lines observed in the track visualisation (**Figure 4A**). Saponin-exposed fish spent most time in intermediate zone (29°C) compared to control fish that spent most of the time in the coldest zone (24°C) (**Figure 4B**, heatmap and % cumulative duration).

Inflammation increases cytokines expression, which is partially reversed by dexamethasone

To characterize saponin-induced inflammation pro- and anti-inflammatory cytokine expression was quantified. First, we show that saponin-exposed fish presented significantly higher expression of *il1b, mmp9, il10, cxcl8a, ccl38 (ccl38.5), tnfa* and *tnfb* compared to control fish (**Figure 5,** white boxes). Then we co-exposed the zebrafish larvae to saponin and dexamethasone: a commonly used immuno-suppressive drug that binds to glucocorticoid receptors and alters the expression of target genes (Schaaf and Cidlowski, 2003). Dexamethasone is used in humans, mice and zebrafish (Tsurufuji, Sugio and Takemasa, 1979; Abraham *et al.*, 2006; Yang *et al.*, 2014; Cholan *et al.*, 2020)). After saponin and dexamethasone co-exposure gene expression of of *il1b, il10, ccl38 (ccl38.5), tnfa* and *tnfb* tended to decrease compared to saponin-only exposed fish, however not significantly (**Figure 5,** grey boxes). Noticeably, dexamethasone only reduced the expression of *cxcl8a* significantly compared to saponin-only exposed fish (back to control levels).

Inflammation increases neutrophil presence and dexamethasone reverses it to control levels

As *cxcl8a* is a very potent chemoattractant, macrophages and neutrophils were imaged *in vivo* and quantified as area percentage of the total fish as well as individual cell counts. Saponin-exposed fish presented higher fluorescent area as well as counts for neutrophils and macrophages compared to control fish (**Figure 6A, 6B** white boxes). After saponin and dexamethasone co-exposure the levels of fluorescent area for neutrophils decreased significantly compared to saponin-only exposed fish and were comparable to control fish, whereas no differences were found for the macrophages when compared to controls or saponin-exposed fish (**Figure 6B** grey boxes).

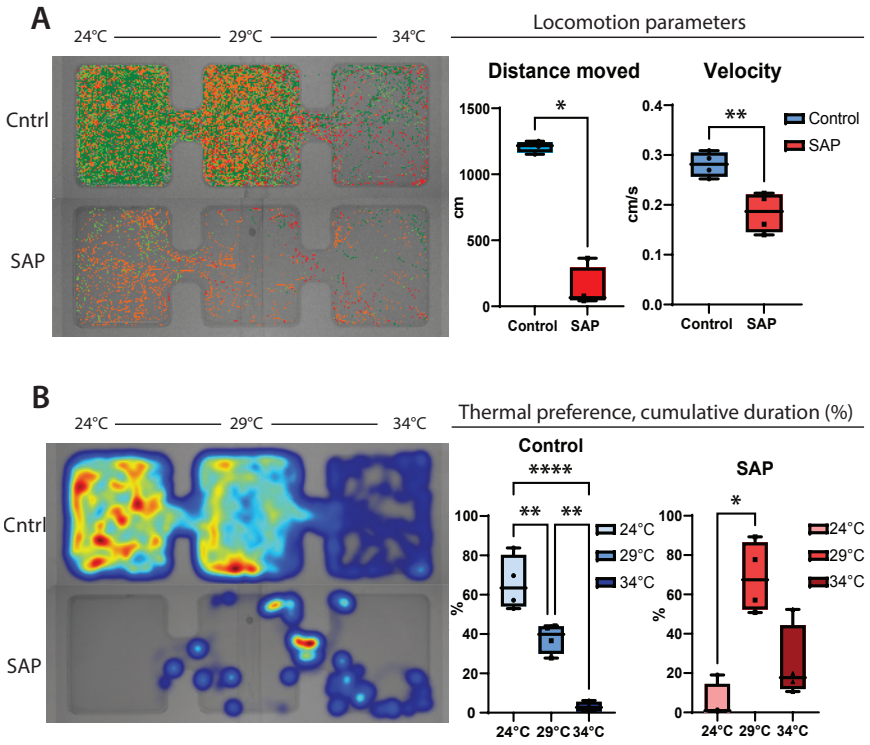


Figure 4: **(A)** Left: Fish track visualization (n=4 per arena; top: Tg control fish, bottom: Tg saponin-exposed fish). Right: Locomotion parameters; distance moved in centimeters and velocity in centimeters/seconds. **(B)** Left: Fish location heatmap (n=4 per arena; top: Tg control fish, bottom: Tg saponin-exposed fish) color scale blue (less time spent) to red (more time spent). Right: Individual fish cumulative duration (%) the 24°C zone, 29°C and the 34°C zone. 10 minutes acclimatization + 2h trial. Man-Whitney t-test for distance moved, un-paired t-test for velocity, one way ANOVA test with Tukey's multiple comparison post-hoc test. *p<0.05, **p<0.005, ****p<0.0001.

Dexamethasone reverses zebrafish thermal preference upon inflammation

After assessing the specific anti-inflammatory effects of dexamethasone (reduced *cxcl8a* expression and neutrophil presence) we studied whether there were any differences in fish locomotion and thermal preference. Since 34°C was not informative in our previous experiments (fish did not spent time in water of that temperature) (**Figure 3, 4**) and we kept the temperature in the middle compartment around 28.5°C (as a standard reference) the temperature range was narrowed down: 27°C (left) to 28.5°C (middle) and 30°C (right). Again, saponin-exposed fish (4-7dpf; 0.5mg/ml) moved less and slower (lower velocity)

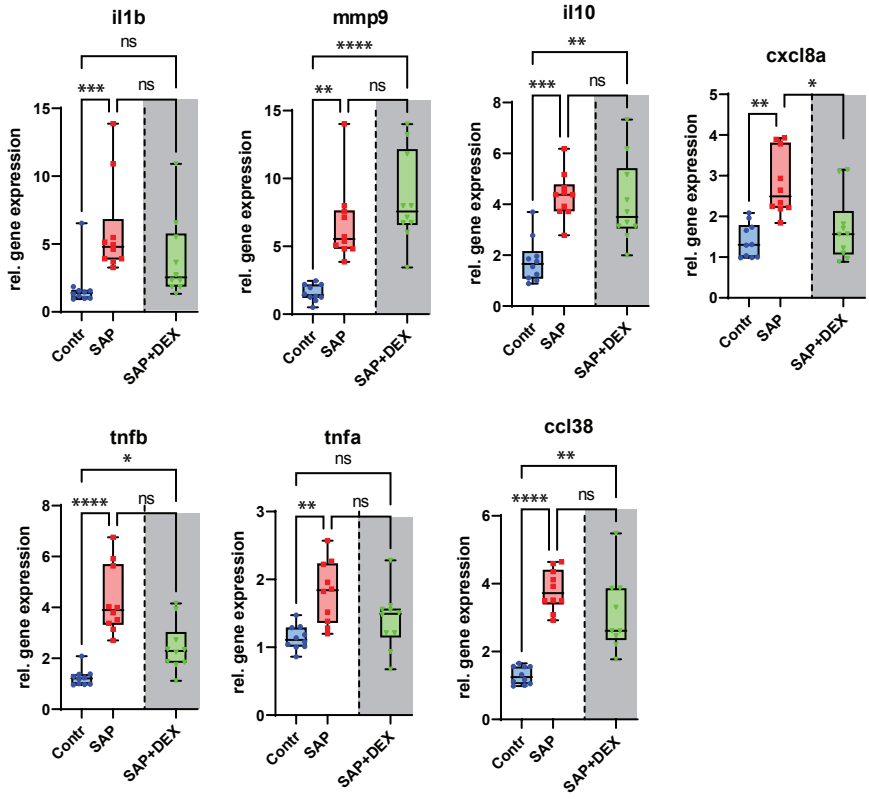


Figure 5: Relative gene expression of control fish (7dpf), fish exposed to saponin (0.5mg/ml, 4-7dpf) and fish co-exposed to saponin + dexamethasone (0.5mg/ml saponin + 1.1μM dexamethasone, 4-7dpf) of the following genes corelated with inflammation: il1b, mmp9, il10, cxcl8a, ccl38 (ccl38.5), tnfa and tnfb. One way ANOVA test with Tukey's multiple comparison post-hoc test. *p<0.05, **p<0.005, ***p<0.001, ****p<0.0001.

compared to controls (**Figure 7A, 7B**). When we co-exposed fish to saponin and dexamethasone locomotion remained similar to saponin-only exposed fish (**Figure 7A, 7B**). Fish exposed to saponin spent more time in warmer waters (28.5°C) compared to controls that spent most of their time in the coldest zone (27°C), heatmaps and cumulative duration (%) of the trial time (**Figure 7C, 7D**). Fish co-exposed to saponin and dexamethasone reversed their thermal preference back to control fish values, preferring again colder waters (27°C) compared to saponin-only exposed fish that preferred 28.5°C (**Figure 7C, 7D**).

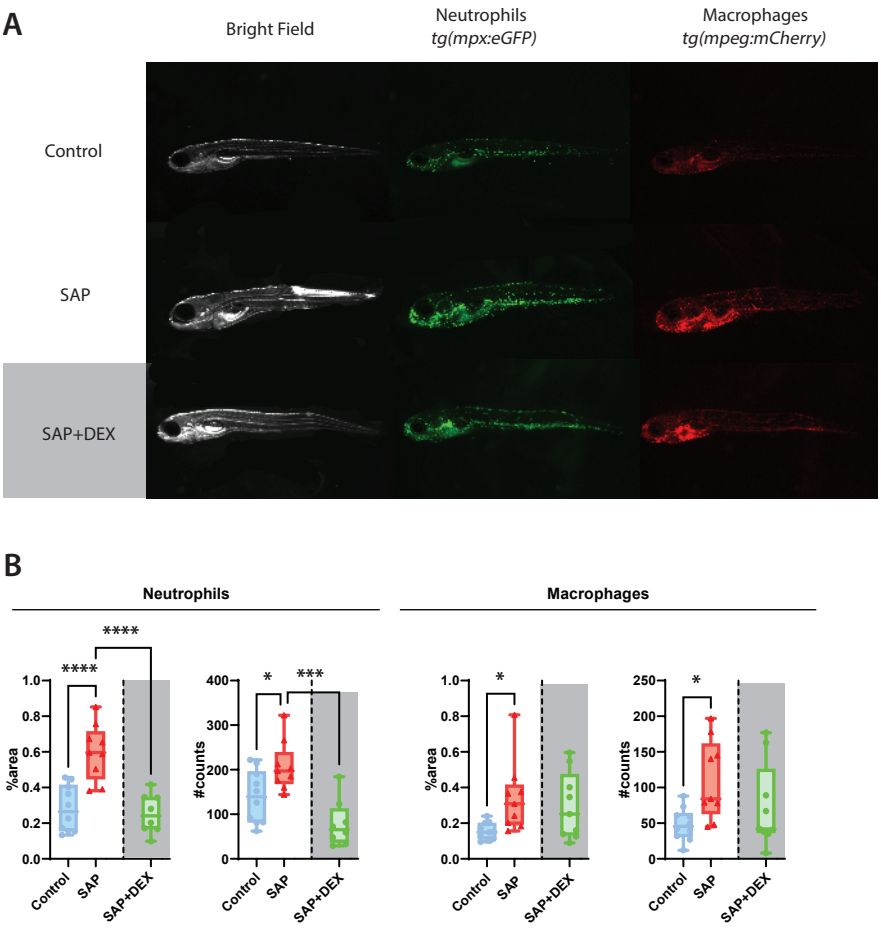


Figure 6: **(A)** Representative pictures of 7 dpf Tg (mpeg1:mCherry / mpx:eGFPi[114]) control fish (7dpf), fish exposed to saponin (0.5mg/ml, 4-7dpf) and fish co-exposed to saponin + dexamethasone (0.5mg/ml saponin + 1.1µM dexamethasone, 4-7dpf) in the bright field, green channel and red channel. **(B)** Quantification of the percentage of area corresponding to neutrophils and macrophages from the overall fish area by suing ImageJ (n=8-10 fish per group). One way ANOVA test with Tukey's multiple comparison post-hoc test. *p<0.05, ***p<0.001, ****p<0.0001.

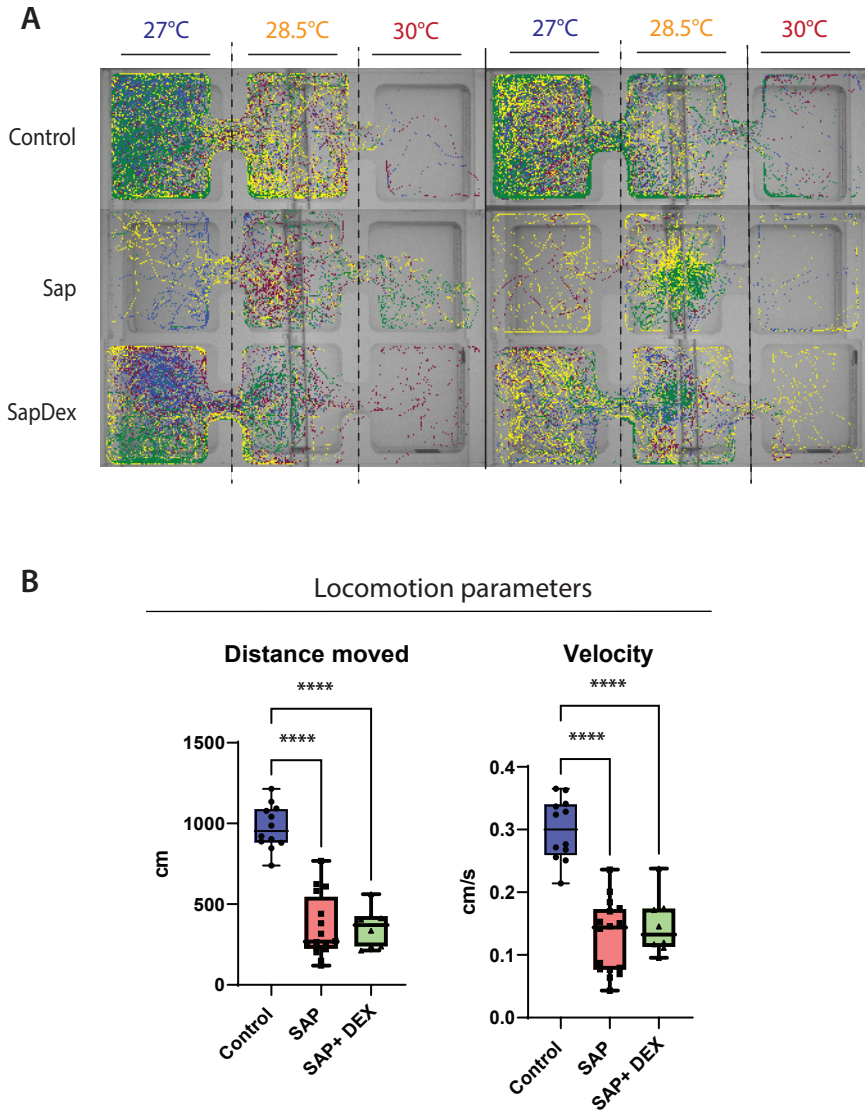


Figure 7: **(A)** Fish track visualization (n=4 per arena; top: Tg control fish, middle: Tg saponin-exposed fish, bottom: saponin and dexamethasone-exposed fish). **(B)** Locomotion parameters; distance moved in centimeters and velocity in centimeters/seconds. **(C)** Fish location heatmap (n=4 per arena; top: Tg control fish, middle: Tg saponin-exposed fish, bottom: saponin and dexamethasone-exposed fish) color scale blue (less time spent) to red (more time spent). **(D)** Individual fish cumulative duration (%) the 27°C zone, 28.5°C and the 30°C zone. 10 minutes acclimatization + 1h trial. 3 trials combined. One way ANOVA test with Tukey's multiple comparison post-hoc test. *p<0.05, ***p<0.001, ****p<0.0001.

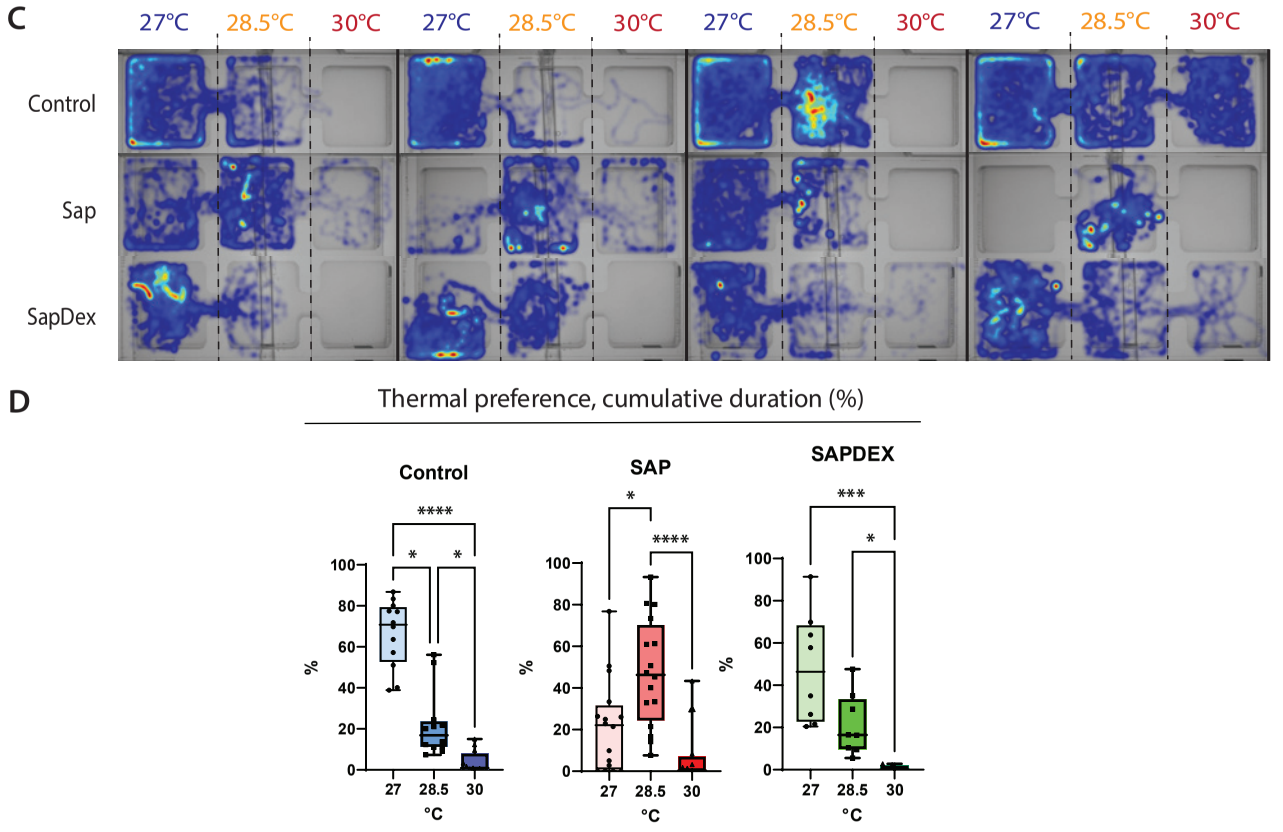


Figure 7: Continued.

Cxcl8a expression is needed for fish preference for warm waters upon inflammation

Since dexamethasone reverted saponin-associated fish thermal preference, *cxcl8a* expression and neutrophil presence to control levels, we hypothesised that c*xcl8a* could play a role in thermal preference in zebrafish larvae. To explore the implications of *cxcl8a* expression in the thermal preference behaviour upon inflammation we engineered a *cxcl8a* knockout (KO) zebrafish. The founder displayed a 141 bp deletion in DNA (29 bp deletion in exon 1 and 2 in mRNA) of *cxcl8a* (**Supplementary Figure 3**).

Unchallenged c*xcl8a-/-* fish moved much less than unchallenged *cxcl8a+/+* siblings (distance moved **Figure 7B, 8B**). *Cxcl8a-/-* saponin-exposed fish (4-7dpf; 0.5mg/ml) moved in a similar fashion as *cxcl8a-/-* unchallenged fish, suggesting that the absence of *cxcl8a* was enough to impair fish locomotion (**Figure 8A, 8B**). However, saponin-exposed *cxcl8a-/-* showed increased velocity compared to control *cxcl8a-/-* fish, indicating shorter but faster movements than unchallenged *cxcl8a-/-* (**Figure 8B**). Unchallenged *cxcl8a-/-* fish showed a clear preference for 28.5°C water (**Figure 8C, 8D**) while unchallenged *cxcl8a+/+* siblings preferred 27°C water (**Figure 7C**, **7D**). *Cxcl8a-/-* saponin-exposed fish did not present an increased preference for warmer waters compared to unchallenged *cxcl8a-/-* fish (**Figure 8C, 8D**) as observed previously in wildtype (**Figure 7C, 7D**). These data indicate *cxcl8a* expression influences thermal preference upon induction of saponin-induced inflammation.

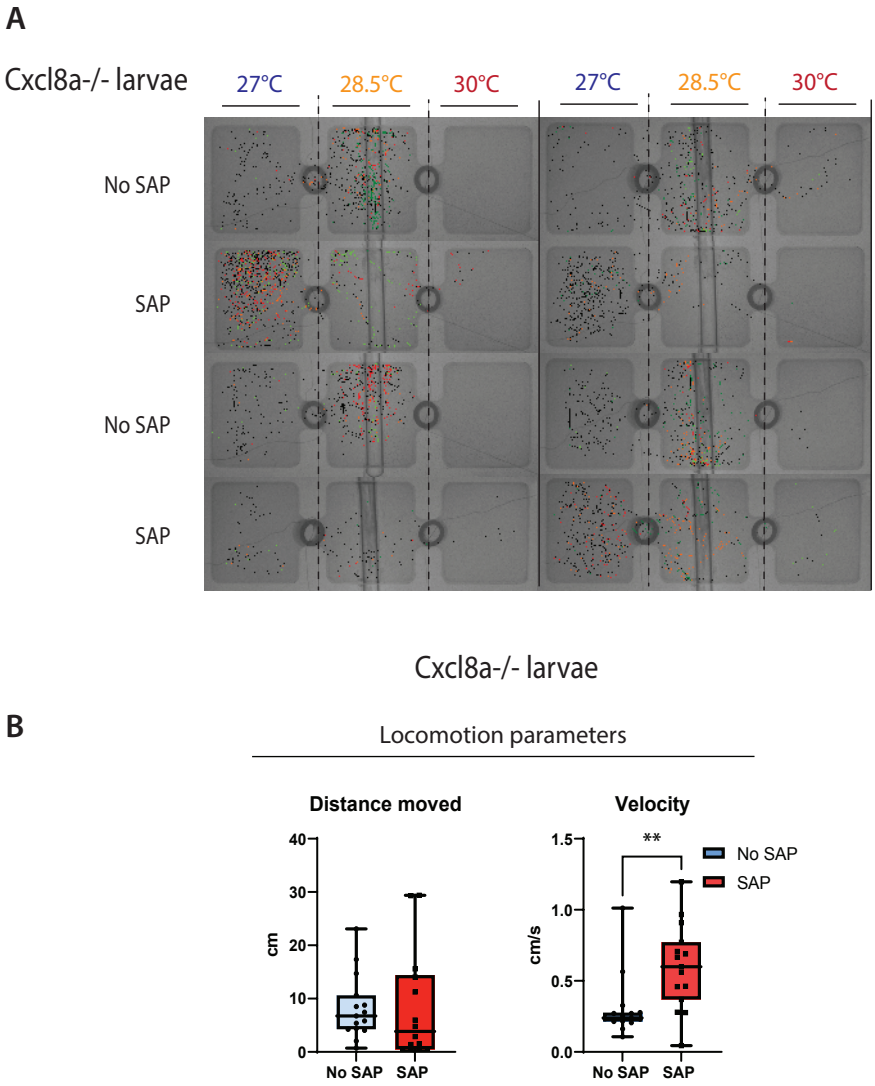


Figure 8: **(A)** Fish track visualization (n=4 per arena; up: cxcl8a-/- control fish, down: cxcl8a-/- saponin-exposed fish). **(B)** Locomotion parameters; distance moved in centimeters and velocity in centimeters/seconds. **(C)** Fish location heatmap (n=4 per arena; top: cxcl8a-/- control fish, bottom: cxcl8a-/- saponin-exposed fish) color scale blue (less time spent) to red (more time spent). **(D)** Individual fish cumulative duration (%) the 27°C zone, 28.5°C and the 30°C zone. 10 minutes acclimatization + 1h trial. 3 trials combined. One way ANOVA test with Tukey's multiple comparison post-hoc test. *p<0.05, ***p<0.001, ****p<0.0001.

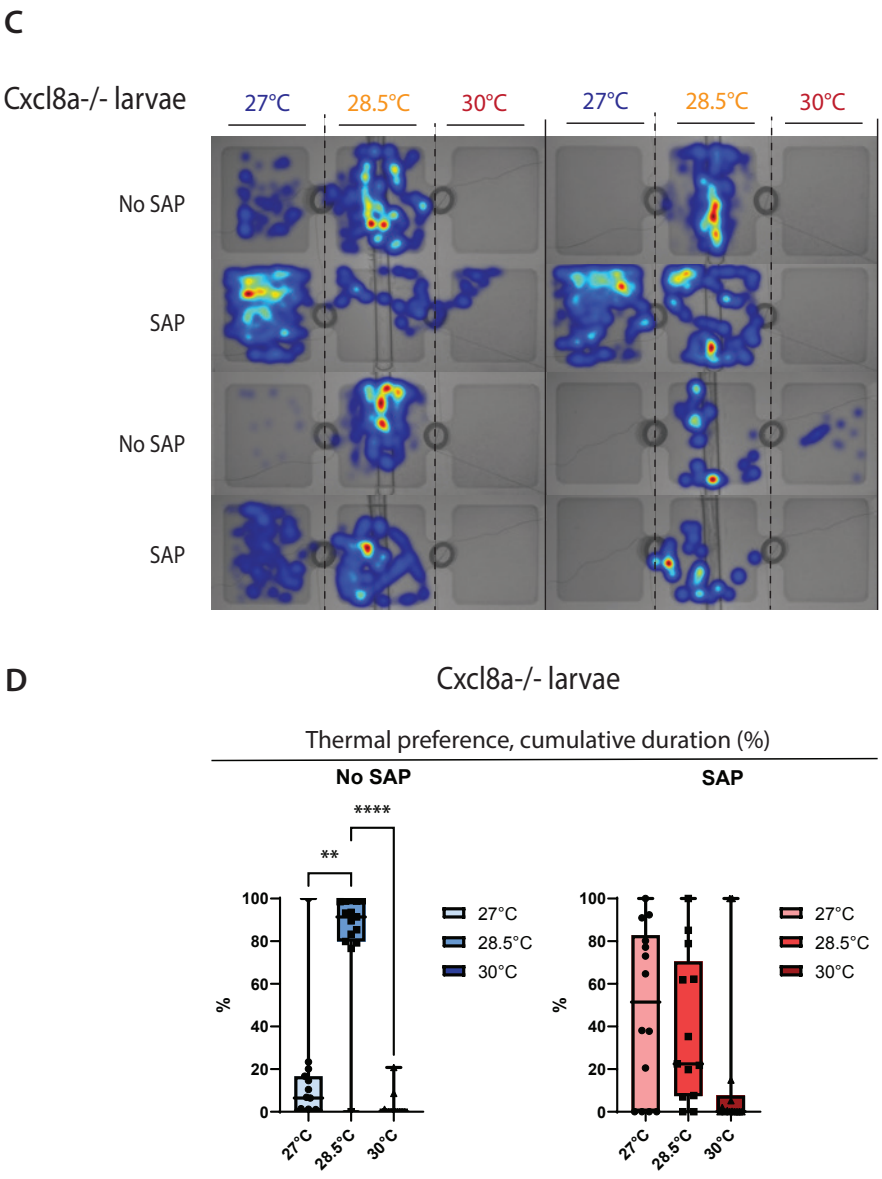


Figure 8: Continued.

Discussion

In the present study we investigated whether zebrafish larvae display thermal preference for warm waters upon saponin-induced inflammation, or whether fever-like behaviour is only seen during infections. Here, we show that saponin-exposed zebrafish spent more time in warmer waters compared to controls, which coincided with increased pro-inflammatory gene expression and neutrophil and macrophage presence in the intestinal area. When saponin-treated fish were co-treated with dexamethasone (glucocorticoid with anti-inflammatory properties) macrophage presence as well as the expression of several pro-inflammatory genes (*il1b, mmp9, il10, tnfb, tnfa* and *ccl38.5*) was not significantly affected compared to saponin-only exposed fish. Interestingly, dexamethasone did reverse the increased number of neutrophils as well as the expression of *cxcl8a* of the saponin-exposed fish to control levels. Lastly, engineered *cxlc8a-/-* fish exposed to saponin do not display selective thermal preference, implicating *cxcl8a* and associated neutrophil recruitment in the thermal preference response during saponin-induced inflammation in zebrafish larvae.

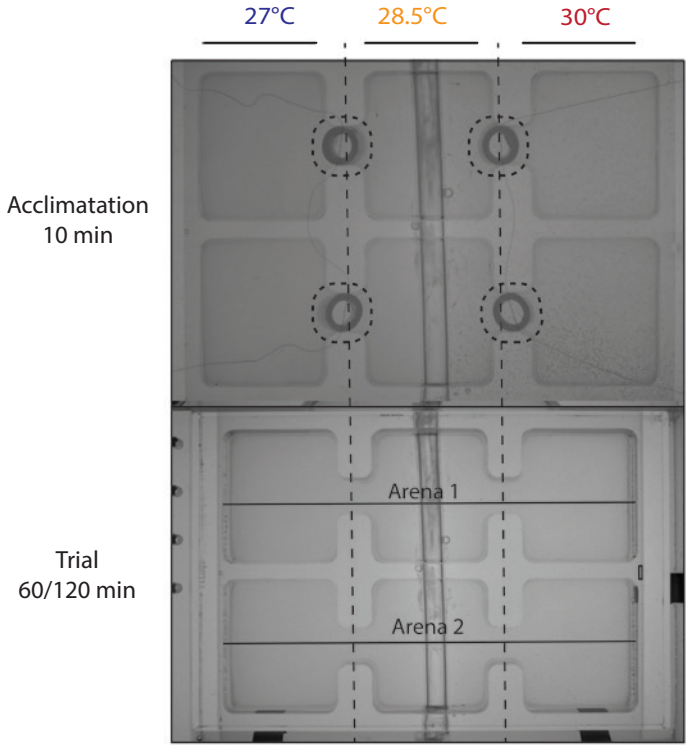
In the present study, we first characterized the locomotion parameters (distance moved and velocity) and thermal preference of zebrafish larvae and showed that zebrafish larvae (WT and Tg) preferred lower temperatures (24°C) than the previously established 28.5°C (Kimmel *et al.*, 1995; Schaefer and Ryan, 2006). Such findings were consistent when analysing individual and group fish behaviour (**Figure 3**). Since it is believed that the preferred fish temperature optimizes their growth and physiological processes (Schaefer and Ryan, 2006) more research is warranted to understand the optimal temperature for zebrafish rearing and first life stages under laboratory conditions. Our results do indicate that zebrafish larvae of 5dpf prefer slightly cooler rearing conditions than the actual golden standard temperatures (Ripley *et al.*, 2022).

Upon infection, endotherms increase their body temperature to promote vasodilation and increase cell recruitment to the site of infection, a process commonly known as fever (reviewed in (Evans, Repasky and Fisher, 2015). Ectotherms (among which fish) lack intrinsic thermogenesis and rely on environmental temperature sensing to select a species-specific thermal preferendum. Upon infection, (zebra)fish display behavioural fever by swimming towards warmer waters to have better chances to fight the infectious agent (Rakus, Ronsmans and Vanderplasschen, 2017; Rey *et al.*, 2017). However, it has not been previously reported whether fish present a fever-like behaviour upon saponin-induced inflammation. Our data showed that saponin-induced inflamed fish displayed reduced distance moved and velocity as well as altered thermal preference: fish spent more time in warmer waters than controls, mimicking a fever-like behaviour. To the best of our knowledge, this is the first observation of a fish-specific behaviour associated to

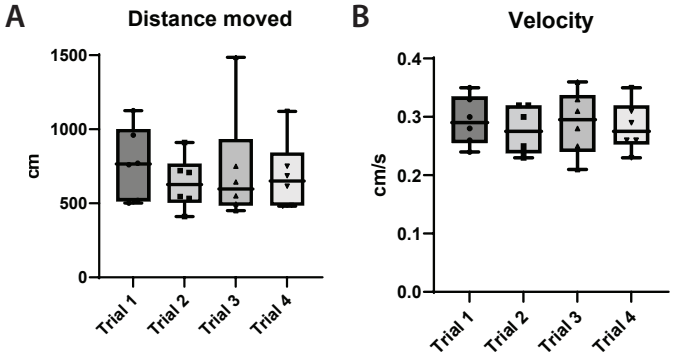
non-infectious inflammation. Most probably, inflamed fish spent more time in warm waters to increase physiological processes to resolve the inflammatory event (Campos-Sánchez and Esteban, 2021). When dexamethasone, an anti-inflammatory drug was used together with saponin, thermal preference was reversed together with neutrophil presence and *cxcl8a* expression to control levels. Interestingly, it has been reported that injection of CXCL-8 induced fever (measured as increase in body temperature) in rats (Zampronio *et al.*, 1994)and rabbits (Zampronio *et al.*, 1995)independently of the classical pathway of fever induction and maintenance after infection: the IL6-COX2-PGE2 fever axis. For canonical initiation of the fever response pathogen-associated molecular patterns (PAMPs) are recognized by Toll-like receptors (TLRs) and activate dendritic cells and macrophages that release prostaglandin E2 (PGE2) and pyrogenic cytokines (IL-1, IL-6 and TNF). IL-1 induces the synthesis of cyclooxygenase 2 (COX2), an enzyme that contributes to the additional production of PGE2 (considered the major pyrogenic mediator of fever) (reviewed in Evans, Repasky and Fisher, 2015). Research in mammals supported the observation that heat-induced non-canonical CXCL8 expression initiate neutrophil infiltration in the lungs (Rice *et al.*, 2005). Heat-induced neutrophil infiltration depends on the non-canonical chemotactic heat shock protein (HSP) and requires the expression of CXC-chemokine ligand 8 (CXCL8) which is controlled by the heat-inducible transcription factor heat shock factor protein 1 (HSF1) (Singh *et al.*, 2008; Tulapurkar *et al.*, 2012; also reviewed in Evans, Repasky and Fisher, 2015). In fish, it is well documented that neutrophils migrate and infiltrate the gut epithelium in response to *cxcl8a* expression which encodes a very potent chemoattractant cytokine (Oehlers *et al.*, 2010; de Oliveira *et al.*, 2013).

Our study provides evidence that the induction of (behavioural) fever by *cxcl8a* expression is a mechanism that may be conserved between endotherms and ectotherms: upon saponin-induced inflammation fish spent more time in warmer waters and *cxcl8a* expression increased together with neutrophil presence compared to controls. Furthermore, with the engineered *cxcl8a* knockout fish we showed that *cxcl8a* expression is necessary for the induction of a fever-like behaviour upon inflammation, although lack of cxcl8a also affected general swimming behaviour. Further characterisation of *cxlc8a-/-* mutants is needed to understand possible deleterious effects which may result in multiple downstream differentially expressed genes compared to WT fish. The implications of a conserved role of *cxcl8a* in a fever-like behaviour during inflammation in fish and mammals contributes to the global knowledge of the fever mechanisms across vertebrates.

Supplementary figures



Supplementary Figure 1: Set-up of the 2 arenas custom-made plate with the gates (dotted circles). During the acclimatization period, fish are kept in the middle zone with the gates that are removed at the start of the trial to allow fish swimming freely.



Supplementary Figure 2: **(A)** Distance moved (cm) and **(B)** velocity (cm/s) of 4 separate trials of 1.5h in the same day (Trial 1: 9h-10:30h; Trial 2: 11h-12:30h; Trial 3: 13h-14:30h; Trial 4: 15h-16:30h).

Crispr-Cas9 deletion in the *cxcl8a*

A

```
1                    WT CTTAGGCAAAATGACCAGCAAAATCATTTCAGTGTGTGTTATTGTTTTCCTGGCATTTCT    180
EF32692720_EF32692720 KO CTTAGGCAAAATGACCAGCAAAATCATTTCAGTGTGTGTTATTGTTTTCCTGGCATTTCT    180
                         ************************************************************

1                    WT GACCATCATTGAAGGTAAGTGAACATGCAAAACCATATACTGCTATAACTATCAGTAACC    240
EF32692720_EF32692720 KO GACCATCATTGAAGGTAA----------------------------------------    198
                         ******************

1                    WT ATAGATAGTTATGCATATATGTGGTAGAATGATATGAACTAATCCCTGTGATTTTTTGC    300
EF32692720_EF32692720 KO ------------------------------------------------------------    198

1                    WT TTTCAGGAATGAGCTTGAGAGGTCTGGCTGTAGATCCACGCTGTCGCTGCATTGAAACAG    360
EF32692720_EF32692720 KO ----------------------------------------GCTGTCGCTGCATTGAAACAG    219
                                                                   ********************

1                    WT AAAGCCGACGCATTGGAAAACACATAAAGAGTGTGGAGCTCTTCCCTCCAAGCCCACACT    420
EF32692720_EF32692720 KO AAAGCCGACGCATTGGAAAACACATAGAGAGTGTGGAGCTCTTCCCTCCAAGCCCACACT    279
                         ************************** **********************************
```

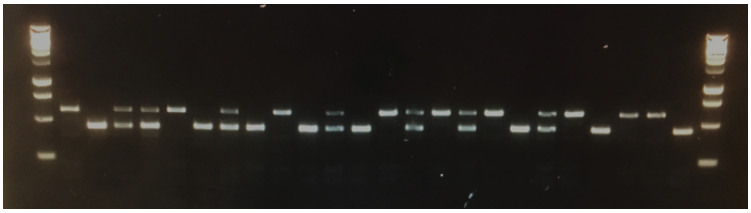
B

```
Protein cxcl8a          1 MTSKIISVCVIVFLAFLTIIEG-MSLRGLAVDPRCRCIETESRRIGKHIK     49
                            ||||||||||||||||||.. :||.
Protein mutated cxcl8a  1 MTSKIISVCVIVFLAFLTIIRSTLSLH----------------------     27


Protein cxcl8a          50 SVELFPPSPHCKDLEIIATLMTTGQEICLDPSAPWVKKIIDRIIVNRKP     98

Protein mutated cxcl8a  28 --------------------------------------------------     27
```

C



Supplementary Figure 3: CRISPR-Cas generated mutation of *cxcl8a*. **(A)** DNA sequence of a wild-type (WT) fish and a the founder mutated fish (KO). **(B)** Protein alignment of the WT *cxcl8a* compared to the mutation (deletion) in the *cxcl8a* (KO). **(C)** Agarose gel with the PCR product performed with 5'UTR FR primer (TAAGCGCTACGGCTTCAACA) and the mid reverse (GGAGATCT-GTCTGGACCCCT) for *cxcl8a*. 1 kb ladder. WT is top band 595 bp, KO band below 454 bp.

References

Abraham, S.M. *et al.* (2006) 'Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1', *The Journal of experimental medicine*, 203(8), pp. 1883–1889.

Aleström, P. *et al.* (2020) 'Zebrafish: Housing and husbandry recommendations.', *Laboratory animals*, 54(3), pp. 213–224. Available at: https://doi.org/10.1177/0023677219869037.

Arunachalam, M. *et al.* (2013) 'Natural history of zebrafish (Danio rerio) in India', *Zebrafish*, 10(1), pp. 1–14.

Boltaña, S. *et al.* (2013) 'Behavioural fever is a synergic signal amplifying the innate immune response', *Proceedings of the Royal Society B: Biological Sciences*, 280(1766). Available at: https://doi.org/10.1098/rspb.2013.1381.

Campos-Sánchez, J.C. and Esteban, M.Á. (2021) 'Review of inflammation in fish and value of the zebrafish model', *Journal of Fish Diseases*, 44(2), pp. 123–139.

Cholan, P.M. *et al.* (2020) 'Conserved anti-inflammatory effects and sensing of butyrate in zebrafish', *Gut microbes*, 12(1), p. 1824563.

David Jonah Grunwald, J.S.E. (2002) 'Hearwaters of the zebrafish - emergence of a new model vertebrate', *Nature Reviews Genetics*, 3, pp. 717–724.

Engeszer, R.E. *et al.* (2007) 'Zebrafish in the wild: a review of natural history and new notes from the field.', *Zebrafish*, 4(1), pp. 21–40. Available at: https://doi.org/10.1089/zeb.2006.9997.

Evans, S.S., Repasky, E.A. and Fisher, D.T. (2015) 'Fever and the thermal regulation of immunity: The immune system feels the heat', *Nature Reviews Immunology* [Preprint]. Available at: https://doi.org/10.1038/nri3843.

Gray, C. *et al.* (2011) 'Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish', *Thrombosis and haemostasis*, 105(05), pp. 811–819.

Henry, K.M. *et al.* (2013) 'Zebrafish as a model for the study of neutrophil biology', *Journal of leukocyte biology*, 94(4), pp. 633–642.

Kimmel, C.B. *et al.* (1995) 'Stages of embryonic development of the zebrafish', *Developmental dynamics*, 203(3), pp. 253–310.

Krogdahl, Å. *et al.* (2015) 'Soya Saponins Induce Enteritis in Atlantic Salmon (Salmo salar L.)', *Journal of Agricultural and Food Chemistry*, 63(15), pp. 3887–3902. Available at: https://doi.org/10.1021/jf506242t.

López Nadal, A. *et al.* (2018) 'Exposure to Antibiotics Affects Saponin Immersion-Induced Immune Stimulation and Shift in Microbial Composition in Zebrafish Larvae', *Frontiers in Microbiology*, 9(October), pp. 1–16. Available at: https://doi.org/10.3389/fmicb.2018.02588.

López Nadal, A. *et al.* (2020) 'Feed, Microbiota, and Gut Immunity: Using the Zebrafish Model to Understand Fish Health ', *Frontiers in Immunology* . Available at: https://www.frontiersin.org/article/10.3389/fimmu.2020.00114.

Oehlers, S.H.B. *et al.* (2010) 'Expression of zebrafish cxcl8 (interleukin-8) and its receptors during development and in response to immune stimulation', *Developmental and Comparative Immunology*, 34(3), pp. 352–359. Available at: https://doi.org/10.1016/j.dci.2009.11.007.

de Oliveira, S. *et al.* (2013) 'Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response.', *Journal of immunology (Baltimore, Md. : 1950)*, 190(8), pp. 4349–4359. Available at: https://doi.org/10.4049/jimmunol.1203266.

Rakus, K. *et al.* (2017) 'Conserved Fever Pathways across Vertebrates: A Herpesvirus Expressed Decoy TNF-α Receptor Delays Behavioral Fever in Fish', *Cell Host and Microbe*, 21(2), pp. 244–253. Available at: https://doi.org/10.1016/j.chom.2017.01.010.

Rakus, K., Ronsmans, M. and Vanderplasschen, A. (2017) 'Behavioral fever in ectothermic vertebrates', *Developmental and Comparative Immunology* [Preprint]. Available at: https://doi.org/10.1016/j.dci.2016.06.027.

Rey, S. *et al.* (2017) 'Behavioural fever in zebrafish larvae', *Developmental and Comparative Immunology* [Preprint]. Available at: https://doi.org/10.1016/j.dci.2016.09.008.

Reynolds, W.W., Casterlin, M.E. and Covert, J.B. (1976) 'Behavioural fever in teleost fishes', *Nature* [Preprint]. Available at: https://doi.org/10.1038/259041a0.

Rice, P. *et al.* (2005) 'Febrile-range hyperthermia augments neutrophil accumulation and enhances lung injury in experimental gram-negative bacterial pneumonia', *The Journal of Immunology*, 174(6), pp. 3676–3685.

Ripley, D.M. *et al.* (2022) 'Thermal Preference does not align with optimal temperature for aerobic scope in zebrafish (Danio rerio)', *Journal of Experimental Biology* [Preprint].

Schaaf, M.J.M. and Cidlowski, J.A. (2003) 'Molecular determinants of glucocorticoid receptor mobility in living cells: the importance of ligand affinity', *Molecular and cellular biology*, 23(6), pp. 1922–1934.

Schaefer, J. and Ryan, A. (2006) 'Developmental plasticity in the thermal tolerance of zebrafish Danio rerio', *Journal of Fish Biology* [Preprint]. Available at: https://doi.org/10.1111/j.1095-8649.2006.01145.x.

Singh, I.S. *et al.* (2008) 'Heat shock co-activates interleukin-8 transcription', *American journal of respiratory cell and molecular biology*, 39(2), pp. 235–242.

Spence, R. *et al.* (2008) 'The behaviour and ecology of the zebrafish, Danio rerio', *Biological Reviews*, 83(1), pp. 13–34. Available at: https://doi.org/10.1111/j.1469-185X.2007.00030.x.

Traver, D. *et al.* (2003) 'The zebrafish as a model organism to study development of the immune system.', *Advances in immunology*, 81(February), pp. 253–330. Available at: https://doi.org/10.1016/S0065-2776(03)81007-6.

Tsurufuji, S., Sugio, K. and Takemasa, F. (1979) 'The role of glucocorticoid receptor and gene expression in the anti-inflammatory action of dexamethasone', *Nature*, 280(5721), pp. 408–410.

Tulapurkar, M.E. *et al.* (2012) 'Febrile-range hyperthermia modifies endothelial and neutrophilic functions to promote extravasation', *American journal of respiratory cell and molecular biology*, 46(6), pp. 807–814.

Westerfield, M. (2007) *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio).* 5th edn. Eugene: University of Oregon Press, Eugene.

Yang, L.-L. *et al.* (2014) 'Endotoxin molecule lipopolysaccharide-induced zebrafish inflammation model: a novel screening method for anti-inflammatory drugs', *Molecules*, 19(2), pp. 2390–2409.

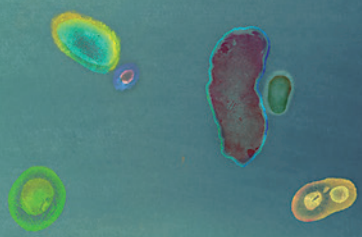
Yoder, J.A. *et al.* (2002) 'Zebrafish as an immunological model system.', *Microbes and infection*, 4(14), pp. 1469–78. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12475637.

Zampronio, A.R. *et al.* (1994) 'Interleukin-8 induces fever by a prostaglandin-independent mechanism', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 266(5), pp. R1670–R1674.

Zampronio, A.R. *et al.* (1995) 'Indomethacin blocks the febrile response induced by interleukin-8 in rabbits', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 269(6), pp. R1469–R1474.

Chapter 5

Feed, microbiota, and gut immunity: using the zebrafish model to understand fish health



Adrià López Nadal, Wakako Ikeda-Ohtsubo, Detmer Sipkema, David Peggs, Charles McGurk, Maria Forlenza, Geert F. Wiegertjes and Sylvia Brugman

Frontiers in Immunology, 05 February 2020
https://doi.org/10.3389/fimmu.2020.00114

Abstract

Aquafeed companies aim to provide solutions to the various challenges related to nutrition and health in aquaculture. Solutions to promote feed efficiency and growth, as well as improving the fish health or protect the fish gut from inflammation may include dietary additives such as prebiotics and probiotics. The general assumption is that feed additives can alter the fish microbiota which, in turn, interacts with the host immune system. However, the exact mechanisms by which feed influences host-microbe-immune interactions in fish still remain largely unexplored. Zebrafish rapidly have become a well-recognized animal model to study host-microbe-immune interactions because of the diverse set of research tools available for these small cyprinids. Genome editing technologies can create specific gene-deficient zebrafish that may contribute to our understanding of immune functions. Zebrafish larvae are optically transparent, which allows for in vivo imaging of specific (immune) cell populations in whole transgenic organisms. Germ-free individuals can be reared to study host-microbe interactions. Altogether, these unique zebrafish features may help shed light on the mechanisms by which feed influences host-microbe-immune interactions and ultimately fish health. In this review, we first describe the anatomy and function of the zebrafish gut: the main surface where feed influences host-microbe-immune interactions. Then, we further describe what is currently known about the molecular pathways that underlie this interaction in the zebrafish gut. Finally, we summarize and critically review most of the recent research on prebiotics and probiotics in relation to alterations of zebrafish microbiota and immune responses. We discuss the advantages and disadvantages of the zebrafish as an animal model for other fish species to study feed effects on host-microbe-immune interactions.

Zebrafish as a model for immunity

In late 1960s, the Hungarian molecular biologist George Streisinger obtained zebrafish (*Danio rerio*) to investigate molecular mechanisms applying forward genetics in a vertebrate model (reviewed in Grunwald and Eisen, 2002). Initially, researchers used zebrafish to study developmental biology followed by the employment of zebrafish in numerous other fields. Among these, zebrafish stood-out as a model to study immunity due to the high presence (~70%) of human orthologous genes in the zebrafish genome (Howe *et al*., 2013) and its intrinsic characteristics. Zebrafish are small (<5 cm), highly prolific (200–300 new progeny per week) and fast growing compared to mice. Zebrafish develop *ex-utero* which, combined with the embryos' transparency, enables investigation of ontogeny *in vivo* from an early time point in development (reviewed in Yoder *et al*., 2002). Moreover, the use of transgenic fish facilitates *in vivo* visualization of specific immune cell populations such as neutrophils (Renshaw *et al*., 2006) based on expression of the neutrophil-associated enzyme myeloperoxidase (Bunchan *et al*., 2019) using fluorescent microscopy. In addition, their well-annotated genome eased the generation of mutant zebrafish lines, some of which contributed to elucidate immune gene functions (reviewed in Yoder *et al*., 2002). In the last decade, genome editing techniques based on Zinc finger nuclease (reviewed in Urnov *et al*., 2010), TALENs (Bedell *et al*., 2012) and the highly successful CRISPR-Cas technique (Hwang *et al*., 2013; Jao *et al*., 2013) changed the speed at which single gene functions can be addressed in this model organism. Currently gene insertion still appears more challenging than gene knock-out, something that will undoubtedly change in the near future (Albadri *et al*., 2017). Zebrafish characteristics combined with these unique research tools established these small cyprinids as an important animal model to study immune processes and underlying molecular mechanisms.

Zebrafish intestine: structure, function, and microbiota

Zebrafish do not have a stomach and their digestive tract is anatomically divided into separate sections: the mouth, the esophagus, three gut segments (anterior, middle, and posterior) and the anus. The zebrafish esophagus is connected with the anterior gut segment, where the nutrient absorption predominantly occurs due to a high presence of digestive enzymes. Nutrient uptake gradually diminishes from the anterior to the posterior gut segments. Ion transport, water reabsorption, fermentation processes as well as certain immune functions occur in the middle and posterior gut segment (Wallace and Pack, 2003; Wallace *et al*., 2005). Wang *et al*. investigated the gene expression of the adult zebrafish gut and compared it to the gut of mice which is anatomically divided into: mouth, esophagus, stomach, three small intestine sections [duodenum, jejunum, and ileum), cecum, large intestine, rectum and anus (Nguyen *et al*., 2015)]. In this study the

zebrafish gut was divided into equal-length segments (called S1–S7, from anterior to posterior) and, based on subsequent transcriptomic analysis, regrouped into three main segments: S1–S5, S6, and S7 corresponding to small and large murine gut (Wang *et al.*, 2010). Subsequently, Lickwar *et al.* performed transcriptomics on adult intestinal epithelial cells (IECs) from zebrafish, stickleback, mouse and human. They specified that the segments S1-S4 of the zebrafish gut presented 493 highly expressed genes from which 70 were also upregulated in the mouse anterior gut (duodenum and ileum-like segments). Next to this, the authors found a core set of genes present in all vertebrate IECs as well as conservation in transcriptional start sites and regulatory regions, independent of sequence similarity (Lickwar *et al.*, 2017).

Besides all the similarities described above, there are clear anatomical differences between zebrafish and the murine digestive tract. Zebrafish do not have a stomach, intestinal crypts, Peyer's patches nor Paneth cells (reviewed in Brugman *et al.*, 2016). In addition, there are dissimilarities in feeding habits, environmental conditions, body sizes and/or specific metabolic requirements. The fact that for instance, lipid metabolism is regulated by similar gut segments between zebrafish and mouse does not imply homology since their metabolism differs greatly: i.e., zebrafish do not have brown fat (Nguyen *et al.*, 2015). Still it remains striking that IECs of different species are more similar in gene expression and regulation (regardless of species intestinal anatomy or feeding habits) than different cell types of the same species (Lickwar *et al.*, 2017). The evidence that gene expression and regulation of this expression in the gut is so highly conserved between species suggests the potential of zebrafish as a valid model for other fish species such as other cyprinids or salmonids when investigating intestinal function.

It has been shown in mice that colonization of the gut with specific microbes induces immune system function. For example, colonization of germ-free (GF) mice with segmented filamentous bacteria induced activation of CD4+ T cells as well as IgA production (Talham *et al.*, 1999). Rawls *et al.* generated a GF zebrafish larval model to study the function of the gut microbiota (Rawls *et al.*, 2004). Using this model they examined the effect of colonization on the host transcriptional response (6 dpf -days post fertilization-larvae) by DNA microarray analysis. Similarly to mice or humans, microbiota-associated gene expressions clustered in several canonical pathways mainly related to four physiological functions: epithelial cell turn-over, nutrient metabolism, xenobiotic metabolism, and innate immune responses (Rawls *et al.*, 2004). In mammals, microbiome colonization may occur during birth (Perez-Muñoz *et al.*, 2017) or prenatally in the womb (Walker *et al.*, 2017). In zebrafish, microbiome colonization is thought to occur at hatching although vertical transmission of microbiome components during oviposition has also been suggested (Llewellyn *et al.*, 2014). Recently, the colonization cycle of microbial species into the gut of zebrafish larvae has been studied in more detail using several

generations of GF zebrafish larvae mono-associated with *Aeromonas veronii* (Robinson *et al.*, 2018). The colonization cycle was found to be divided in four steps: (1) immigration of environmental microbes into the fish, (2) gut adaptation of such microbes, (3) microbe emigration from the host to the environment, and (4) environmental adaptation of the microbes. Both environmental and host gut microbial adaptation were assessed by microbial growth rate, abundance and persistence within the gut or the environment. When comparing four evolved isolates (undergone multiple cycles through the host) and the ancestral strain the authors observed that the evolved isolates were more abundantly present in the fish gut, emphasizing the role of immigration and further adaptation of species into the zebrafish gut.

Earlier colonization studies showed that immigration into the host and gut adaptation are found to be time-specific for each microbe: γ-Proteobacteria were highly abundant in environmental samples as well as in the gut of zebrafish larvae while β-Proteobacteria were mostly abundant in environmental samples and in the gut of juvenile zebrafish, indicating a delayed colonization by certain species of β-Proteobacteria after initial exposure (Stephens *et al.*, 2016). Further research may clarify the specific species involved in the colonization process and whether the colonization delay is due to low microbe immigration to or adaptation to the host gut. During colonization, two major microbial shifts in colonization of zebrafish were described: a first shift at 10 dpf from embryo to larvae and a second shift between 35 and 75 dpf, from juvenile to early adult (Stephens *et al.*, 2016). During the first shift at 10 dpf some individuals had high taxa an richness samples (resembling embryos) while others showed low taxa richness and diversity (resembling juveniles). This distribution could be the result of different developing speed among the larvae. Since feeding generally commences at 6 dpf and zebrafish larvae actively hunt for the (live) feed some fish grow and develop faster than others. In support of the zebrafish observations, studies in other fish species also describe an age-dependent decrease in species density and diversity of the gut microbial community from larval to adult stages (reviewed in de Bruijn *et al.*, 2018). The embryo-to-larva shift could be due to the consumption of exogenous feed (Paramecium) and the juvenile-to-early-adult shift could be due to physiological processes such as sexual maturation (Stephens *et al.*, 2016). Nonetheless, it cannot be excluded that microbiota may adapt and expand due to certain feed components or that the live feed itself brings along microbes and microbial analysis of feed samples could further clarify gut colonization dynamics. Most significantly, so far a putative contribution of a maturing immune system regarding microbiota composition has hardly been addressed in zebrafish.

Larval zebrafish have functional and well-developed organs but their immune system is not completely mature yet. Adaptive immune maturation in zebrafish is an active research topic within the scientific field. In a relatively small study, we showed that T cells

control Proteobacteria *(Vibrio)* abundance in the zebrafish gut, providing evidence that like in mice the adaptive immune system plays a role in shaping the microbiota composition (Brugman *et al.*, 2014). T cells are present in the thymus by 4 dpf as shown by using CD4-1:mCherry transgenic zebrafish (Dee *et al.*, 2016) and CD8a+ antibody staining (Miyazawa *et al.*, 2018). It was shown that T cells egress from the thymus as early as 10 dpf. This suggests that from that time point onwards systemic adaptive responses could be mounted in the zebrafish. However, more in depth studies on the exact timing (the variability thereof) and functionality of these thymic emigrants are warranted.

After the initial colonization period, important for both host and microbe development, the microbiota is believed to enter a stable state. Comparison of gut microbiota of wild-caught zebrafish and zebrafish raised in two separate laboratory facilities revealed that there is a shared so-called core gut microbiota (Roeselers *et al.*, 2011; Stephens *et al.*, 2016). High quality 16S rRNA gene analysis showed common and abundant bacterial groups represented by 21 operational taxonomic units (OTUs), dominated by members of the Proteobacteria phylum (genera *Aeromonas* and *Shewanella*) followed by Fusobacteria or Firmicutes (class Bacilli), Actinobacteria and Bacteroidetes phyla (Roeselers *et al.*, 2011).

In conclusion, all organisms on earth are colonized with bacterial species from their environment. The host and colonizing microbes adapt to ensure fitness of both the host and microbiota. It is important to realize that only performing colonization studies using zebrafish larvae may not represent the complete picture. Especially the maturation of the host immune system can have a profound effects on shaping the intestinal microbiota and, therefore, extrapolation of larval results to juveniles or adults should be carefully examined. Nonetheless, the fact that zebrafish can be reared GF and are still optically transparent at 10 dpf together with the possibility of transgenesis of immune cell populations make zebrafish a very powerful organism to study the timing of microbial colonization and immune system maturation.

Shaping the microbiota: environmental and host factors

Microbes can establish symbiotic relationships with their host by, for instance, facilitating nutrient digestion of diets. Host (biotic) and environmental (abiotic) factors play a role in the modulation of the (intestinal) microbiota. For example, zebrafish larvae exposed to naturally found concentrations of antibiotics together with an antinutritional factor (soy saponin) showed an increased neutrophil recruitment in the gut as well as dysbiosis in the overall microbiome composition (López Nadal *et al.*, 2018). A meta-analysis of 16S rRNA gene sequence data from 25 individual fish gut communities (Sullam *et al.*, 2012) integrated five already published zebrafish data-sets (Rawls *et al.*, 2006; Roeselers *et al.*, 2011). Microbial intestinal communities from different species clustered together and separately from

environmental samples. Within the intestinal microbial cluster different gut bacterial communities exist depending on trophic level (herbivores, carnivores, or omnivores), habitats (saltwater, freshwater, estuarine, or migratory fish), and sampling methods (Sullam *et al*., 2012). Taking the observations together, the symbiotic process between host and bacteria is highly conserved and partly depends on diet and natural habitat.

So which host mechanisms influence the gut microbiota composition? In order to study to what extend the gut selects the microbial community, GF mice were colonized with gut microbiota of conventionally-raised (CONV) zebrafish and vice-versa, GF zebrafish were colonized with gut microbiota of CONV mice. The mouse microbiota generally contains a higher proportion of Firmicutes and Bacteroides compared to the zebrafish microbiota which is dominated by Proteobacteria. Interestingly, after transfer of the mouse microbiota into GF zebrafish, the relative abundance of the Proteobacteria increased toward a microbiota composition of zebrafish. Vice-versa, when zebrafish microbes (dominated by Proteobacteria) were transferred to mice recipient the Firmicutes from this zebrafish content flourished up to >50% compared to the Firmicutes abundance of 1% in original zebrafish microbiota (Rawls *et al*., 2006). Therefore, it seems that the host gut environment shapes the microbiota.

The immune system is part of this host gut environment. For example, zebrafish gut macrophages can shape the microbiota via interferon regulatory factor *irf8*. Adult *irf8*-deficient zebrafish displayed a reduced number of macrophages (mpeg1.1 promoter), presented reduced *c1q* genes expression (*c1qa, c1qb, c1qc*, and *c1ql*) and severe dysbiosis (Fusobacteria, α- and γ-Proteobacteria diminished in favor of δ-Proteobacteria) compared to controls. Downregulation of *c1q* genes may imply an ineffective complement system which could contribute to the observed dysregulation of commensal microbiota. Restauration of *irf8* expression reversed *c1q* genes expression and the levels of commensal microbes (Earley *et al*., 2018). However, a recent study showed that the mpeg1.1 promoter is not only marking macrophages but also phagocytic B lymphocytes in adult zebrafish (Ferrero *et al*., 2019). This might indicate that B cells might also play a role in shaping the microbiota.

In addition to the influence of the fish innate immune system on shaping the microbial communities, there is evidence that the adaptive immune system also plays a role in this process. Adult wild-type zebrafish displayed a decreased abundance of Proteobacteria (*Vibrio)* compared to zebrafish lacking adaptive immunity (rag1-/-), indicating that the innate immune system alone cannot fully regulate all members of the microbiota in the gut. Also, adoptive transfer of T and non-T cells (B and NK-like cells) from wild-types to rag1-/- fish showed that transfer of T cells, but not B/NK-like cells, in the rag1-/- fish diminished *Vibrio* spp. outgrowth 1 week after transfer, suggesting that T cells could regulate the abundance of certain intestinal microbial species. Furthermore, the

lack of adaptive immune response together with altered microbiota induced an inflamed state in the gut of aged zebrafish (14 weeks post feralization): *il-1*β and *cxcl2-l2* were upregulated and *il10, ifn*γ, and *il17f2* downregulated compared to controls. These aged rag1-/- zebrafish developed dropsy (edema caused by bacterial infection) or became anorexic, confirming the physiological effects of an absence of adaptive immunity and possibly a dysregulated microbiota (Brugman *et al.*, 2014). Others also tested the contribution of the adaptive immune system to gut microbiota in adult zebrafish. In this study, rag1-/- or wild-type zebrafish were either housed separately or were co-housed. In segregated genotypes, rag1-/- microbial communities differed from that of wild-types, suggesting a selective pressure of the adaptive immune system. However, such effect was lost when rag1-/- and wild-type zebrafish were housed together (Stagaman *et al.*, 2017). This study suggested that housing could have more influence on microbial diversity than (the absence of the) adaptive immunity. The observation seems to contradict an earlier meta-analysis where different rearing conditions did not result in phylogenetically divergent gut microbiota although cohousing of distinct genotypes was not included in their study (Sullam *et al.*, 2012). Even though the exact extent to which the host immune system affects the microbiota is not completely elucidated, the aforementioned studies (Rawls *et al.*, 2006; Brugman *et al.*, 2014; Stagaman *et al.*, 2017; Earley *et al.*, 2018) suggest selective pressures of the innate and adaptive immune system on the composition of the host gut microbiota.

Contrary to the putative selective pressure of the gut immunity on the microbiota, chance and random distribution (neutral model) was also investigated as explanation for the initial/early assembly of the zebrafish gut microbial community (Burns *et al.*, 2016). Non-neutral processes, such as immune system or feed could become more important for microbial modulation at older stages. Gut bacterial communities in zebrafish could be modulated mostly by ecological dynamics outside of the host, on a broader scale (Burns *et al.*, 2016; Burns *et al.*, 2017). Although microbial ecology processes outside the host certainly play a role in the assembly of the host-gut microbiota, it seems unlikely that chance and random microbial dispersion could vastly explain the similarities of gut microbial compositions across species (Sullam *et al.*, 2012). The fact that gut microbial communities of mammals and fish cluster together suggests that specific pressures to the intestinal environment shape the intestinal microbiota. The earlier mentioned colonization cycle proposed by Robinson *et al.* (Robinson *et al.*, 2018) already takes into account a broader perspective of the environmental ecology including extra- and intra-host factors, such as gut adaptation of the microbes, but only non-fed larvae were analyzed. Taken together these observations, it is highly probable that the intestinal microbiota is, at least partly, modulated by the innate and adaptive host-immune system.

Microbe-host interaction in the zebrafish intestine: molecular immune mechanisms

The host gut exerts selective pressure on the microbiota (reviewed in the section above), which in turn influences host immune responses. In **Figure 1**, we summarized the host-microbe molecular pathways in the zebrafish gut cells. Commensal gram-negative microbes produce low quantities of lipopolysaccharide (LPS) which activate intestinal alkaline phosphatase (Iap) (Bates *et al.*, 2006). Iap is an endogenous protein located in the apical intestinal epithelium and secretes surfactant-like particles to the intestinal lumen (Alpers *et al.*, 1995). Activated Iap counteracts LPS-associated intestinal inflammation, as quantified by neutrophil infiltration in the gut of zebrafish larvae (Bates *et al.*, 2007). In mammals, after Toll like receptor (TLR)-microbial recognition and Myd88 adaptor protein

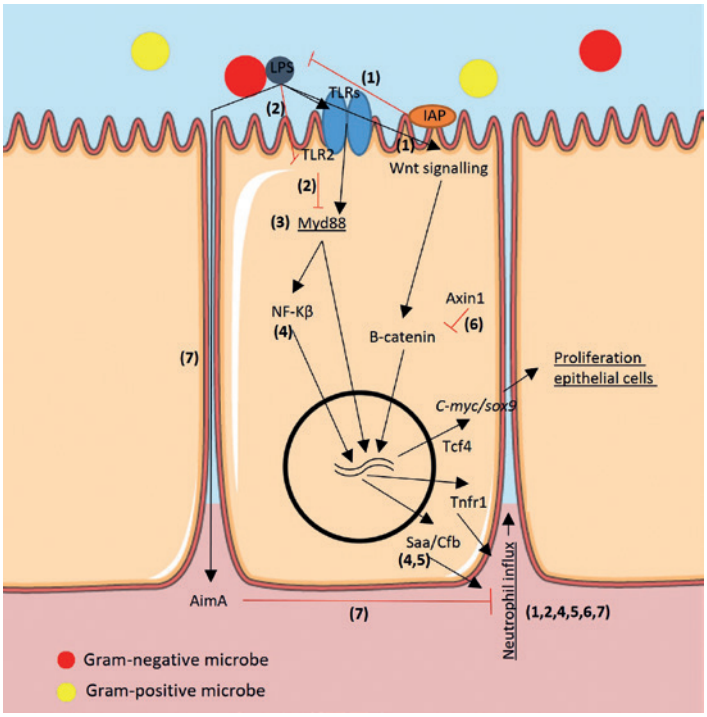


Figure 1: Immuno-modulatory molecular pathways regarding the microbe-host interaction in the epithelium of the zebrafish intestine. We depicted the molecules involved in the proliferation of epithelial cells and in the neutrophil influx as a host-responses to microbiota in the zebrafish gut. In black arrows activation processes, in red inhibition processes. Genes are in italics and host-associated responses are underlined. Numbers correspond to articles proving such molecular interactions: 1: Bates *et al.* 2007; 2: Koch *et al.* 2018; 3: Troll *et al.* 2018 4: Kanther *et al.* 2011, 5: Murdoch *et al.* 2019, 6: Cheesman *et al.* 2011, and 7: Rolig *et al.* 2018.

activation, a downstream signaling cascade follows, including nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signal transduction to the nucleus (reviewed in Janssens and Beyaert, 2002 and in Banerjee *et al*., 2007).

Recently, a TLR2-Myd88-dependent transcriptional feedback mechanism was described upon microbial colonization by using *myd88* deficient zebrafish larvae (Koch *et al*. 2018). The proposed mechanism involves microbial stimuli being recognized by TLR2, partly suppressing *myd88* but enabling enough *myd88* transcriptional activity to possibly induce protective mucin secretion in the apical intestinal epithelium. However, downstream TLR-*myd88* induction of mucin has only been demonstrated in *ex-vivo* mice experiments (Birchenough *et al*. 2016) and not yet in zebrafish. In GF zebrafish, TLR2 cannot suppress *myd88* expression and its elevated levels leads to stimulation of activator protein 1 (AP-1) transcription factors, which resulted in an overall increase in leukocytes (macrophages) in the gut (Koch *et al*. 2018). Nonetheless, GF zebrafish did not show enhanced inflammation as could be expected from AP-1 over-expression. Thus, other mechanisms perhaps absent in larval stages–i.e., adaptive immunity- must be involved in myd88 regulation. Knock-out myd88-/- juveniles or adult zebrafish could be used to further investigate the role of adaptive immunity in regulating microbe-host interaction.

In line with the observation that Myd88 is a key regulator of host-microbe interaction in the gut of larval zebrafish, microbiota determined secretory or absorptive differentiation of IECs via inhibiting Myd88-Notch signaling (Troll *et al*. 2018). Notch signaling is a crucial mechanism for intestinal stem cell differentiation into secretory intestinal cells in zebrafish (Crosnier, 2005). The study focused more on the downstream Myd88 signaling rather than on the recognition of the microbes via TLRs. TLRs have been thoroughly studied in zebrafish (reviewed in Kanwal *et al*. 2014) yet to our knowledge there are no studies showing a direct link of feed components to subsequent TLR-myd88-Notch signaling and increased secretory fate of IECs (goblet cell differentiation) via changes in the microbiota. In the future, several TLR knock-out zebrafish could be engineered to understand how specific feed components and/or the microbiota trigger relevant molecular pathways.

Single microbial species can also influence the zebrafish larval immune system. Gram-negative *Pseudomonas aeruginosa* stimulated NF-κB-dependent expression of innate immune genes such as complement factor b (*cfb)* and serum amyloid a (*saa)* which enhanced neutrophil influx (Kanther *et al*. 2011). In a recent article, *saa*-deficient zebrafish displayed aberrant neutrophil responses to wounding but increased clearance of pathogenic bacteria. Interestingly, *saa* function depended on microbial colonization of GF individuals. To prove that *saa* produced in the gut can systemically affect neutrophil recruitment, they created a transgenic zebrafish expressing *saa* specifically in IECs by using the *cldn15la* promoter fragment to drive mCherry fluorescence, located in the IECs.

Saa produced in the gut in response to microbiota systemically prevented excessive inflammation (tested by tail amputations) as well as reduced bactericidal potential and neutrophil activation (Murdoch *et al.* 2019). Thus, besides the aforementioned functions (Koch *et al.* 2018; Troll *et al.* 2018), Myd88 activation after TLR-microbial recognition orchestrates neutrophil migration to inflamed tissues as previously shown by Kanther *et al.* (Kanther *et al.* 2011) and also pathogenic bacterial clearance in a *saa*-dependent manner (Murdoch *et al.* 2019) in zebrafish larvae in response to microbiota.

Further molecular pathways have been studied by generating specific gene mutations in zebrafish, such as a*xin1*. *Axin1* mutant zebrafish showed upregulated Wnt signaling and β-catenin protein levels (Cheesman *et al.* 2011). It was previously shown in mice that β-catenin accumulates in the cytoplasm and, at a threshold concentration, translocates to the nucleus where (with cofactors such as intestine-specific transcription factor Tcf4) it switches on expression of pro-proliferative genes like *c-myc* or *sox9* (Wetering *et al.* 2002; Blache *et al.* 2004). Induction of *c-myc* and *sox9* in turn increases IEC proliferation. Similarly, *axin1* mutant zebrafish showed increased cell proliferation in the intestine but not when *axin1* mutant zebrafish were reared GF, indicating that the microbiota triggers this increased cell proliferation, confirming earlier results showing increased epithelial turn-over upon microbial colonization (Rawls *et al.* 2004). Interestingly, mono-association of resident bacteria *Aeromonas veronii* was enough to increase intestinal cell proliferation in *axin1* mutant zebrafish by the same mechanisms: upregulating Wnt signaling and β-catenin protein expression. It can be concluded that the microbiota plays a role in the proliferation of epithelial cells in the zebrafish gut during microbial colonization via two mechanisms: TLR recognition with Myd88 downstream signaling and Wnt signaling with β-catenin protein accumulation and pro-proliferative gene activation (Rawls *et al.* 2004). Increased intestinal cell turnover in the developing zebrafish larvae may be beneficial for the host to renew damaged epithelial cells and to shed potentially pathogenic bacteria attached to the epithelium.

To quantify host immune responses to multi-species rather than mono-association, a species quantitative model was created. Two variables were assessed in the zebrafish larvae model: the neutrophil response to individual strains and the absolute abundances of community members. Specific microbes, regardless of their relative abundances, played a major role in the neutrophil influx. GF zebrafish were colonized with different species (*Aeromonas, Vibrio*, and *Shewanella*) and neutrophil influx into the gut was investigated. *Shewanella* partly inhibited the *Vibrio* induction of neutrophil influx in the gut via cell-free supernatant (CFS). However, *Shewanella* CFS did not alter neutrophil influx in combination with *Aeromonas* mono-association (Rolig *et al.* 2015). This study stresses the fact that mono-association experiments may be important to understand molecular mechanisms, however they may not reflect the *in vivo* situation where microbial species

affect each other. Here, the authors used zebrafish larvae and neutrophil influx as the immune parameter, it would be interesting to see effects on other immune mediators, such as eosinophils which are abundantly present in the zebrafish gut. Although the knowledge of immunomodulatory factors produced by fish gut microbiota is limited, a recent study discovered a unique protein AimA ("Aeromonas immune modulator") secreted by *Aeromonas veronii*, which benefit both host and microbe. While AimA protects the host by preventing chemically and bacterially-induced intestinal inflammation, it protects *A. veronii* from host immune response and enhances colonization (Rolig *et al.* 2015). Further studies are needed to understand how specific bacterial species and their associated secreted molecules are involved in overall immune modulation in the zebrafish intestine and systemically. For further reading on the modulation of innate immunity to commensal bacteria, we refer to a recently published review (Murdoch and Rawls, 2019) and for a more extensive review on hematopoiesis in the developing zebrafish to the review of Musad and coworkers (Masud *et al.* 2017).

Impact of prebiotics and probiotics on the zebrafish microbiota and gut immunity

In their natural environment, adult zebrafish eat zooplankton and insects. Analysis of the zebrafish gut content also revealed the presence of phytoplankton, spores and filamentous algae, among others (reviewed in Spence *et al.* 2008). There is not a standard diet for zebrafish in captivity and feeding practices include feeding a mixture of live feeds such as rotifers, ciliates, *Artemia nauplii* and formulated dry feeds (Westerfield, 2007). Supplementary ingredients have been investigated in several commercially relevant fish species in order to increase growth and control aquaculture related diseases (Hoseinifar *et al.* 2018). More specifically, fish microbial communities may influence the immune system and decrease aquaculture-related diseases (reviewed in de Bruijn *et al.* 2018). An overall summary of key operational taxonomic units (OTUs) in various tissues (skin, gut, gills, and digesta) have been associated with fish diseases and infections compared to the wild-type individuals (reviewed in Legrand *et al.* 2019). The use of zebrafish as experimental model to develop novel feeds for farmed fish has gained interest, especially for the development of prebiotics and probiotics as immune and microbiome modulators (reviewed in Ulloa *et al.* 2018). Although most of the prebiotics and probiotics assure benefits for the host, a careful assessment of their effects remains important, as shown for effects of human probiotics uncovering problematic research design, incomplete reporting, lack of transparency or under-reported safety were described (reviewed in Lerner *et al.* 2019). In the next section, we review the current literature on the effects of prebiotics and probiotics on the immune system and microbiota of zebrafish.

Prebiotics

Prebiotics can be defined as non-digestible feed ingredients that have a beneficial effect toward the host by selectively stimulating the growth or the activity of commensal gut bacteria and thus improving host health (reviewed in Gibson and Roberfroid, 1995). Prebiotics most often consist of small carbohydrate chains that are commercially available as oligosaccharides of glucose (like β-glucans), galactose, fructose, or mannose. The use of prebiotics as immuno-stimulants in farmed fish feed has been reviewed elsewhere (Song *et al.* 2014), however the effect of prebiotics on zebrafish (gut) health and on microbiota composition needs further examination. We summarized such studies in **Table 1**. Most of the studies have been performed in larval zebrafish and only very few studies have been performed in adults. The most employed prebiotics in zebrafish research were fucoidans (sulphated polysaccharides mainly present in brown algae and brown seaweed), β-glucans (β-D-glucose polysaccharides extracted from cell walls of bacteria and fungi) and sometimes others, such as galactooligosaccharides. It is of note that not much is known about the modulation of the microbiota by prebiotics since most of the reviewed studies only investigated their immune stimulatory effects.

Fucoidans extracted from several brown algae; *Eklonia cava* (Lee *et al.* 2013), *Chnoospora minima* (Fernando *et al.* 2017), and *Turbinaria ornata* (Jayawardena *et al.* 2019) were administrated to zebrafish larvae in the water. In all three studies, larvae exposed to fucoidans displayed reduced levels of reactive oxygen species (ROS), inducible nitric oxygen synthase (iNOS) and improved cell viability in whole larvae after LPS challenge (Lee *et al.* 2013; Fernando *et al.* 2017; Jayawardena *et al.* 2019). However, in these studies the candidate prebiotics were diluted in the water when the embryos were 8 h post-fertilization. Since the mouth of the zebrafish embryo does not open until 3 dpf and the complete digestive tract is not fully developed until 6 dpf (Wallace and Pack, 2003) such studies do not prove a prebiotic effect on gut immunity. Preferably, zebrafish larvae with a fully developed digestive tract (6 dpf or older) are employed to study such interactions. Furthermore, prebiotics should be tested at physiologically relevant concentrations. Testing a prebiotic in zebrafish larvae may uncover a prebiotic function but often the overall goal would be to formulate novel diets containing the optimal concentration of prebiotic. For this aim, juvenile or adult zebrafish would be more suitable. We investigated the effect of fucoidan derived from the brown alga *Cladosiphon okamuranus* on microbiota composition in whole larvae (water exposure) and in adult zebrafish gut (feeding with flakes). In the gut of adult zebrafish, gene expression of *il-1*β was reduced and the dominant *Escherichia coli* (Proteobacteria) decreased in favor of *Rhizobiaceae* and *Burkholderiaceae* after feeding with fucoidan, while in larvae *il-1*β*, il-10, tnfb*, and *mmp9* increased but no microbial changes were observed (Ikeda-Ohtsubo, this issue).

Table 1 Summary of prebiotics, probiotics, and synbiont studies performed in zebrafish regarding immunity and microbiota.

Feed component	Specie(s)/ Strain(s) tested	Zebrafish age (dpf)	Microbiota composition
Prebiotic	Fucoidan from *Eklonia cava*	Embryos (not specified)	-
Prebiotic	Fucoidan from *Turbinaria ornata*	3 dpf	-
Prebiotic	Fucoidan from *Chnoospora minima*	3 dpf	-
Prebiotic	β-glucan from oats	5 dpf	-
Prebiotic	β-glucan	4 hpf-6dpf	-
Prebiotic	Fucoidan from *Cladosiphon okamuranus*	6-9 dpf and adult zebrafish	- Decreased *E coli* and favoured Rhizobiaceae and Burkholderiaceae in adults but not larvae.
Prebiotic	Galactooligosaccharide supplemented in diet (0.5%, 1% nd 2%)	Adult zebrafish (8 weeks feeding)	-
Probiotic	2 yeast species: *Debaryomyces* (Db) and *Pseudozyma* (Ps)	2 dpf–3 dpf yeast exposure, gut sampling at 14 dpf	- Core microbiota differed from controls. - Reduced Bacteroidetes abundance. - Db increased species richness. - Db increased abundance of *Pediococcus* and *Lactococcus*.
Probiotic	*Lactobacillus casei BL23*	From 3 dpf to 25 dpf	-
Probiotic	Yeasts*: Yarrowia lipolytica 242* (Yl242) and *Debaryomyces hansenii 97* (Dh97)	At 4 dpf, 2 h exposure	- Germ-free (GF) larvae and conventionally raised (CONV) larvae.
Probiotic	*Lactobacillus plantarum ST-III* (LAB) and bile salt hydrolase (BSH). Exposure to Triclosan (TCS) alone or with LAB (TL) or BSH (TB).	From 4 hpf to 90 dpf	- Gut microbiota clustered: LAB> Control > TL and BSH > TB> TCS. - TCS shifted the microbiota and when LAB or BSH co-exposed microbiota resembled more to controls.
Probiotic	15 yeast strains	At 4 dpf, 2 h exposure	-
Probiotic	*L. plantarum* WCFS1 and *NA7* and *L. fermentum ATCC9338*, *NA4* and *NA6*.	At 5 dpf, 24 h exposure	- GF larvae
Probiotic	37 commensal or probiotic Gram-positive and Gram-negative bacteria	6-9 dpf	-

	Immune-modulatory effects	Other relevant parameters
	- Reduced the levels of ROS and NO after challenge with LPS and tail cutting	-
	- Reduced LPS-induced levels of *COX2*, *iNOS* and ROS.	- Improved cell viability
	- Reduced LPS-induced levels of *COX2*, *iNOS* and ROS.	- Improved cell viability
	- Upregulation of *tnfa*, *il-1β, il10, il12, defb1, lyz, c-rel.*	- Increased survival after *E. tarda* challenge.
	- Upregulation of *tnfa, mpo, trf, lyz*	- Increased survival after *Vibrio anguillarum* challenge
	- No change in expression levels of *il-1β* and *tnfb* in the zebrafish adult gut	-
	- Upregulation of *tnfa* and *lyz* - Increase in total immunoglobulin concentration.	-
	-	-
	- Upregulated expression of *il-1β, C3a* and *il-10* after 8 or 24h post-challenge with A. hydrophila.	- Increased survival after *A. hydrophila* challenge
	- Upregulation of *il-1β*, *c3, tnfa, mpx* and *il10* in CONV larvae after *V. anguillarum* challenge - Pre-treatment with Dh97 and Yl242 prevented gene upregulation in CONV and GF larvae.	- Increased survival of CONV and GF larvae due to yeast after challenge with *V. anguillarum (*GF higher mortality than CONV).
	- LAB and TL reduced malonaldehyde in the gut. - TCS upregulated *NF-kB* and *il-1β, tnfa* expression. - TCS increased CD4+T cells in the lamina propria. - TCS thinned intestinal mucosa, destructed epithelia and increased goblet cells.	- TCS induced fibrosis, increased lipid droplet, increased triglycerides and total cholesterol concentrations in the liver compared to controls and LAB/TL treated fish.
	- Larvae after *V. anguillarum* displayed more neutrophils outside the caudal hematopoietic tissue	- All yeast except Mv15 and Csp9 increased survival after *V. anguillarum* challenge.
	- NA4 exposure prior to TNBS challenge lowered levels *trfa* and *il-1β* - *Il-10* expression was higher in larvae exposed to NA4	-
	-	- Increased survival by *V. parahaemolyticus, E. coli ED1a-sm* and *E. coli MG1655* F' upon *E. ictaluri* infection.

Table 1 Continued.

Feed component	Specie(s)/ Strain(s) tested	Zebrafish age (dpf)	Microbiota composition
Probiotic	*Lactobacillus rhamnosus*	96 hfp, 6 dpf and 8 dpf	- Increased the rel. abundance of Firmicutes
Probiotic	B. *coagulans*, *L. plantarum*, *L. rhamnosus*, *Streptococcus thermophilus*, *Bifidobacterium infantis*.	Adult zebrafish (28 days feeding)	-
Probiotic	*Lactobacillus plantarum*	Adult zebrafish (30 days feeing)	- *L. plantarum* clustered gut microbiota independently - Reduced rel. abundance of Vibrionaceae, Pseudoalteromonadaceae and Leuconostrocaceae and increased Lactobacillaceae, Stenotrophomonas and Catenibacterium.
Probiotic	*Lactobacillus rhamnosus*	Adult fish (10 days feeding)	-
Probiotic	8 probiotic strains were lyophilized and mixed with a commercial diet	Adult fish (30 days feeding)	-
Probiotic	*Bacillus amyloliquefaciens*	Adult fish (30 days feeding)	-
Probiotic	*E. coli 40*, *E. coli Nissle* and *E. coli MG 1655* ΔptsG.	Adult zebrafish	
Probiotic & Prebiotic	*Lactobacillus casei BL23* and exopolysaccharide-protein complex (EPSP)	3-12 dpf	- Microbiota did not change due to *L. casei BL23*.
Probiotic & prebiotic	*Ecklonia cava* (EC) Celluclast enzymatic EC (ECC) 100% ethanol extract EC (ECE).	Adult zebrafish (21 days feeding)	- E. cava induced *L. brevis*, *L. pentosus* and *L. plantarum* growth.

Differently from fucoidans, β-glucans can act as immunostimulators in zebrafish. Beta-glucans from oats, upregulated gene expression of *tnfa*, *il-1*β, *il-10*, *il-12*, *defb1*, *lyz*, and *c-rel* in a dose-dependent manner in 5 dpf whole zebrafish larvae (Udayangani *et al.* 2017). In a similar study, β-glucan exposure from 4 hpf until 6 dpf upregulated *tnfa*, *mpo*, *tlf*, and *lyz* gene expression (Oyarbide *et al.* 2012). In both studies, β-glucan administration in the

	Immune-modulatory effects	Other relevant parameters
	- Enlarged enterocytes and microvilli on the apical surface of the epithelium.	- Increased total length and wet weight at 8dpf.
	- *B. coagulans* and *L. plantarum* reduced the number of Masts cells in the gut after *A. hydrophila* challenge. - *B. coagulans* and *L. plantarum* reduced expression of *tnfa* and *il10* and increased *il-1β* in the gut.	- *B. coagulans* and *L. plantarum* reduced mortality after *A. hydrophila* challenge.
	- Not clear effect of *L. plantarum*	- Upregulated canonical pathways related with energy metabolism and vitamin biosynthesis.
	- Upregulated expression of *il1b*, *tnfa* and *becn1* in the gut.	
	- Downregulated *casp4* and *baxa* and upregulated *bcl2a* in the gut. - Upregulated *il-1β*, *tnfa*, *myd88*, *il10*, *casp1*, *nos2a, tgfb1a, nfkb, tlr1, tlr2, tlr3* and *tlr9* (also in protein level, expect for Tlr2).	- Upregulated *cnr1/2* and *abhd4* and downregulated *faah* and *mgll* in the gut compared to controls.
	- Upregulated expression of *il-1β*, *il6*, *il21, tnfa, lyspzyme*, *tlr1, tlr3 and tlr4.*	- Increased survival after *A. hydrophila* and *S. agalactiae* challenges.
	- *E. coli 40* and *E. coli Nissle* decreased mucin found in water after *V. cholerae O395* or *V. cholerae El Tor* strain *N16961* challenge.	
	- *L. casei* upregulated *tnfa, il-1β, il-10* and *Saa* after 24h infection with *A. veronii* but downregulated after 48h. ESPS increased *tlr1*, *tlr2*, *il10*, *tnfa* expression and decreased *il-1β* exp.	- *L. casei BL23* and EPSP increased survival after *Aeromonas veronii* infection.
	- EC combined with *L. plantarum* increased *iNOS* and *COX2* in the gut after *E. tarda* challenge.	- EC, ECC and ECE diminished colony counts of *E. tarda, S. iniae* and *V. harveyi.* - EC reduced mortality *after E. tarda* challenge

water hampers its uptake quantification by the fish and again the exposure of very young larvae probably does not lead to gut-related effects. Oligosaccharides such as galactooligosaccharides (GOS) and fructooligosaccharides (FOS) are frequently used as prebiotics in agriculture and human infant nutrition to boost health via increased production of suggested beneficial bacterial fermentation products (Song *et al.* 2014). Adult zebrafish fed

with GOS for 8 weeks at 0.5, 1, and 2% inclusion levels displayed upregulation of *tnfa* and *lyz* expression and an increase in total immunoglobulins in the whole zebrafish (Yousefi *et al.* 2018). However, no gut specific read-outs were assessed.

It is clear that prebiotics can act on the immune system in a specific manner depending on their source of origin. Fucoidans can decrease inflammation markers whereas β-glucans and GOS increase gene expression of pro-inflammatory cytokines. Despite the promising outcomes, the vast majority of studies exposed undeveloped larvae to prebiotics which are unable to ingest the additive via free feeding. Prebiotics research should carefully evaluate gut health because is the organ where feed can potentially modulate the microbiota and the host immune system. If such candidate prebiotics are included within dry pellets and administrated to fish slightly before satiation (ensuring fish eat all the pellets), it is feasible to estimate the prebiotic gut levels and assess effects on gut microbiota and immunity with more clarity.

Several methods not yet extensively employed in the previously mentioned prebiotic studies may also be suitable for prebiotics gut health research in zebrafish. Firstly, histology and immunohistochemistry staining is needed to understand the immuno-modulatory effects in the gut tissue (i.e., disruption of the normal gut architecture). Transgenic zebrafish could potentially help to clarify which subpopulations of immune cells infiltrate the gut using fluorescently-activated cell sorting (FACS) and imaging. Furthermore, cell sorting of these sub-populations together with transcriptomics would depict the real effect of the prebiotic. Omics technologies (genomics, transcriptomics, proteomics, etc.) play an increasing important role in understanding the immune effects of aqua-feeds (reviewed in Martin and Król, 2017) and omics-based read-outs should become more popular as their costs decrease.

Comparing the limited number of studies performed on zebrafish with a much larger number of studies performed in aquaculture species confirms that supplementation of β-glucans to feed of Atlantic salmon, trout or sea bass increases immune activity (reviewed in Ringø *et al.* 2012) and trained immunity (reviewed in Petit and Wiegertjes, 2016). However, only a limited number of studies have been performed on GOS supplementation. Dietary supplementation to Atlantic salmon of GOS at 1 g/kg feed for 4 months did not show effects on reactive oxygen species (ROS) production or lysozyme activity. Research on the use of seaweed is increasing, for example testing 10% inclusion levels of *Laminaria digitata* in feed of Atlantic salmon (Palstra *et al.* 2018). The dietary seaweed improved chemokine-mediated signaling but the study only assessed transcriptional responses after LPS challenge so further research into the health effects of elevated or reduced gene expression is warranted. This last example nicely supports the use of zebrafish model, not to replace testing in aquaculture target species, but to prescreen feed components and further dissect the mechanism of action by live imaging and

assessment of health parameters for prolonged periods, something difficult to achieve in large and costly aquaculture species.

Probiotics

Already in 1907, Elie Metchnikoff related the use of probiotics to elongation of life expectancy. For the purpose of this review we define probiotics as a live or inactivated microorganism, such as bacterium or yeast, that when administrated via feed or water, confers a benefit to the host, such as improved disease resistance or enhanced immune responses (adapted from Merrifield *et al.* 2010; Brugman *et al.* 2018). Probiotics can influence the health of the host in several ways: secreting secondary metabolites that inhibit growth of microbial pathogens and/or directly stimulating immune responses to downregulate gut inflammation (reviewed in Hai, 2015). Here we focused on the probiotic studies in zebrafish concerning (gut) immune and microbiota modulation (summarized in **Table 1**).

To assess potential health benefits of live probiotics it is important to understand their optimal environment inside the host (oxygen levels, pH, etc.) and their colonization route. Probiotic-host interaction was addressed by a model of oro-intestinal pathogen colonization in GF zebrafish (Rendueles *et al.* 2012). Firstly, 6 dpf zebrafish were exposed by immersion to 25 potential enteric fish pathogens after which mortality was recorded during 3 days. *Edwardsiella ictaluri* caused the highest larvae mortality and was further selected to challenge the fish. Then, larvae were pre-colonized with single strains of 37 possible probiotics prior to *E. ictaluri* challenge. From this extensive screening, *Vibrio parahaemolyticus, E. coli* ED1a-sm and *E. coli* MG1655 F' provided a significant increase in survival upon *E. ictaluri* infection. *V. parahaemolyticus* protected the host by inhibiting *E. ictaluri* growth whereas *E. coli* protected via specific adhesion factors, such as F pili involved in biofilm and conjugation formations offering niches to other probiotic bacteria in the host (Rendueles *et al.* 2012). It is of note that zebrafish gills, although they are active in gas exchange 2 weeks after fertilization (Pelster and Bagatto, 2010), provide a potential portal of entry for pathogens. Regretfully, gills were not included in the aforementioned study. Interestingly, in the same study, *Vibrio parahaemolyticus* was assessed as a possible probiotic whereas *Vibrio ichthyoenteri* was considered as a possible pathogen. The majority of the microbiota studies associate immune responses to taxonomic levels such as genera or families (i.e., *Vibrio* spp.) rather than species or strains. As a consequence, there is a generalization of an entire genus to a functions that could be species or even strain-specific. Such widely used generalizations may come from the difficulty to generate amplicons that are long enough to discriminate between closely related organisms. Besides, transcriptomics and shot gun approaches are preferred over 16S rRNA gene analysis to depict the active microbiota because they more informative regarding the fish health status (Llewellyn *et al.* 2014). Adult zebrafish were also used to test probiotics as a

model for human probiotic consumption. Adult zebrafish were exposed to two *E. coli* strains (Nissle and MG 1655 ΔptsG*)* and challenged with species of *Vibrio choleae* (strain El Tor). *E. coli* spp. decreased the mucin content found in the tank water, indicator of diarrhea (Nag *et al.* 2018) although these mucins could perhaps also result from skin shedding. It might be interesting to assess whether these *E. coli* spp. increase secretory cell development and therefore mucus secretion via reduction of Myd88-Notch signaling as previously reviewed (Troll *et al.* 2018). In addition, while in humans administration of bacteria via a solutions orally ingested is an efficient way of ensuring ingestion, addition of probiotics to the water may not guarantee uptake by fish and may affect overall fish mucosa (skin, gills, gut) and not only uptake in the gut. Besides, the environment of the fish gut is more aerobic than the human gut environment (Llewellyn *et al.* 2014) and lactic acid bacteria may be outcompeted by other bacteria in these aerobic conditions. This rationale may explain why human probiotics (*Lactobacillus* spp.) tested in zebrafish by immersion did not confer protection against *E. ictaluri* infection (Rendueles *et al.* 2012). Several studies reported Lactic Acid Bacteria (LAB) as good probiotic candidates due to their ability to withstand and adhere to the gut, their lactic acid production which inhibits the growth of pathogenic bacteria and their strengthening of the mucosal barrier (Ringø and Gatesoupe, 1998). Zebrafish immersed with *Lactobacillus casei* BL23 from 3-25 dpf displayed an increased survival compared to controls after an immersion challenge with *Aeromonas hydrophila*. Gut gene expression of *il-1*β, *C3a*, and *il-10* was upregulated after 8 and 24 h after *A. hydrophila* challenge compared to controls (Qin *et al.* 2018). Interestingly, potential probiotics from the genera *Lactobacillus* modulated gene regulation in a strain-specific fashion. As a matter of fact, GF larvae immersed with *Lactobacillus fermentum* NA4 displayed an increased *il-10* expression and a decreased *il-1*β and *tnfa* expression after chemically-induced inflammation compared to controls. However, in the same study, larvae immersed with several strains of *Lactobacillus plantarum* (WCFS1 and NA7] or other *Lactobacillus fermentum* strains (ATCC9338 and NA6) did not show these differences in gene expression (Aoudia *et al.* 2016). Dissimilarities in gene expression among the aforementioned studies (Aoudia *et al.* 2016; Qin *et al.* 2018) could be due to fish age (3–25 vs. 7 dpf), tissue analyzed (gut vs. whole larvae) challenge applied (live pathogen vs. chemical) and the specific *Lactobacillus* strain used as a probiotic candidate. *Bacillus amyloquefaciens* supplemented twice a day for 30 days in a commercial diet upregulated *il-1*β, *il-6, il-21 tnfa, lysozyme, tlr1, tlr3.* and *tlr4* expression in adult zebrafish whole body and increased survival during *A. hydrophila* and *S. agalactiae* challenge (Lin *et al.* 2019). Upregulation of gene expression appeared related to enhanced innate immunity although no other immune parameters were taken into account. In another study in adult zebrafish, a commercial diet was supplemented with multiple lyophilized probiotic strains for 30 days. The probiotic mix upregulated *il-1*β, *tnfa, myd88, il-10, casp1, nos2a, tgfb1a, nfkb, tlr1,*

tlr2, tlr3, and *tlr9* expression in the gut. Furthermore, the probiotic mix increased the protein levels encoded by all the upregulated genes (except for Tlr2 protein) (Gioacchini *et al.* 2017). On the one hand, certain bacteria of the probiotic mix may have inhibited Tlr2, which in turn could have partly suppressed *myd88* (Koch *et al.* 2018). On the other hand, other bacteria of the probiotic mix may have enhanced expression of other TLRs that upregulated *myd88* and the overall Myd88-balance orchestrated innate immune responses. As previously reviewed, microbial species can influence host immunity irrespective of their abundance (Rolig *et al.* 2015) and when using mix of probiotics the effects of each individual species are harder to disentangle. Other studies using LAB as probiotics did not only examined gene expression but also microbiota (Falcinelli *et al.* 2015, Davis *et al.* 2016; Zang *et al.* 2019) and histological changes (Falcinelli *et al.* 2015; Wang *et al.* 2015) in the zebrafish gut (**Table 1**). Some studies investigated the potential of yeast as a probiotic for zebrafish. GF and CONV zebrafish larvae were immersed from 2–3 dpf in solutions of two yeasts after which gut microbiota were sampled at 14 dpf (Siriyappagouder *et al.* 2018). Although microbial changes were observed, immune-related outcomes where not measured so the probiotic effect of the yeasts in this study remains undefined. In another study, 4 dpf zebrafish were exposed to 15 fluorescently labeled yeast strains for 2 h prior to *Vibro anguillarum* challenge (Caruffo *et al.* 2015). Most of the yeast strains conferred increased survival after challenge. In a later experiment, the same group further studied two of the yeast strains in GF and CONV larvae using a similar set-up. Exposure to either yeast strain significantly increased survival in GF and CONV larvae after *V. anguillarum* challenge (Caruffo *et al.* 2015). CONV zebrafish challenged with *V. anguillarum* displayed an upregulation of *il-1*β, *c3, tnfa, mpx*, and *il-10* expression. Pre-treatment with either yeast strain prevented such gene upregulation in CONV and GF larvae, indicating that these yeast strains might prevent or reduce the effects of *V. anguillarum* (Caruffo *et al.* 2015).

Zebrafish have also been employed for synbiotic studies which typically combine the use of prebiotics and probiotics. *Lactobacillus casei* BL23 and an exopolysaccharide complex (ESPS) were studied in combination in GF and CONV larvae from 3 to 12 dpf. *L. casei* exposure upregulated *tnfa, il-1*β, *il-10*, and *saa* expression after 24 h in a challenge with *Aeromonas veronii* and downregulated expression of these genes after a 48 h challenge. It is of note that the ESPS alone upregulated *tlr1, tlr2, il-10*, and *tnfa* and downregulated *il-1*β after 24 h challenge. Synbiotically, *L. casei* BL23 and EPSP improved survival dose-dependently after *A. veronii* challenge (Qin *et al.* 2017). The combined supplementation of *E. cava* enzymatic digest, with enhanced biological activity, as prebiotic together with *L. plantarum* as a probiotic in adult zebrafish for 21 days reduced the level of *iNOS* and *cyclooxygenase 2* (*cox2*) in the gut. Moreover, when prebiotics and probiotics were administrated together, they increased survival compared to *L. plantarum*-treated fish alone after a challenge with *E. tarda* (Lee *et al.* 2016). Interestingly these studies suggest

that certain extracts and/or biologically active compounds rather than the whole prebiotic may cause immune-modulation.

A large number of studies (co)exposed potential prebiotics and/or probiotics to zebrafish to improve their immune condition via microbial modulation (**Figure 2**). Remarkably, in most of these studies, gene expression was assumed a conclusive immunological read-out. Apart from the fact that gene expression does not always translate to protein functionality, often pro- and anti-inflammatory cytokines are upregulated or downregulated depending on the dynamics and the timing of the response. The gene expression may reflect the balance in the host during an immune response: specific and strong enough to fight potentially pathogenic bacteria but at the same time able to tolerate commensal host microbiota (Kelly and Salinas, 2017). This balance is also dependent on different cell types that work in concert to prevent excessive damage to the host when acting against an invading pathogen or ongoing inflammation. We need to understand the role and presence of different immune cell types that are involved in the different responses in much more detail before we can try to modulate the response to the benefit of the host. To this end, the zebrafish remains the ideal candidate model organism. To date, more studies could have made use of the unique tools in zebrafish such as live imaging of different transgenic reporter zebrafish (cytokines as well as immune cell populations) to get a much broader understanding of the complex dynamic interactions of host-feed-microbe interactions.

Concluding remarks

In this review we focused on the zebrafish as an animal model to study the effect of feed on host-microbe-immune interactions (summarized in **Figure 2**). Zebrafish are now widely used as models to study fundamental and evolutionary processes that might uncover pathways relevant for both fish and mammals. The studies on microbial composition development summarized in this review reveal that although the gut microbial composition is dependent on salinity, trophic level and host phylogeny, mammalian, fish and insect gut microbiota still cluster together and separately from environmental samples. Thus, although mammals and fish live in distinct environments and clearly have different physiology, gene expression and regulation of gene expression in the gut is highly similar. IEC transcriptional profiles are more similar between species than responses of different cell types of the same species. Therefore, experimentation with zebrafish seems suitable to elucidate conserved molecular mechanisms.

Using zebrafish as a model for aquaculture species is of interest. Eighty percent of farmed fish are other cyprinids and therefore close relatives. We argue that using the zebrafish as a model for aquaculture species brings several advantages yet may never fully replace studies performed in the target species for validation. Nevertheless, using

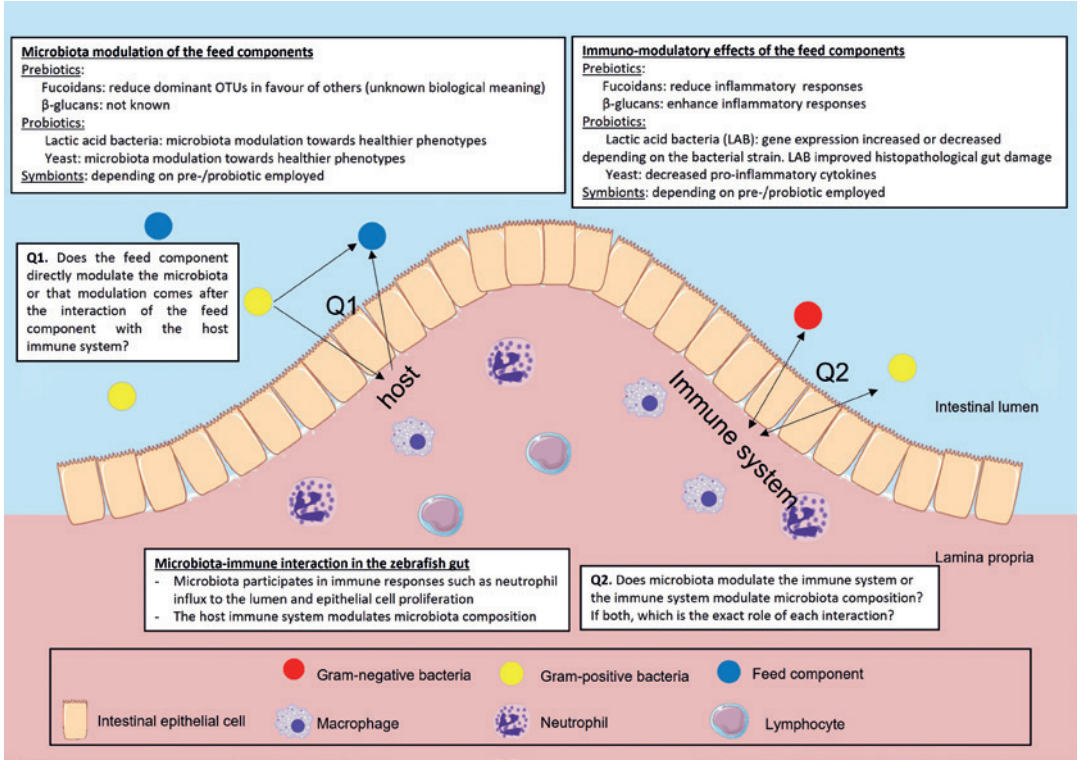


Figure 2: Overview of the interaction of pre- and probiotics, immune system and microbiota in the zebrafish intestine. We summarized the interactions of microbiota and feed components, immune system and feed components and microbiota and immune system. We highlighted the questions that still remain unsolved in the field.

zebrafish as a pre-screen model to guide studies in aquaculture species might contribute to elucidate mechanisms underlying feed and host-microbe-immune interactions.

Recently, exiting new research using *in vivo* mice models has shown that the microbial community can influence the severity of viral infections (Kuss *et al.* 2011; Kane *et al.* 2011). Moreover, *in vitro* data using RAW264.7 cells showed antiviral activity of several *Lactobacillus* strains to murine norovirus (MNV) infection through IFN-β upregulation (Lee and Ko, 2016). Currently, it is unknown whether microbes can also alter fish-specific viral infectivity. This is an exciting new avenue of research that might lead to novel vaccination strategies, combining virus-targeting vaccines with prebiotic or probiotic treatment to change the microbiota as well as target the virus itself. A fundamental field in which zebrafish are most probably will contribute due to its unique advantages.

The studies published in the field using zebrafish will continue to increase and by combining existing technologies (omics, immunohistochemistry, FACS, *in vivo* imaging) or by emerging novel technology knowledge gaps will surely be filled. For future experiments it would greatly benefit our understanding if more holistic approaches would be taken. We need to combine read-out parameters such as gene expression, survival after challenges, gut architecture, immune cell recruitment, microbiota composition, metabolite production and behavioral data within each experiment to provide a broader picture of the consequences of certain treatments on the health of the fish. Only by carefully determining cause and effect by interrogating possible molecular pathways through gene editing we can provide a solid rationale for the design of novel immunomodulatory strategies. As it becomes clear from this literature survey next to a more holistic approach, there is a need to study the effect of feed and microbes at different life stages of the fish. The immune system develop sequentially in zebrafish presenting adaptive immunity only from 14 dpf onwards. An substantial part of zebrafish literature is built on zebrafish larvae around 5 dpf of age when fish only rely on innate immunity which may occasionally lead to a an incomplete picture of the biological processes studied. Therefore, in the subsequent chapters of this thesis we included experimentation with juvenile and adult zebrafish to explore the effects of novel feeds and feed supplements in fully immuno-competent individuals.

Authors contributions

AL drafted the manuscript and the figures. WI-O, DS, DP, CM, MF, GW, and SB edited and contributed to writing the manuscript. SB, GW, and DS obtained the funding.

Funding

Our work is generously funded by TTW-NWO (project number 15566). This work was partially supported by the Japan Society for the Promotion of Science (JSPS) through JSPS

Core-to-Core Program (Advanced Research Networks) entitled "Establishment of international agricultural immunology research-core for a quantum improvement in food safety". WI-O was supported by a WIAS fellowship provided by the Graduate School of Animal Science of Wageningen University & Research.

Conflict of interest statement

DP and CM are employed by Skretting Aquaculture Research Center. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

The authors thank Ángel Chacón Orozco for editing the figures of this paper.

References

Albadri S, Del Bene F, Revenu C. Genome editing using CRISPR/Cas9-based knock-in approaches in zebrafish. *Methods*. (2017) 121–2:77–85. doi: 10.1016/j.ymeth.2017.03.005

Alpers DH, Zhang Y, Ahnen DJ. Synthesis and parallel secretion of rat intestinal alkaline phosphatase and a surfactant-like particle protein. *Am J Physiol Endocrinol Metab.* (1995) 268:E1205–14. doi: 10.1152/ajpendo.1995.268.6.E1205

Aoudia N, Rieu A, Briandet R, Deschamps J, Chluba J, Jego G, et al. Biofilms of *Lactobacillus plantarum* and *Lactobacillus fermentum*: effect on stress responses, antagonistic effects on pathogen growth and immunomodulatory properties. *Food Microbiol.* (2016) 53:51–9. doi: 10.1016/j.fm.2015.04.009

Banerjee A, Gerondakis S. Coordinating TLR-activated signaling pathways in cells of the immune system. *Immunol Cell Biol.* (2007) 85:420–4. doi: 10.1038/sj.icb.7100098

Bates JM, Akerlund J, Mittge E, Guillemin K. Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe.* (2007) 2:371–82. doi: 10.1016/j.chom.2007.10.010

Bates JM, Mittge E, Kuhlman J, Baden KN, Cheesman SE, Guillemin K. Distinct signals from the microbiota promote different aspects of zebrafish gut differentiation. *Dev Biol.* (2006) 297:374–86. doi: 10.1016/j.ydbio.2006.05.006

Bedell VM, Wang Y, Campbell JM, Poshusta TL, Starker CG, Krug RG, et al. *In vivo* genome editing using a high-efficiency TALEN system. *Nature.* (2012) 491:114–8. doi: 10.1038/nature11537

Birchenough GMH, Nystrom EEL, Johansson MEV, Hansson GC. A sentinel goblet cell guards the colonic crypt by triggering Nlrp6-dependent Muc2 secretion. *Science.* (2016) 352:1535–42. doi: 10.1126/science.aaf7419

Blache P, Van De Wetering M, Duluc I, Domon C, Berta P, Freund JN, et al. SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. *J Cell Biol.* (2004) 166:37–47. doi: 10.1083/jcb.200311021

Brugman S, Ikeda-Ohtsubo W, Braber S, Folkerts G, Pieterse CMJ, Bakker PAHM. A comparative review on microbiota manipulation: lessons from fish, plants, livestock, and human research. *Front Nutr.* (2018) 5:80. doi: 10.3389/fnut.2018.00080

Brugman S, Schneeberger K, Witte M, Klein MR, van den Bogert B, Boekhorst J, et al. T lymphocytes control microbial composition by regulating the abundance of Vibrio in the zebrafish gut. *Gut Microbes.* (2014) 5:737–47. doi: 10.4161/19490976.2014.972228

Brugman S. The zebrafish as a model to study intestinal inflammation. *Dev Comp Immunol.* (2016) 64:82–92. doi: 10.1016/j.dci.2016.02.020

Buchan KD, Prajsnar TK, Ogryzko NV, De Jong NWM, Van Gent M, Kolata J, et al. A transgenic zebrafish line for *in vivo* visualisation of neutrophil myeloperoxidase. *PLoS ONE.* (2019) 14:e0215592. doi: 10.1371/journal.pone.0215592

Burns AR, Guillemin K. The scales of the zebrafish: host–microbiota interactions from proteins to populations. *Curr Opin Microbiol.* (2017) 38:137–41. doi: 10.1016/j.mib.2017.05.011

Burns AR, Stephens WZ, Stagaman K, Wong S, Rawls JF, Guillemin K, et al. Contribution of neutral processes to the assembly of gut microbial communities in the zebrafish over host development. *ISME J.* (2016) 10:655–64. doi: 10.1038/ismej.2015.142

Caruffo M, Navarrete N, Salgado O, Díaz A, López P, García K, et al. Potential probiotic yeasts isolated from the fish gut protect zebrafish (*Danio rerio*) from a *Vibrio anguillarum* challenge. *Front Microbiol.* (2015) 6:1093. doi: 10.3389/fmicb.2015.01093

Caruffo M, Navarrete NC, Salgado OA, Faúndez NB, Gajardo MC, Feijóo CG, et al. Protective yeasts control *V. anguillarum* pathogenicity and modulate the innate immune response of challenged zebrafish (*Danio rerio*) larvae. *Front Cell Infect Microbiol.* (2016) 6:127. doi: 10.3389/fcimb.2016.00127

Cheesman SE, Neal JT, Mittge E, Seredick BM, Guillemin K. Epithelial cell proliferation in the developing zebrafish intestine is regulated by the Wnt pathway and microbial signaling via Myd88. *Proc Natl Acad Sci USA.* (2011) 108(Suppl_1):4570–7. doi: 10.1073/pnas.1000072107

Crosnier C. Delta-Notch signalling controls commitment to a secretory fate in the zebrafish intestine. *Development.* (2005) 132:1093–104. doi: 10.1242/dev.01644

Davis DJ, Doerr HM, Grzelak AK, Busi SB, Jasarevic E, Ericsson AC, et al. *Lactobacillus plantarum* attenuates anxiety-related behavior and protects against stress-induced dysbiosis in adult zebrafish. *Sci Rep.* (2016) 6:33726. doi: 10.1038/srep33726

de Bruijn I, Liu Y, Wiegertjes GF, Raaijmakers JM. Exploring fish microbial communities to mitigate emerging diseases in aquaculture. *FEMS Microbiol Ecol.* (2018) 94:fix161. doi: 10.1093/femsec/fix161

Dee CT, Nagaraju RT, Athanasiadis EI, Gray C, Fernandez del Ama L, Johnston SA, et al. CD4-Transgenic zebrafish reveal tissue-resident Th2- and regulatory T cell–like populations and diverse mononuclear phagocytes. *J Immunol.* (2016) 197:3520–30. doi: 10.4049/jimmunol.1600959

Earley AM, Graves CL, Shiau CE. Critical role for a subset of intestinal macrophages in shaping gut microbiota in adult zebrafish. *Cell Rep.* (2018) 25:424–36. doi: 10.1016/j.celrep.2018.09.025

Falcinelli S, Picchietti S, Rodiles A, Cossignani L, Merrifield DL, Taddei AR, et al. *Lactobacillus rhamnosus* lowers zebrafish lipid content by changing gut microbiota and host transcription of genes involved in lipid metabolism. *Sci Rep.* (2015) 5:8–10. doi: 10.1038/srep09336

Fernando IPS, Sanjeewa KKA, Samarakoon KW, Lee WW, Kim HS, Kang N, et al. A fucoidan fraction purified from Chnoospora minima; a potential inhibitor of LPS-induced inflammatory responses. *Int J Biol Macromolecules.* (2017) 104:1185–93. doi: 10.1016/j.ijbiomac.2017.07.031

Ferrero G, Gomez E, Iyer S, Rovira M, Miserocchi M, Langenau DM, et al. The macrophage-expressed gene (mpeg) 1 identifies a subpopulation of B cells in the adult zebrafish. *bioRxiv.* (2019) 836098. doi: 10.1101/836098

Gibson GR, Roberfroid MB. Dietary modulation of the human colonie microbiota: introducing the concept of prebiotics. *J Nutr.* (1995) 1401–12. doi: 10.1093/jn/125.6.1401

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

Gioacchini G, Rossi G, Carnevali O. Host-probiotic interaction: New insight into the role of the endocannabinoid system by *in vivo* and *ex vivo* approaches. *Sci Rep.* (2017) 7:1–12. doi: 10.1038/s41598-017-01322-1

Grunwald DJ, Eisen JS. Hearwaters of the zebrafish - emergence of a new model vertebrate. *Nat Rev Genet.* (2002) 3:717–24. doi: 10.1038/nrg892

Hai NV. The use of probiotics in aquaculture. *J Appl Microbiol.* (2015) 119:917–35. doi: 10.1111/jam.12886

Hoseinifar SH, Sun YZ, Wang A, Zhou Z. Probiotics as means of diseases control in aquaculture, a review of current knowledge and future perspectives. *Front Microbiol.* (2018) 9:2429. doi: 10.3389/fmicb.2018.02429

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature.* (2013) 496:498–503. doi: 10.1038/nature12111

Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, et al. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol.* (2013) 31:227–9. doi: 10.1038/nbt.2501

Janssens S, Beyaert R. A universal role for MyD88 in TLR/IL-1R-mediated signaling. *Trends Biochem Sci.* (2002) 27:474–82. doi: 10.1016/S0968-0004(02)02145-X

Jao LE, Wente SR, Chen W. Efficient multiplex biallelic zebrafish genome editing using a CRISPR nuclease system. *Proc Natl Acad Sci USA.* (2013) 110:13904–9. doi: 10.1073/pnas.1308335110

Jayawardena TU, Fernando IPS, Lee WW, Sanjeewa KKA, Kim HS, Lee DS, et al. Isolation and purification of fucoidan fraction in *Turbinaria ornata* from the Maldives; inflammation inhibitory potential under LPS stimulated conditions in *in-vitro* and *in-vivo* models. *Int J Biol Macromolecules.* (2019) 131:614–23. doi: 10.1016/j.ijbiomac.2019.03.105

Kane M, Case LK, Kopaskie K, Kozlova A, MacDearmid C, Chervonsky AV, et al. Successful transmission of a retrovirus depends on the commensal microbiota. *Science.* (2011) 334:245–9. doi: 10.1126/science.1210718

Kanther M, Sun X, Mhlbauer M, MacKey LC, Flynn EJ, Bagnat M, et al. Microbial colonization induces dynamic temporal and spatial patterns of NF-κB activation in the zebrafish digestive tract. *Gastroenterology.* (2011) 141:197–207. doi: 10.1053/j.gastro.2011.03.042

Kanwal Z, Wiegertjes GF, Veneman WJ, Meijer AH, Spaink HP. Comparative studies of Toll-like receptor signalling using zebrafish. *Dev Comp Immunol.* (2014) 46:35–52. doi: 10.1016/j.dci.2014.02.003

Kelly C, Salinas I. Under pressure: interactions between commensal microbiota and the teleost immune system. *Front Immunol.* (2017) 8:559. doi: 10.3389/fimmu.2017.00559

Koch BEV, Yang S, Lamers G, Stougaard J, Spaink HP. Intestinal microbiome adjusts the innate immune setpoint during colonization through negative regulation of MyD88. *Nat Commun.* (2018) 9:4099. doi: 10.1038/s41467-018-06658-4

Kuss SK, Best GT, Etheredge CA, Pruijssers AJ, Frierson JM, Hooper LV, et al. Intestinal microbiota promote enteric virus replication and systemic pathogenesis. *Science.* (2011) 334:249–52. doi: 10.1126/science.1211057

Lee H, Ko GP. Antiviral effect of Vitamin A on norovirus infection via modulation of the gut microbiome. *Sci Rep.* (2016) 6:1–9. doi: 10.1038/srep25835

Lee SH, Ko CI, Jee Y, Jeong Y, Kim M, Kim JS, et al. Anti-inflammatory effect of fucoidan extracted from Ecklonia cava in zebrafish model. *Carbohydr. Polym.* (2013) 92:84–9. doi: 10.1016/j.carbpol.2012.09.066

Lee WW, Oh JY, Kim EA, Kang N, Kim KN, Ahn G, et al. A prebiotic role of *Ecklonia cava* improves the mortality of *Edwardsiella tarda*-infected zebrafish models via regulating the growth of lactic acid bacteria and pathogen bacteria. *Fish Shellfish Immunol.* (2016) 54:620–8. doi: 10.1016/j.fsi.2016.05.018

Legrand TPRA, Wynne JW, Weyrich LS, Oxley APA. A microbial sea of possibilities: current knowledge and prospects for an improved understanding of the fish microbiome. *Rev Aquac.* (2019) raq.12375. doi: 10.1111/raq.12375

Lerner A, Shoenfeld Y, Matthias T. Probiotics: if it does not help it does not do any harm. Really? *Microorganisms.* (2019) 7:104. doi: 10.3390/microorganisms7040104

Lickwar CR, Camp JG, Weiser MLCJ, Kingsley DM, Furey TS, Sheikh SZ, et al. Genomic dissection of conserved transcriptional regulation in intestinal epithelial cells. *PLOS Biol.* (2017) 15:e2002054. doi: 10.1371/journal.pbio.2002054

Lin YS, Saputra F, Chen YC, Hu SY. Dietary administration of *Bacillus amyloliquefaciens* R8 reduces hepatic oxidative stress and enhances nutrient metabolism and immunity against *Aeromonas hydrophila* and *Streptococcus agalactiae* in zebrafish (*Danio rerio*). *Fish Shellfish Immunol.* (2019). 86:410–9. doi: 10.1016/j.fsi.2018.11.047

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

López Nadal A, Peggs D, Wiegertjes GF, Brugman S. Exposure to Antibiotics affects saponin immersion-induced immune stimulation and shift in microbial composition in zebrafish larvae. *Front Microbiol.* (2018) 9:2588. doi: 10.3389/fmicb.2018.02588

Martin SAM, Król E. Nutrigenomics and immune function in fish: new insights from omics technologies. *Dev Comp Immunol.* (2017) 75:86–98. doi: 10.1016/j.dci.2017.02.024

Masud S, Torraca V, Meijer AH. Modeling infectious diseases in the context of a developing immune system. *Curr Top Dev Biol.* (2017) 124:277–329. doi: 10.1016/bs.ctdb.2016.10.006

Merrifield DL, Dimitroglou A, Foey A, Davies SJ, Baker RTM, Bøgwald J, et al. The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture.* (2010) 302:1–18. doi: 10.1016/j.aquaculture.2010.02.007

Miyazawa R, Matsuura Y, Shibasaki Y, Imamura S, Nakanishi T. Cross-reactivity of monoclonal antibodies against CD4-1 and CD8α of ginbuna crucian carp with lymphocytes of zebrafish and other cyprinid species. *Dev Comp Immunol.* (2018) 80:15–23. doi: 10.1016/j.dci.2016.12.002

Murdoch CC, Espenschied ST, Matty MA, Mueller O, Tobin DM, Rawls JF. Intestinal serum amyloid a suppresses systemic neutrophil activation and bactericidal activity in response to microbiota colonization. *PLoS Pathog.* (2019) 15:e1007381. doi: 10.1371/journal.ppat.1007381

Murdoch CC, Rawls JF. Commensal microbiota regulate vertebrate innate immunity-insights from the zebrafish. *Front Immunol.* (2019) 10:2100. doi: 10.3389/fimmu.2019.02100

Nag D, Breen P, Raychaudhuri S, Withey JH. Glucose metabolism by *Escherichia coli* inhibits vibrio cholerae intestinal colonization of zebrafish. *Infect Immun.* (2018) 86:e00486-18. doi: 10.1128/IAI.00486-18

Nguyen TLA, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? *Dis Models Mech.* (2015) 8:1–16. doi: 10.1242/dmm.017400

Oyarbide U, Rainieri S, Pardo MA. Zebrafish (*Danio rerio*) larvae as a system to test the efficacy of polysaccharides as immunostimulants. *Zebrafish.* (2012) 9:74–84. doi: 10.1089/zeb.2011.0724

Palstra AP, Kals J, Garcia AB, Dirks RP, Poelman M. Immunomodulatory effects of dietary seaweeds in LPS challenged Atlantic Salmon Salmo salar as determined by deep RNA sequencing of the head kidney transcriptome. *Front Physiol.* (2018) 9:625. doi: 10.3389/fphys.2018.00625

Pelster B, Bagatto B. Respiration. In: Perry SF, Ekker M, Farrell AP, Brauner CJ. *Fish Physiology: Zebrafish*. Vol. 29. 1st ed. Burlington, MA: Elsevier Inc. (2010). p. 289–305.

Perez-Muñoz ME, Arrieta MC, Ramer-Tait AE, Walter J. A critical assessment of the "sterile womb" and *"in utero* colonization" hypotheses: implications for research on the pioneer infant microbiome. *Microbiome.* (2017) 5:48. doi: 10.1186/s40168-017-0268-4,

Petit J, Wiegertjes GF. Long-lived effects of administering β-glucans: indications for trained immunity in fish. *Dev Comp Immunol.* (2016) 64:93–102. doi: 10.1016/j.dci.2016.03.003

Qin C, Xie Y, Wang Y, Li S, Ran C, He S, et al. Impact of *Lactobacillus casei* BL23 on the host transcriptome, growth and disease resistance in larval zebrafish. *Front Physiol.* (2018) 9:1245. doi: 10.3389/fphys.2018.01245

Qin C, Zhang Z, Wang Y, Li S, Ran C, Hu J, et al. EPSP of *L. casei* BL23 protected against the infection caused by *Aeromonas veronii* via enhancement of immune response in zebrafish. *Front Microbiol.* (2017) 8:2406. doi: 10.3389/fmicb.2017.02406

Rawls JF, Mahowald MA, Ley RE, Gordon JI. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell.* (2006) 127:423–33. doi: 10.1016/j.cell.2006.08.043

Rawls JF, Samuel BS, Gordon JI. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *PNAS.* (2004) 101:4596–601. doi: 10.1073/pnas.0400706101

Rendueles O, Ferrières L, Frétaud M, Bégaud E, Herbomel P, Levraud JP, et al. A new zebrafish model of oro-intestinal pathogen colonization reveals a key role for adhesion in protection by probiotic bacteria. *PLoS Pathog.* (2012) 8:12. doi: 10.1371/journal.ppat.1002815

Renshaw SA, Loynes CA, Trushell DMI, Elworthy S, Ingham PW, Whyte MKB. Plenary paper A transgenic zebrafish model of neutrophilic inflammation. *Blood J.* (2006) 108:3976–9. doi: 10.1182/blood-2006-05-024075

Ringø E, Erik Olsen R, Gonzalez Vecino JL, Wadsworth S. Use of immunostimulants and nucleotides in aquaculture: a review. *J Mar Sci Res Dev.* (2012) 2:104. doi: 10.4172/2155-9910.1000104

Ringø E, Gatesoupe F-J. Lactic acid bacteria in fish: a review. *Aquaculture.* (1998) 160:177–203. doi: 10.1016/S0044-8486(97)00299-8

Robinson CD, Klein HS, Murphy KD, Parthasarathy R, Guillemin K, Bohannan BJM. Experimental bacterial adaptation to the zebrafish gut reveals a primary role for immigration. *PLoS Biol.* (2018) 16:e2006893. doi: 10.1371/journal.pbio.2006893

Roeselers G, Mittge EK, Stephens WZ, Parichy DM, Cavanaugh CM, Guillemin K, et al. Evidence for a core gut microbiota in the zebrafish. *ISME J.* (2011) 5:1595–608. doi: 10.1038/ismej.2011.38

Rolig AS, Parthasarathy R, Burns AR, Bohannan BJM, Guillemin K. Individual members of the microbiota disproportionately modulate host innate immune responses. *Cell Host Microbe.* (2015) 18:613–20. doi: 10.1016/j.chom.2015.10.009

Rolig AS, Sweeney EG, Kaye LE, DeSantis MD, Perkins A, Banse AV, et al. A bacterial immunomodulatory protein with lipocalin-like domains facilitates host–bacteria mutualism in larval zebrafish. *ELife.* (2018) 7:e37172. doi: 10.7554/eLife.37172

Siriyappagouder P, Galindo-Villegas J, Lokesh J, Mulero V, Fernandes JMO, Kiron V. Exposure to yeast shapes the intestinal bacterial community assembly in zebrafish larvae. *Front Microbiol.* (2018) 9:1868. doi: 10.3389/fmicb.2018.01868

Song SK, Beck BR, Kim D, Park J, Kim J, Kim HD, et al. Prebiotics as immunostimulants in aquaculture: a review. *Fish Shellfish Immunol.* (2014) 40:40–8. doi: 10.1016/j.fsi.2014.06.016

Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of the zebrafish, Danio rerio. *Biol Rev.* (2008) 83:13–34. doi: 10.1111/j.1469-185X.2007.00030.x

Stagaman K, Burns AR, Guillemin K, Bohannan BJM. The role of adaptive immunity as an ecological filter on the gut microbiota in zebrafish. *ISME J.* (2017) 11:1630–9. doi: 10.1038/ismej.2017.28

Stephens WZ, Burns AR, Stagaman K, Wong S, Rawls JF, Guillemin K, et al. The composition of the zebrafish intestinal microbial community varies across development. *ISME J.* (2016) 10:644–54. doi: 10.1038/ismej.2015.140

Sullam KE, Essinger SD, Lozupone CA, Connor MPO, Rosen GL, Knight R, et al. Environmental and ecological factors that shape the gut 2 bacterial communities of fish: a meta-analysis - Supplementary. *Mol Ecol.* (2012) 21:3363–78. doi: 10.1111/j.1365-294X.2012.05552.x

Talham GL, Jiang HQ, Bos NA, Cebra JJ. Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. *Infect Immun.* (1999) 67:1992–2000.

Troll JV, Hamilton MK, Abel ML, Ganz J, Bates JM, Stephens WZ, et al. Microbiota promote secretory cell determination in the intestinal epithelium by modulating host Notch signaling. *Development.* (2018) 145:dev155317. doi: 10.1242/dev.155317

Udayangani RMC, Dananjaya SHS, Fronte B, Kim CH, Lee J, De Zoysa M. Feeding of nano scale oats β-glucan enhances the host resistance against *Edwardsiella tarda* and protective immune modulation in zebrafish larvae. *Fish Shellfish Immunol.* (2017) 60:72–7. doi: 10.1016/j.fsi.2016.11.035

Ulloa PE, Medrano JF, Feijo CG. Zebrafish as animal model for aquaculture nutrition research. *Front Genet.* (2014) 5:313. doi: 10.3389/fgene.2014.00313

Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet.* (2010) 11:636–46. doi: 10.1038/nrg2842

Walker RW, Clemente JC, Peter I, Loos RJF. The prenatal gut microbiome: are we colonized with bacteria *in utero*? *Pediatric Obes.* (2017) 12:3–17. doi: 10.1111/ijpo.12217

Wallace KN, Akhter S, Smith EM, Lorent K, Pack M. Intestinal growth and differentiation in zebrafish. *Mech Dev.* (2005) 122:157–73. doi: 10.1016/j.mod.2004.10.009

Wallace KN, Pack M. Unique and conserved aspects of gut development in zebrafish. *Dev Biol.* (2003) 255:12–29. doi: 10.1016/S0012-1606(02)00034-9

Wang Y, Ren Z, Fu L, Su X. Two highly adhesive lactic acid bacteria strains are protective in zebrafish infected with *Aeromonas hydrophila* by evocation of gut mucosal immunity. *J Appl Microbiol.* (2015) 120:441–51. doi: 10.1111/jam.13002

Wang Z, Du J, Lam SH, Mathavan S, Matsudaira P, Gong Z. Morphological and molecular evidence for functional organization along the rostrocaudal axis of the adult zebrafish intestine. *BMC Genomics.* (2010) 11:392. doi: 10.1186/1471-2164-11-392

Westerfield M. *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio).* 5th ed. Eugene: University of Oregon Press, Eugene (2007).

Wetering M, van de Sancho E, Verweij C, Lau W, de Oving I, Hurlstone A, et al. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell.* (2002) 111:241–50. doi: 10.1016/S0092-8674(02)01014-0

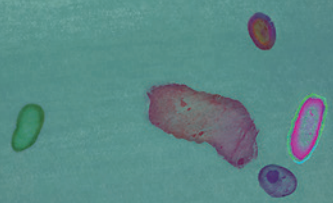
Yoder JA, Nielsen ME, Amemiya CT, Litman GW. Zebrafish as an immunological model system. *Microbes Infect.* (2002) 4:1469–78. doi: 10.1016/S1286-4579(02)00029-1

Yousefi S, Hoseinifar SH, Paknejad H, Hajimoradloo A. The effects of dietary supplement of galactooligosaccharide on innate immunity, immune related genes expression and growth performance in zebrafish (*Danio rerio*). *Fish Shellfish Immunol.* (2018) 73:192–6. doi: 10.1016/j.fsi.2017.12.022

Zang L, Ma Y, Huang W, Ling Y, Sun L, Wang X, et al. Dietary *Lactobacillus plantarum* ST-III alleviates the toxic effects of triclosan on zebrafish (*Danio rerio*) via gut microbiota modulation. *Fish Shellfish Immunol.* (2019) 84:1157–69. doi: 10.1016/j.fsi

Chapter 6

Intestinal Microbiota and Immune Modulation in Zebrafish by Fucoidan From Okinawa Mozuku (*Cladosiphon okamuranus*)



Wakako Ikeda-Ohtsubo, Adrià López Nadal, Edoardo Zaccaria, Masahiko Iha, Haruki Kitazawa, Michiel Kleerebezem and Sylvia Brugman

Frontiers in Nutrition, 24 June 2020
https://doi.org/10.3389/fnut.2020.00067

Abstract

Fucoidan represents fucose-rich sulfated polysaccharides derived from brown seaweeds, which exerts various biological activities applicable for functional foods and therapeutic agents. The objective of the present study was to investigate *in vivo* effects of fucoidan extracted from Okinawa mozuku (*Cladosiphon okamuranus*), common edible seaweed in Japan, on immune responses and microbiota composition in zebrafish. We treated larvae and adult zebrafish with Okinawa mozuku (OM) fucoidan by immersion (100 and 500 µg/mL, 3 days) and by feeding (3 weeks), respectively. The effect of OM fucoidan on immune responses in zebrafish larvae was evaluated by live imaging of neutrophils and macrophages as well as quantitative polymerase chain reaction of pro- and anti-inflammatory cytokine genes. Whole microbiota of zebrafish larvae and intestinal microbiota of adult zebrafish treated with OM fucoidan were analyzed by Illumina MiSeq pair-end sequencing of the V3–V4 region of 16S rRNA genes. Fucoidan treatment only slightly affected the composition of the larvae microbiota and the number of neutrophils and macrophages, while pro- and anti-inflammatory cytokine gene expression levels were upregulated in the larvae treated with 500 µg/mL OM fucoidan. In contrast, feeding of OM fucoidan clearly altered the intestinal microbiota composition of adult zebrafish, which was characterized by the emergence and predominance of multiple bacterial operational taxonomic units (OTUs) affiliated with *Rhizobiaceae* and *Comamonadaceae* at the expense of *E. coli*-related *Enterobacteriaceae*, the dominant OTUs throughout the studied samples. These changes were accompanied by decreased expression levels of pro-inflammatory cytokine *il1b* in the intestines of the adult zebrafish. Our current study provides the first insights into *in vivo* modulatory effects of fucoidan on microbiota and immune responses of unchallenged zebrafish, which underscores the potential of fucoidan to play a modulatory role in the diet–microbiota–host interplay.

Introduction

Fucoidan represents polysaccharides consisting of α-(1 → 3) or α-(1 → 4) -linked L-fucose residues with sulfate substitutions, which occasionally contain acetate, glucuronic acid, and monosaccharides such as mannose and galactose (Ale and Meyer, 2013). Fucoidan from different algal origins has been reported to exhibit unique properties such as anti-inflammatory, antiallergic, antitumor, or antiviral effects (Vo and Kim, 2012; Fitton *et al.,* 2019) and is therefore recognized as a prospective ingredient for functional foods and for therapeutic agents (Chollet Fitton *et al.,* 2016; Fitton *et al.,* 2019). Although beneficial effects of fucoidan have been well-studied and described, daily intake of fucoidan from brown seaweed is still not common in Western countries. In Japan, daily seaweed consumption can exceed ~5 g/day (Lange *et al.,* 2015) and the brown seaweed *mozuku* represents one of the most common edible seaweeds, which is usually consumed raw. Okinawa mozuku (*Cladosiphon okamuranus*) is exclusively cultivated and used in the traditional cuisine on the Okinawan Islands in Japan, a region that is well-known for its high prevalence of centenarians and the general healthy states of its elderly population (Willcox *et al.,* 2017). Fucoidan extracted from Okinawa *mozuku* (OM fucoidan) has a simple structure with a backbone of α-(1 → 3) fucopyranose, substituted with sulfate and α-glucuronic acid at ~50 and 17% of its residues, respectively (Nagaoka *et al.,* 1999). Similar to what has been shown for fucoidan derived from other origins, OM fucoidan has been reported to exert antitumor and antiviral effects. In a murine model, antitumor activity has been attributed to the fucoidan-mediated stimulation of macrophages and natural killer cells (Takeda *et al.,* 2012; Nagamine *et al.,* 2020), while antiviral activities seem to be more complex and may involve both host–virus and virus–fucoidan interactions. Previous studies have reported antiviral activities of OM fucoidan against human T-cell leukemia virus type 1 (HTLV-1) (Araya *et al.,* 2011; Haneji *et al.,* 2015), dengue virus type 2 (Hidari *et al.,* 2008), hepatitis C (Mori *et al.,* 2012), Newcastle disease virus (DSV) in poultry (Elizondo-Gonzalez *et al.,* 2012; Trejo-Avila *et al.,* 2016), and canine distemper virus (CDV) (Trejo-Avila *et al.,* 2014). Collectively, these studies support the high potential of OM fucoidan as a therapeutic agent in viral infections.

Meanwhile, effects of OM fucoidan on the intestinal microbiota remain poorly understood. Polysaccharides such as fucoidan have a potential to not only mechanistically interfere with host–microbiota interactions but also to serve as nutrition for bacteria constituting the microbiota (Shang *et al.,* 2018 ; Ma *et al.,* 2018). Since no enzymes digesting fucoidan have been found in animal intestinal tracts, fucoidan can reach the lower intestinal tract intact and may confer beneficial effects on microbiota as prebiotics (Michel *et al.,* 1996; de Jesus Raposo *et al.,* 2016; Brugman *et al.,* 2018). Importantly, some studies have suggested that bioactivities of fucoidan may be attributable to its modulatory effects on gut microbiota. A recent study has shown that fucoidan from *Undaria pinnatifida*

can affect host lipid metabolism by modulating the gut microbiota composition (Chen *et al.,* 2019), which may also explain the effect of OM fucoidan to ameliorate dyslipidemia in rodents (Yokota *et al.,* 2016). Other studies have reported that fucoidan from sea cucumber (*Acaudina molpadioides*) and *hijiki* seaweed (*Sargassum fusiforme*) can relieve symptoms of diabetes by modulating gut microbiota (Hu *et al.,* 2019; Cheng *et al.,* 2019).

Considering possible interactions between microbiota and host immune responses, it is crucial to evaluate host immunity and microbiota simultaneously to elucidate the prebiotic potential OM fucoidan (Ma *et al.,* 2018). Zebrafish offer an ideal *in vivo* model to investigate how fucoidan affects host immunity and microbiota under normal (unchallenged) conditions because of their compatibility with live visualization (López Nadal *et al.,* 2020). Using a double-transgenic zebrafish model combined with next-generation sequencing of 16S rRNA genes, we have recently shown that microbiota modulation by antibiotics can significantly affect host inflammatory immune responses in zebrafish larvae immersed in saponin (López Nadal *et al.,* 2018). In this study, we exploited this approach to investigate how OM fucoidan can affect immune response and microbiota composition of zebrafish larvae. We also investigated the effect of OM fucoidan on immune responses and intestinal microbiota of adult zebrafish, which were fed with OM fucoidan for 3 weeks.

Materials and methods

Ethics statement

The present study was approved by the Dutch Committee on Animal Welfare and the Animal Welfare Body (IvD) of Wageningen University, The Netherlands. Furthermore, we adhere to our standard biosecurity and institutional safety procedures at Wageningen University and Research.

Zebrafish and fucoidan

Tg (mpeg1:mCherry/mpx:eGFPi^{114}) (Renshaw *et al.,* 2006; Bernut *et al.,* 2014) and wild-type zebrafish were maintained in Zebtec family tanks (Tecniplast, Buguggiate, Italy) under continuous flow-through at 28°C (14/10-h light/dark cycle) and fed daily with Tetramin Flakes (Tetra, Melle, Germany). For the experiments using zebrafish larvae, embryos were obtained from the adult transgenic zebrafish by natural spawning and raised with embryo medium (E3) water as described previously (López Nadal *et al.,* 2018). OM fucoidan powder (>95% pure fucoidan) extracted from *C. okamuranus* as described previously (Nagamine *et al.,* 2009) was provided by South Product Co., Ltd., Okinawa, Japan. The characteristics of this fucoidan were as follows: average molecular weight of 49.8 kDa, L-fucose content of

52.7%, uronic acid content of 18.0%, and sulfate ion content of 17.6%. The OM fucoidan powder was stored at room temperature until use. Fucoidan treatment of larvae and adult zebrafish was performed as follows: the zebrafish larvae (3 days post fertilization; dpf) were randomly distributed in six-well plates (n = 8 fish/well) and kept in different concentrations (0, 100, and 500 µg/mL of E3 water) of OM fucoidan (immersion) until 6 dpf. Ten adult zebrafish were maintained in two separate tanks in a continuous flow and temperature-controlled (28°C) system and fed once daily with Tetramin Flakes (control group) or a combination of the flakes and OM fucoidan at the ratio of 1:1 (fucoidan group) over 3 weeks.

In vivo imaging of neutrophils and macrophages in zebrafish larvae

Tg (mpeg1:mCherry/mpx:eGFPi[114]) zebrafish larvae were anesthetized with MS-222 (tricaine methane sulfonate) solution and embedded in 1% low melting point agarose (Thermo Fisher Scientific, Waltham, MA, USA), as previously described (López Nadal *et al.,* 2018). Larvae were imaged as whole mounts with a Leica M205 FA Fluorescence Stereo Microscope. Neutrophils and macrophages in the intestinal region of each specimen were quantified by counting the total number of cells per defined area using the cell counter plugin available in ImageJ® software (Abràmoff *et al.,* 2004).

Relative gene expression by quantitative polymerase chain reaction

Zebrafish were euthanized with MS-222 and the whole larvae (five or six fish per 1.5-mL tube) were preserved in RNA later™ at –20°C. Adult zebrafish were anesthetized with MS-222 and intestines were isolated by dissection and were preserved in RNA later™ at –20°C. Total RNA was isolated from larvae or intestinal samples from adult zebrafish using the RNeasy® Micro Kit (QIAGEN, Venlo, The Netherlands) according to the manufacturer's instructions. After quantifying RNA by a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), cDNA was generated from 1 µg of RNA using Superscript™ III First Strand Synthesis Systems (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The diluted cDNA corresponding to 125 ng of RNA was used as a template for each reaction of quantitative polymerase chain reaction (qPCR) using the ABsolute™ qPCR SYBR® Green Mix (Thermo Fisher Scientific, Waltham, MA, USA) as previously described (López Nadal *et al.,* 2018). The sequences of the primer used in this study can be found in **Table S1**. The amplification data of each sample were normalized to the reference gene *elf1*α and calculated using the Pfaffl quantification method with efficiency correction (Pfaffl, 2001), as described by Forlenza et al. (Forlenza *et al.,* 2012). The statistical significance of differences between the control and fucoidan-treated groups was assessed by a one-way analysis of variance (ANOVA) test using R version 3.5.3 (R Core Team, https://www.R-project.org/) where <0.05 was regarded as significant.

16S rRNA-based analyses of zebrafish microbiota

Zebrafish were euthanized with MS-222 and the whole larvae (four fish per 1.5-mL tube) were washed with sterilized phosphate-buffered saline and preserved at –20°C. Adult zebrafish were euthanized with MS-222 and the intestines were isolated by dissection. The intestinal contents were preserved in InhibitEX Buffer supplied in the QIAamp® DNA Fast Stool Mini Kit (QIAGEN, Venlo, The Netherlands) at –20°C. Total DNA was isolated from the whole larvae or intestines of adult zebrafish using the QIAamp® DNA Fast Stool Mini Kit according to the manufacturer's instructions. Pair-end sequencing was performed using Illumina MiSeq (BaseClear, Leiden, The Netherlands) using amplicons generated with the primer pair 341F–785R that target the V3–V4 variable region of the 16S rRNA gene of most bacteria (Klindworth *et al.,* 2013). Raw Illumina sequencing reads were pair-ended, end-trimmed, filtered, and clustered into operational taxonomic units (OTUs) using the microbial genomic module 3.0 implemented in the CLC Bio Genomics Workbench v7.5.1 (QIAGEN, Venlo, The Netherlands), 16S Microbiome Pipeline in the EZBioCloud web server (Yoon *et al.,* 2017), or the MICCA pipeline (Albanese *et al.,* 2015), for which OTU assignment was performed using the SILVA ribosomal RNA reference database [release 128, 97% similarity threshold, (Quast *et al.,* 2013)], the EZBioCloud database (Yoon *et al.,* 2017), and the Ribosomal Database Project (RDP) classifier [version 2.11, 97% identity threshold, (Wang *et al.,* 2007)], respectively. After confirming the reproducibility of the core microbiota composition of each sample, OTU tables in BIOM format generated by the CLC Bio Genomics Workbench was used for statistical analyses of the diversity and richness (alpha- and beta-diversity) implemented in MicrobiomeAnalyst© using the default filtering parameter settings (Dhariwal *et al.,* 2017). Significantly different taxa between control and fucoidan-treated group were identified by differential abundance (DESeq2) analysis by R version 3.5.3 (R Core Team, https://www.R-project.org/) and by Linear Discriminant Analysis Effect Size (LEfSe) analysis implemented with EZBioCloud (Segata *et al.,* 2011; Yoon *et al.,* 2017).

Statistical analysis

The quantified data collected from the fluorescent *in vivo* imaging of the zebrafish larvae and qPCR was analyzed using Student's *t*-test assuming unequal variation as well as one-way analysis of variance (ANOVA) test using Microsoft Excel® and R version 3.5.3 (R Core Team, https://www.R-project.org/), where <0.05 was regarded as significant. The indices of α-diversity and β-diversity for comparing compositional structure of microbiota of each larva and adult zebrafish group were calculated on a species-level summarization of the rarefied OTU tables generated as described in the preceding text. Chao1 and Abundance-based Coverage Estimator (ACE) as well as Shannon and Simpson indices were used to measure the species-level community richness and species level community

evenness, respectively, and each index was calculated using the online module of Microbiomeanalyst© (Dhariwal *et al.,* 2017). The plots of β-diversity indicating dissimilarities between samples were produced by principal coordinates analyses (PCoA) calculated using the Bray–Curtis dissimilarity index implemented in Microbiomeanalyst© (Dhariwal *et al.,* 2017).

Results

Effect of OM fucoidan on innate immunity of zebrafish larvae

Zebrafish larvae (3–6 dpf) treated with OM fucoidan (0, 100, and 500 µg/mL of E3 water) by immersion showed normal development without visible signs of damage relative to untreated controls (data not shown). To investigate whether the treatment with OM fucoidan affected cellular immunity of zebrafish larvae, the numbers of neutrophils (mpx:GFP) and macrophages (mpeg1:mCherry) in the intestinal area of the control and the fucoidan-treated larvae were compared. There was no observable difference between the live-imaged control and the fucoidan-treated larvae (**Figure 1A**). The cell counts of neutrophils and macrophages in fucoidan-treated zebrafish larvae tended to be reduced compared to the control, but the difference was not significant ($P > 0.1$, **Figure 1B**).

Using the same experimental setup, we performed qPCR to measure the relative expression levels of selected genes representing host immune cell responses in the fucoidan (500 µg/mL) -treated zebrafish larvae and the untreated control. The relative gene expression levels of pro- (*il1b, tnfa*) and antiinflammatory (*il10*) cytokines as well as *mmp9* were moderately (1.7–2.2 fold) upregulated in the larvae treated with 500 µg/mL of fucoidan (**Figure 2A**). No significant difference was observed for *cxcl-8a* (**Figure 2A**) and gene transcripts for *il-17f* , *il-22*, and *tnfb* were not detected in our samples (data not shown).

Adult zebrafish fed with OM fucoidan for 3 weeks did not show visible changes in fitness and behavior compared to controls fed the Tetramin Flakes only (data not shown). Immune responses of the adult zebrafish intestines were examined by quantitative PCR using primers specific for *il1b, il-10, cxcl-8a, tnfa*, and *mmp9*. Overall, there was no significant difference between the fucoidan-fed zebrafish and the control, except for *il1b*, which was expressed at slightly decreased levels (0.63-fold) in the fucoidan-fed zebrafish compared to the control (**Figure 2B**).

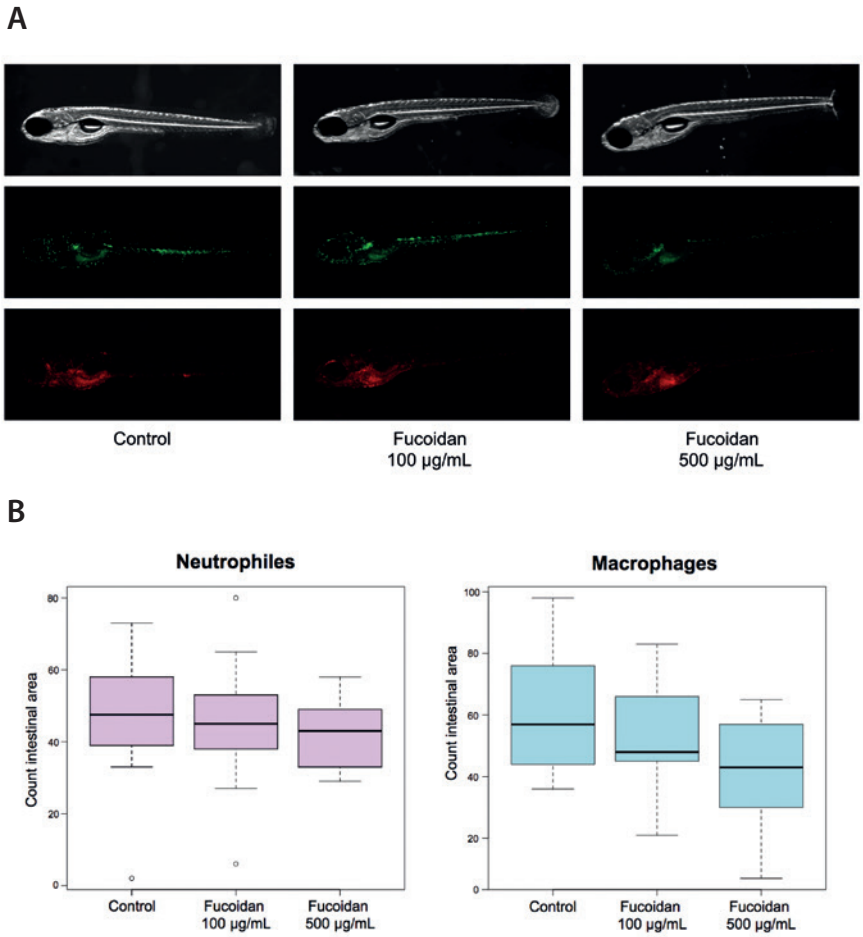


Figure 1: Effect of OM fucoidan on lymphocyte recruitment to the intestinal region of zebrafish larvae. **(A)** Representative pictures of 6 dpf zebrafish larvae (mpeg:mCherry, mpx:GFP) displaying neutrophils (green) and macrophages (red). F100, the zebrafish larvae treated with 100 µg/mL OM fucoidan from 3 dpf for 3 days; F500, the zebrafish larvae treated with 500 µg/mL OM fucoidan from 3 dpf for 3 days. **(B)** Quantification of neutrophils and macrophages in the intestinal area of larval zebrafish. Control, no treatment (n = 14); F100, treated with 100 µg/mL (n = 13); F500, treated with 500 µg/mL (n = 17).

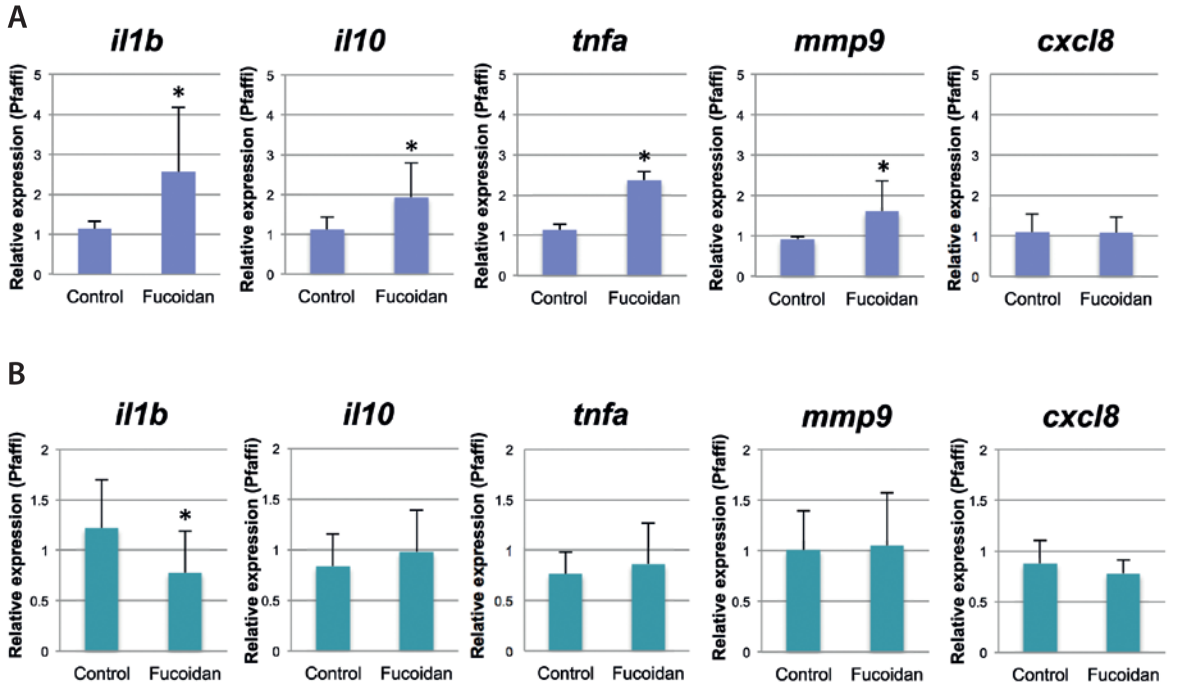


Figure 2: Relative gene expression of immune genes of zebrafish treated with OM fucoidan. **(A)** Control, 6 dpf zebrafish larvae; Fucoidan, 6 dpf zebrafish larvae immersed in 500 µg/mL fucoidan from 3 dpf for 3 days. **(B)** Control, intestinal samples of adult zebrafish fed with standard fish meal for 3 weeks; Fucoidan, intestinal samples of adult zebrafish fed with OM fucoidan meal (Tetramin fish flakes: OM fucoidan = 1:1). The asterisk denotes significant differences from the control samples ($P < 0.05$, 1-way ANOVA).

Effect of OM fucoidan on microbiota diversity and composition of larval and adult zebrafish

The effects of OM fucoidan on diversity of larval and adult zebrafish microbiota were analyzed by 16S rRNA gene amplicon sequencing. Whole-body DNA samples of pools of 6 dpf zebrafish larvae were obtained from the three groups (n = 4 per group; immersion in 0, 100, and 500 µg/mL of OM fucoidan in E3 water) and used for Illumina MiSeq sequencing of 16S rRNA genes. For adult zebrafish, intestinal DNA from each of the two groups (n = 5 per group; control vs. OM-fucoidan fed) was used. A summary of the sequencing results is shown in **Table S2** and the rarefaction curves of all samples, except one sample (WO3, a control sample of zebrafish larvae), reached saturation (**Figure S1**). In microbiota of zebrafish larvae, no significant differences were observed in the species richness (Chao1, P = 0.47265; ACE; P = 0.74339; **Figure 3A**) or the species evenness (Shannon, P = 0.96621; Simpson, P = 0.96058; **Figure 3B**) between the control and fucoidan-treated fish, regardless of the fucoidan concentrations. In intestinal microbiota of adult zebrafish, the species richness was also not affected (Chao1, P = 0.88482; ACE; P = 0.90299; **Figure 3C**), while the species evenness tended to be moderately increased in the fucoidan-fed zebrafish (Shannon, P = 0.079088; Simpson, P = 0.078456; **Figure 3D**). β-Diversity analyses showed no significant association between fucoidan treatment and microbiota composition of zebrafish larvae (P < 0.49845; **Figure 3E**), which was in contrast to adult zebrafish, in which fucoidan-feeding was moderately associated with changes in the species composition of intestinal microbiota (P < 0.023, **Figure 3F**). Collectively, these results indicate that the treatments with OM fucoidan affected the diversity and composition of intestinal microbiota of adult zebrafish, but not the larvae zebrafish microbiota. Taxonomic assignment of OTUs generated from each sample was performed using EzBioCloud database (https://www.ezbiocloud.net), which offers a high genus and species-level resolution (Yoon *et al.,* 2017). Consistent with our previous work (López Nadal *et al.,* 2018), microbiota of zebrafish larvae was predominated (>95%) by Enterobacteriaceae (**Figure 4A**), which were affiliated with the *Escherichia coli* group (**Figure S2, Table S3**). Consistent with the diversity analyses (**Figure 3**), the relative abundance of each bacterial species and OTUs were similar between the larvae samples and did not reflect an effect of OM fucoidan (**Figure 4A**, **Figure S2**). In addition, DESeq2 and LEfSe analyses failed to identify significantly different taxa between the control and fucoidan-treated zebrafish larvae. In contrast, a significant difference was observed in the intestinal microbiota between the control and fucoidan-fed adult zebrafish (**Figures 4B**,**C**, **Figure S3**). The class to species-level composition of intestinal microbiota of fucoidan-fed zebrafish was clearly different from the control (**Figure S3**), and this difference is characterized by the emergence and increase of relative abundance of several bacterial groups affiliated with *Comamonadaceae* and *Rhizobiaceae* (**Figure 4B**, **Figure S3**). At the genus and species

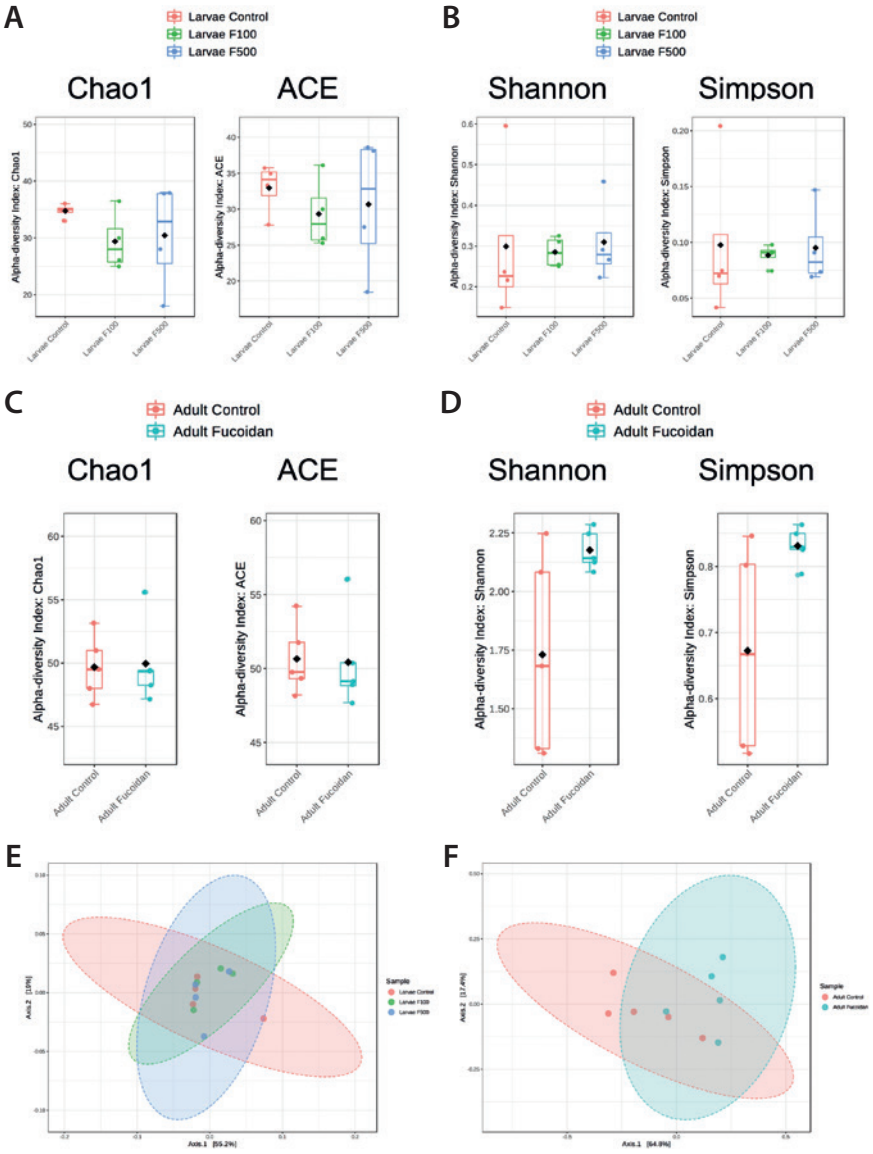


Figure 3: Effect of OM fucoidan on the diversity of larvae zebrafish microbiota and adult zebrafish intestinal microbiota. Species-level community richness **(A,C)** and species level community evenness **(B,D)** were compared between larvae zebrafish samples (A, B, E; F100, the zebrafish larvae treated with 100 μg/mL OM fucoidan from 3 dpf for 3 days; F500, the zebrafish larvae treated with 500 μg/mL OM fucoidan from 3 dpf for 3 days) and adult zebrafish intestinal samples (C, D, F; Fucoidan [Tetramin fish flakes: OM fucoidan = 1:1] for 3 weeks). Beta-diversity of larval **(E)** and adult intestinal **(F)** microbiota compared by the principal coordinates analyses (PCoA) based on Bray–Curtis dissimilarity index.

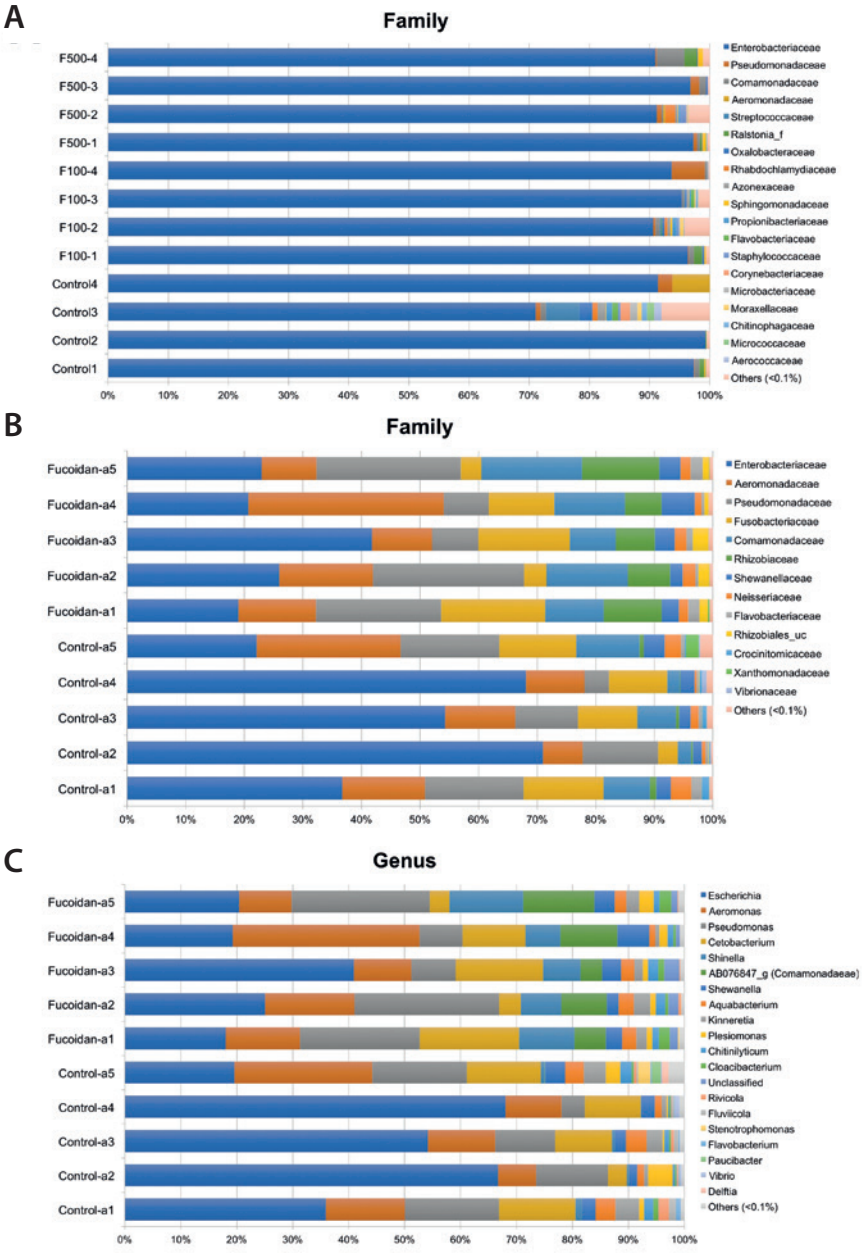


Figure 4: Effects of OM fucoidan on the bacterial community structure of larvae zebrafish microbiota and adult zebrafish intestinal microbiota. **(A)** Composition of family-level bacterial groups in the larvae zebrafish microbiota (F100, the zebrafish larvae treated with 100 μg/mL OM fucoidan from 3 dpf for 3 days; F500, the zebrafish larvae treated with 500 μg/mL OM fucoidan from 3 dpf for

3 days.). **(B)** Composition of family-level bacterial groups in the adult zebrafish intestinal microbiota. **(C)** Composition of genus-level bacterial groups in adult zebrafish intestinal microbiota. (B,C) Fucoidan [Tetramin fish flakes: OM fucoidan = 1:1] for 3 weeks). The taxonomic assignment is based on the latest EZbioCloud database (36). Taxa representing <0.1% of the total community are not visualized.

level, these families were represented by unclassified genus of *Comamonadaceae* [AB076847, (Khan *et al.,* 2012)] and the genus *Shinella granuli* group (Rhizobiaceae) (**Figure 4C**, **Figure S3**). The predominant families *Comamonadaceae* and *Rhizobiaceae* were also identified as the significantly different taxa represented in the fucoidan-fed zebrafish by the DESeq2 and LEfSe analyses (**Figures 5A**,**B**). Interestingly, both analyses revealed that the increase of *Comamonadaceae* and *Rhizobiaceae* were concomitant with the decrease of Enterobacteriaceae (**Figures 5A**,**B**, **Figure S3**). LEfSe analysis also found that unclassified groups of *Rhizobiales* and *Betaproteobacteria* were significantly associated with the fucoidan-feeding, while *Flavobacteriia* (phylum *Bacteroides*) were negatively affected (**Figure 5B**).

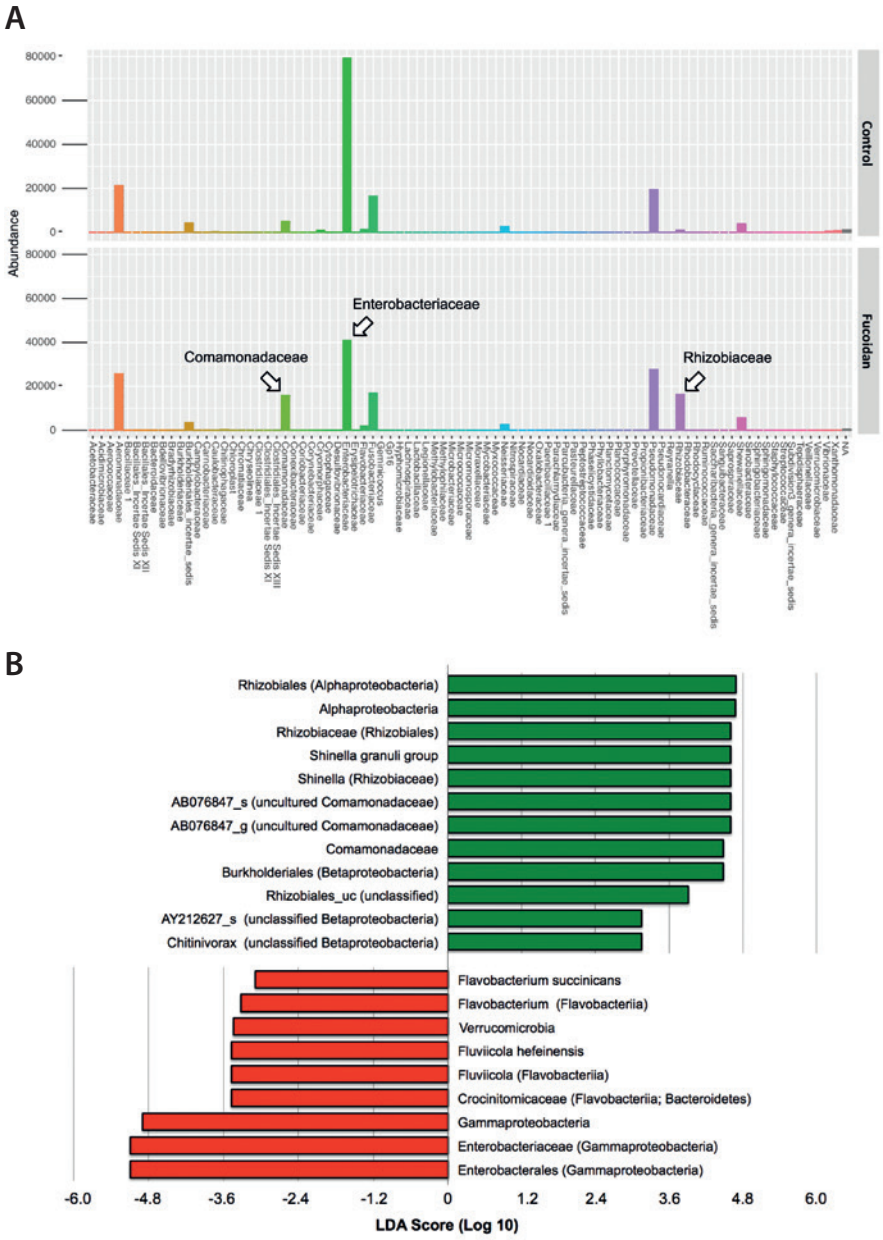


Figure 5: Significantly different taxa in the adult zebrafish intestinal microbiota associated with the OM fucoidan treatment. **(A)** Differently abundant bacterial families between the intestinal microbiota of the control and OM fucoidan-fed adult zebrafish, identified by differential abundance analysis using DESeq2 in R. The taxonomy assignment of the OTU dataset used is based on the RDP classifier (version 2.11, 97% identity threshold, 39). **(B)** Specific bacterial groups positively (green)- and negatively (red)-

associated with OM fucoidan treatment. Identification of the significantly different taxa and LDA score calculation were performed by Linear discriminant analysis effect size (LEfSe) tool implemented with EZBioCloud (36). The taxonomic assignment is based on the latest EZbioCloud database (36).

Discussion

Our current study showed that fucoidan derived from Japanese brown seaweed *C. okamuranus* has the potential to modulate the intestinal microbiota of adult zebrafish. The profound compositional change associated with the fucoidan-feeding in adult zebrafish can be characterized by the increased abundance of bacterial groups affiliated with Comamonadaceae and Rhizobiaceae and a decreased abundance of Enterobacteriaceae. Although non-pathogenic bacteria of the *E. coli* species have been proposed to confer a protective effect on zebrafish larvae via lipopolysaccharide (LPS) tolerance and acid production (Novoa and Figueras, 2012; Nag *et al.,* 2018), numerous studies have reported proinflammatory effects of Enterobacteriaceae in fish (Pressley *et al.,* 2005; Swain *et al.,* 2008). In addition, Enterobacteriaceae are thought to be responsible for the spread of antimicrobial resistance in aquatic environments (Dib *et al.,* 2018). In this context, it is intriguing that the relative expression of *il1b*, a proinflammatory cytokine, was moderately downregulated in the fucoidan- fed adult zebrafish (**Figure 2B**), which suggests that OM fucoidan may have directly or indirectly suppressed the dominance of Enterobacteriaceae that can induce proinflammatory responses. This type of diet–microbiota–host interplay is likely to play a crucial role in pro- and anti-inflammatory states in animal intestines (reviewed in Makki *et al.,*

2018), and it is therefore of great interest further investigate in future studies on the mechanism that OM fucoidan decreases the relative abundance of Enterobacteriaceae in the fish intestine with regard to how it may impact the health of the fish population.

While metabolic and physiological properties of the intestinal bacteria of adult zebrafish that responded to the fucoidan feeding are yet to be determined, the increase of *Comamonadaceae* and *Rhizobiaceae* suggests their involvement in the degradation of OM fucoidan. Interestingly, *Comamonadaceae* and *Rhizobiaceae* have been frequently found in a nitrogen removal process in wastewater treatment systems called solid-phase denitrification (Hiraishi and Khan, 2003), where solid biodegradable polymers are used as carbon sources for denitrifying bacteria (Chu and Wang, 2013). This system is also applicable for nitrogen removal in aquaculture, where increased nitrate concentration poses negative effects on fish (Luo *et al.*, 2020). Indeed, the unclassified Comamonadaceae

[AB076847, Khan *et al*., 2002] and *Shinella* spp., the intestinal abundance of which increased in response to the fucoidan feeding of adult zebrafish in our current study, have been reported to belong to denitrifying bacteria possessing biodegrading abilities of diverse compounds including biopolymers and xenobiotics (Khan *et al*., 2002; Ntougias *et al*., 2015; Wu *et al*., 2016). Therefore, it seems plausible that OM fucoidan may serve as a carbon source for intestinal bacteria of adult zebrafish, and identification of the degradation pathways involved awaits further investigation.

Although a large body of studies has documented that the host innate immunity plays significant roles for shaping microbiota and vice versa (Hooper *et al*., 2012; López Nadal *et al*., 2020), our current results imply that the compositional structure of microbiota is not strongly correlated to the expression patterns of host immune genes. Feeding of OM fucoidan for 3 weeks profoundly altered gut microbiota composition in adult zebrafish; however, the analysis of a selected set of immune genes only showed a slight reduction in the expression of *il1b*. In contrast, the exposure of zebrafish larvae to OM fucoidan resulted in changes in expression of immune genes (*il1b, il10, tnfa*, and *mmp9*) but did not affect the microbiota composition. These results might be partly explained by the timing and duration of the exposure. Since larval feeding starts in the immersion window, it is expected that the immune system of these developing larvae is responding to novel antigens that it is exposed to. In contrast, in adult fish, the immune system has fully developed and a proper homeostasis is reached at the mucosal surfaces such as the intestines (Brugman, 2016). Furthermore, since we only evaluated the immune response in the intestines at 3 weeks after feeding, the initial immune modulatory effect of primary exposure to fucoidan might have been missed, while the microbiota had 3 weeks to adapt to the new substrate provided. Future studies will include multiple time points to address early vs. late immune modulatory effects at different time points in life (of fish).

Furthermore, as has been shown in a study by Burns et al. using innate immune-deficient Myd88 knockout zebrafish (Burns *et al*., 2017), the gut microbiota composition can be better explained by the interhost dispersal effect, i.e., transmission and sharing microbiota among hosts, than immune gene expression patterns. Also, Stagaman et al. have reported that the effects of adaptive immunity on microbiota composition can be overwhelmed by other factors derived from co-housing within the same tank (Stagaman *et al*., 2017). Our study also reflected this phenomenon, since all adult zebrafish fed with OM fucoidan showed the same compositional changes in the relative abundance of specific bacterial groups (**Figures 4B**,**C**, **Figure S3**). The interhost dispersal of *Comamonadaceae* and *Rhizobiaceae* among adult zebrafish associated with the fucoidan-feeding suggests that these specific bacterial groups are subject to filtering by local host environments. Further studies are warranted to determine whether the interhost dispersal and OM fucoidan reciprocally affected the microbial composition.

In contrast to previous studies reporting inhibitory effects of fucoidan on inflammatory responses of injury zebrafish models (Lee *et al*., 2013; Jeong *et al*., 2017), the influence of OM fucoidan treatments on the baseline zebrafish immune responses were rather mild in our current study. In addition, while a previous study has implied a high concentration of fucoidan may be cytotoxic (Kim and Joo, 2008), immersion of zebrafish larvae (3–6 dpf) in OM fucoidan at concentrations of 100 and 500 μg/mL did not affect their fitness. In previous studies using LPS-challenged zebrafish models (Lee *et al*., 2013; Jeong *et al*., 2017), the antiinflammation effects of fucoidan may be rather explained by the interference of LPS–host interaction rather than direct modulation of host immunity. Another interpretation of the less profound effect on the baseline immune response of zebrafish to fucoidan is that zebrafish immunity may have evolved to become tolerant to the constituents of blown algae abundant in their original habitats (Spence *et al*., 2008).

Our current finding that OM fucoidan modulated the gut microbiota composition of zebrafish is in line with studies using rodents (Yokota *et al*., 2016; Chen *et al*., 2019). The improvement of diabetic symptoms attributed to the modulation of gut microbiota by fucoidan that have been shown in previous studies (Hu *et al*., 2019; Cheng *et al*., 2019) implies that fucoidan feeding and the subsequent alteration of intestinal microbiota may also affect metabolic properties of fish. Future studies toward a better understanding of the commonalities between intestinal microbial metabolism and host responses shared by fish and animals (Rawls *et al*., 2006; Ikeda-Ohtsubo *et al*., 2018) will help us to evaluate the potential of OM fucoidan as a new prebiotic in aquaculture (Ringø *et al*., 2010).

Conclusion

Treatment with OM fucoidan moderately modulated the relative expression of innate immune genes in larvae zebrafish, while no change in microbiota composition was observed. In adult zebrafish, feeding OM fucoidan increased the relative abundance of *Comamonadaceae* and *Rhizobiaceae* at the expense of Enterobacteriaceae, which was accompanied by a slight decrease of relative expression of a proinflammatory gene *il1b*, which suggests a potential of OM fucoidan to shift the microbial composition to an anti-inflammatory state by selectively suppressing populations of bacteria that are associated with proinflammatory responses. To our knowledge, this is the first study to describe *in vivo* modulatory effects of fucoidan on microbiota and immune responses of unchallenged zebrafish.

Data availability statement

The raw sequence data files were deposited in the NCBI sequence read archive (SRA) database under BioProject number PRJNA597701 (SRA accession numbers listed in Supplementary File "data sheet 3.xlsx").

Ethics statement

The animal study was reviewed and approved by Dutch Committee on Animal Welfare (2017.W-0034) and the Animal Welfare Body (IvD) of the Wageningen University (Netherlands).

Authors Contribution

WI-O, AL, MK, and SB designed the research. WI-O, AL, and SB conducted the experiments. WI-O, AL, EZ, MK, and SB analyzed the data. WI-O and SB wrote the article. MK and SB provided the funding. All authors critically reviewed the manuscript and approved the manuscript.

Funding

This work was financially supported by the Japan Society for the Promotion of Science (JSPS) through JSPS Core-to-Core Program (Advanced Research Networks) entitled Establishment of International Agricultural Immunology Research-Core for a Quantum Improvement in Food Safety. WI-O was supported by WIAS (Wageningen Institute of Animal Sciences) fellowship (WIAS1807229) provided by Graduate School of Animal Science of Wageningen University and Research and Leading Young Researcher Overseas Visit Program of Tohoku University.

Conflict of interest statement

MI is employed by the company South Product Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

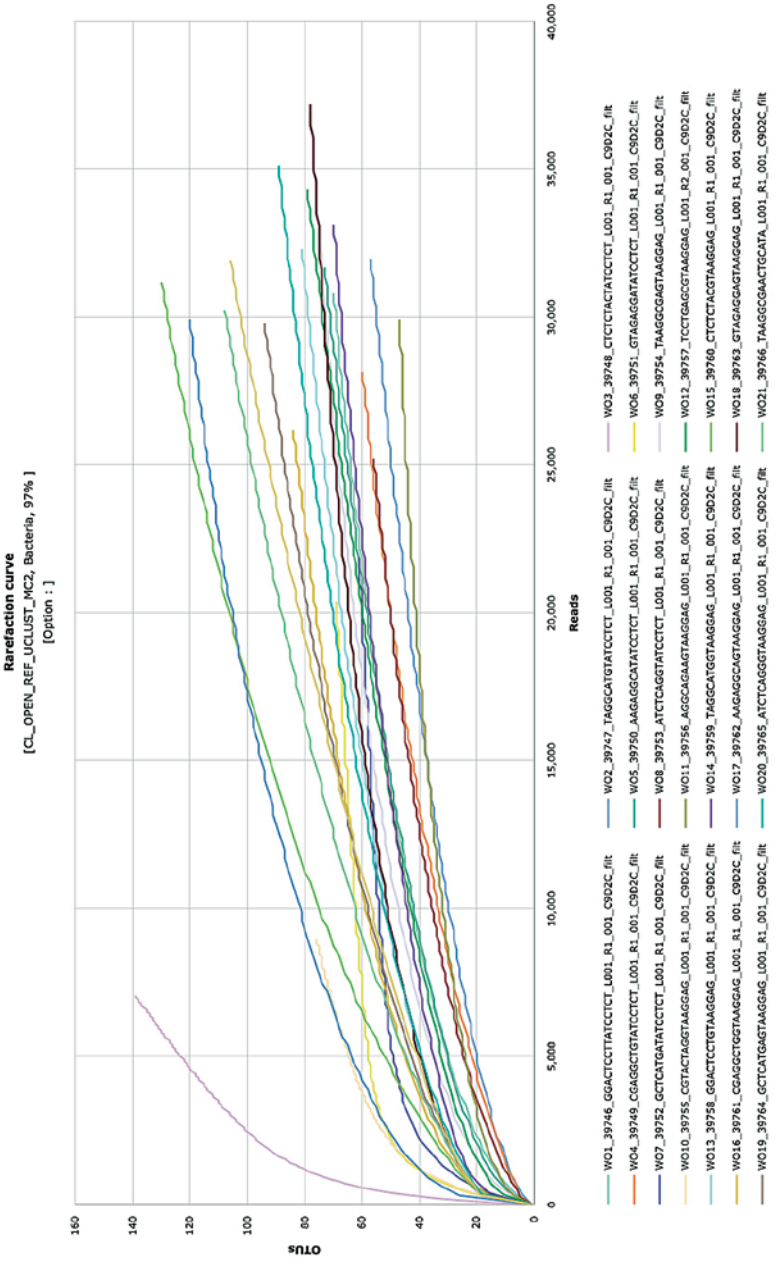


Figure S1: Alpha rarefaction curve of the total number of 16S rRNA gene sequencing from zebrafish samples.

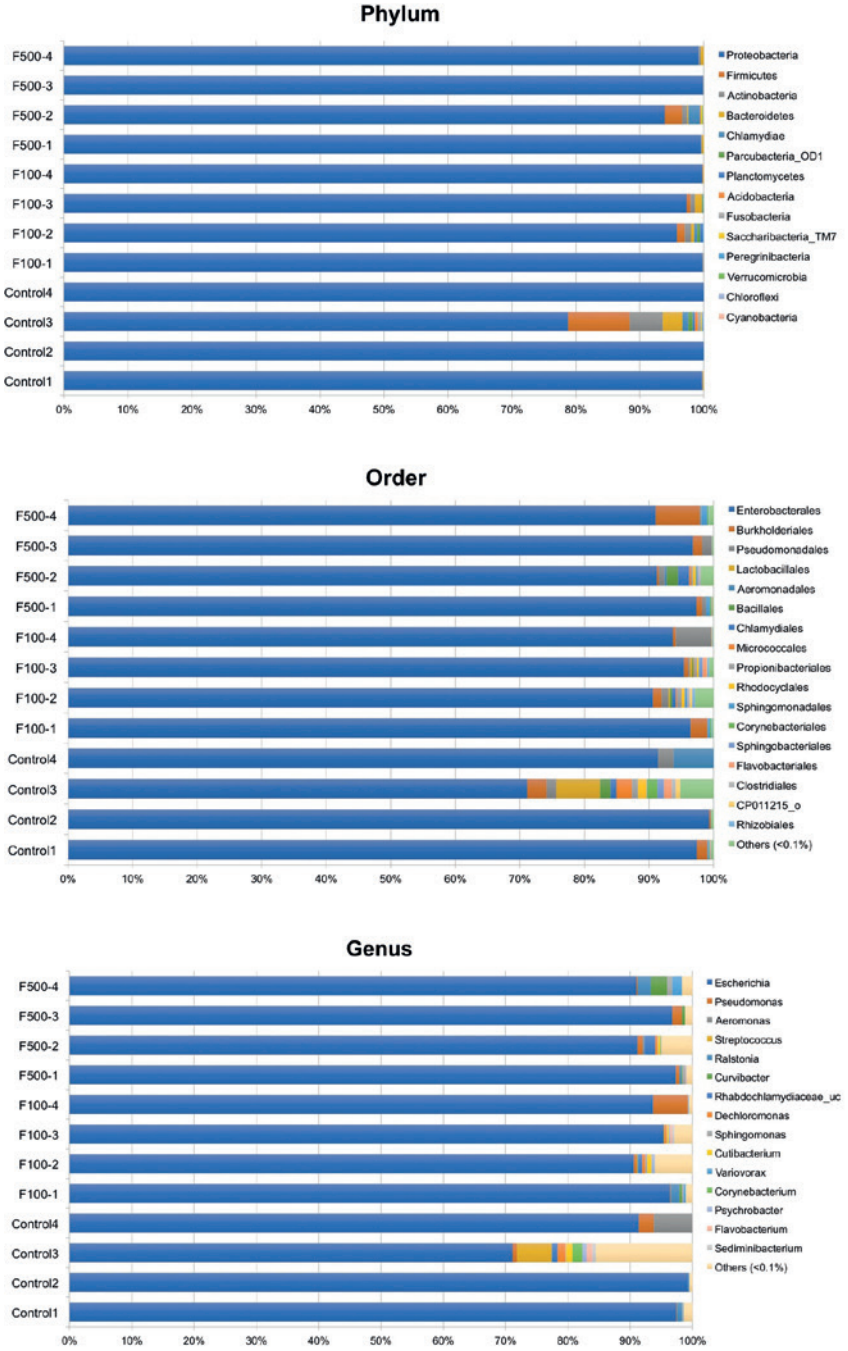


Figure S2: Overview of composition of bacterial groups in the larval microbiota.

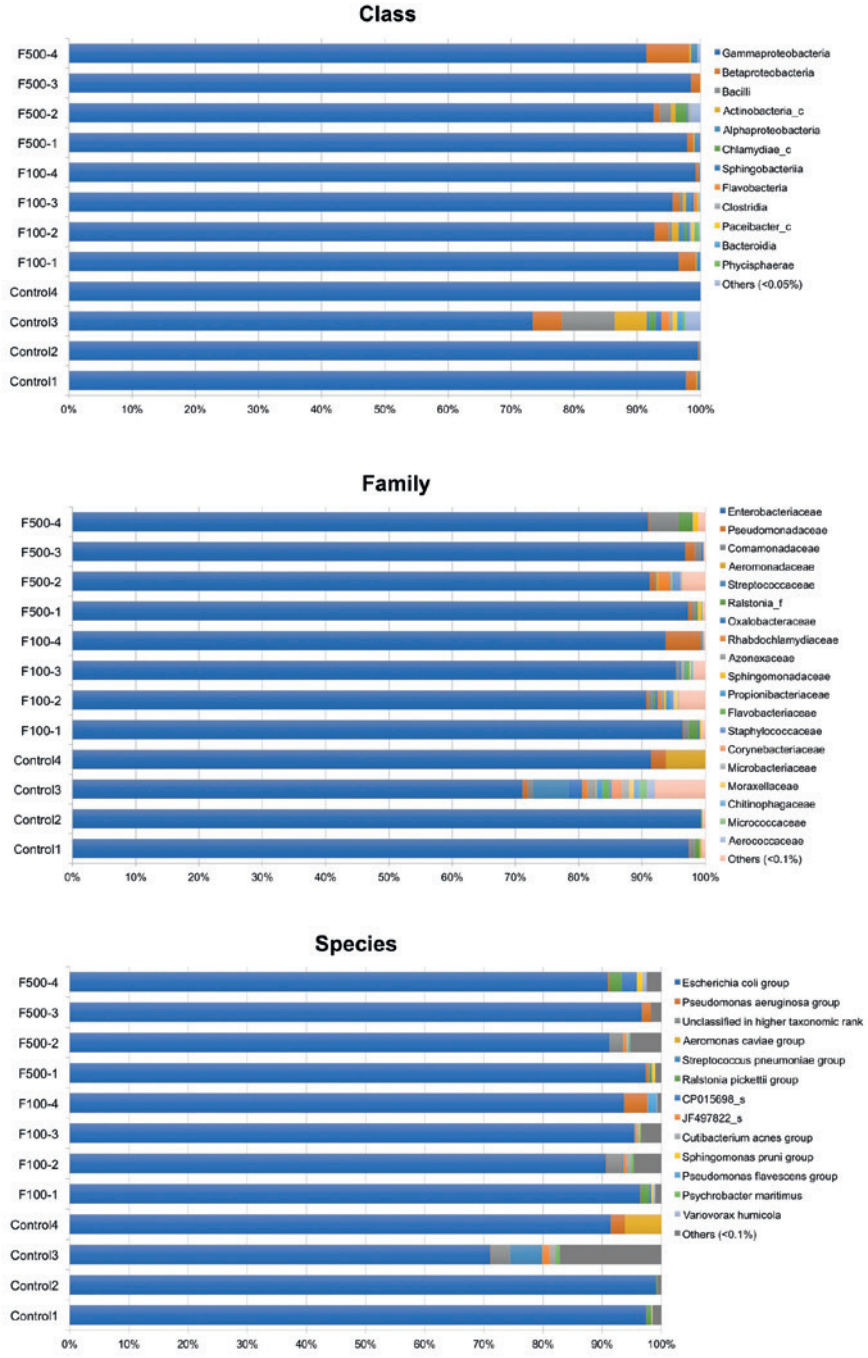


Figure S2: Continued.

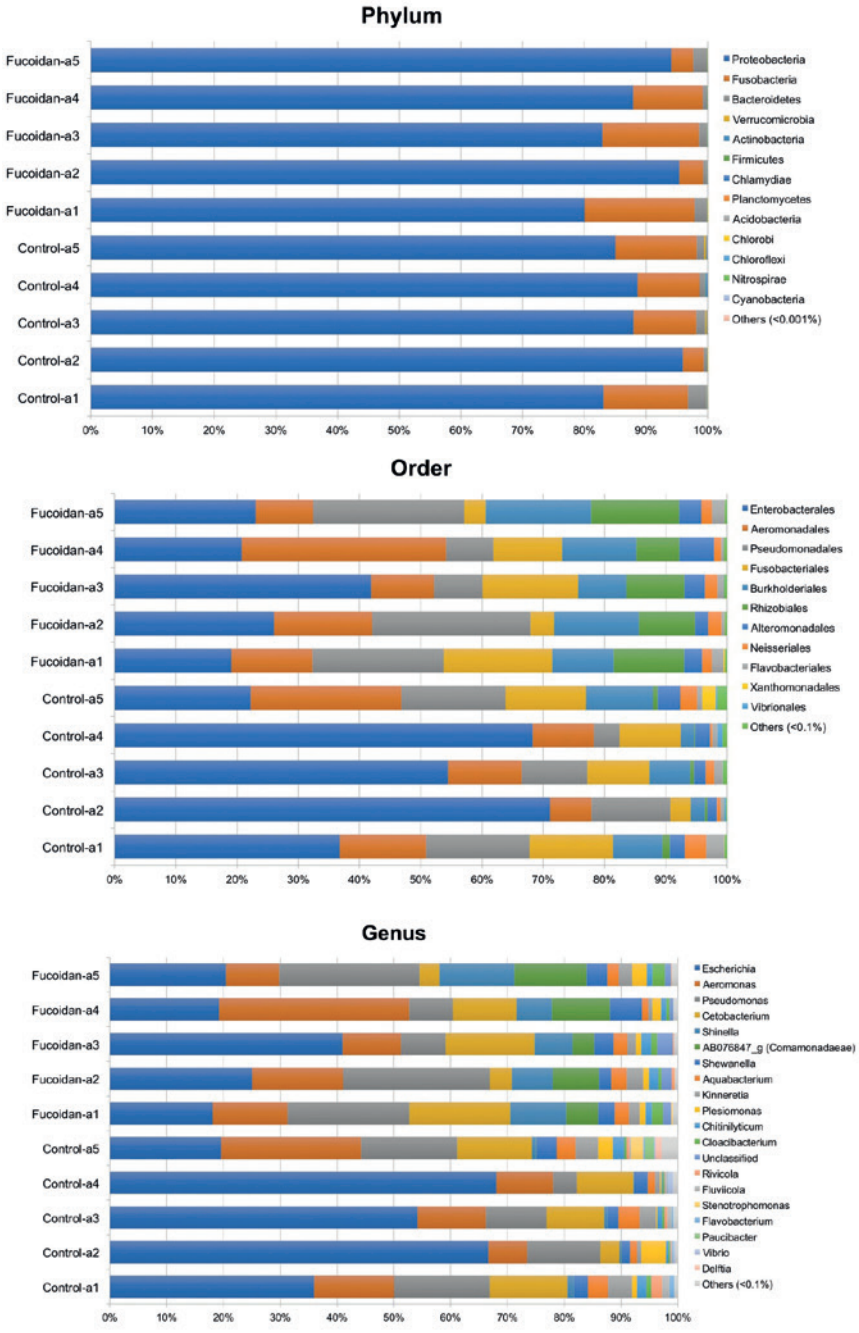


Figure S3: Overview of composition of bacterial groups in the adult intestinal microbiota.

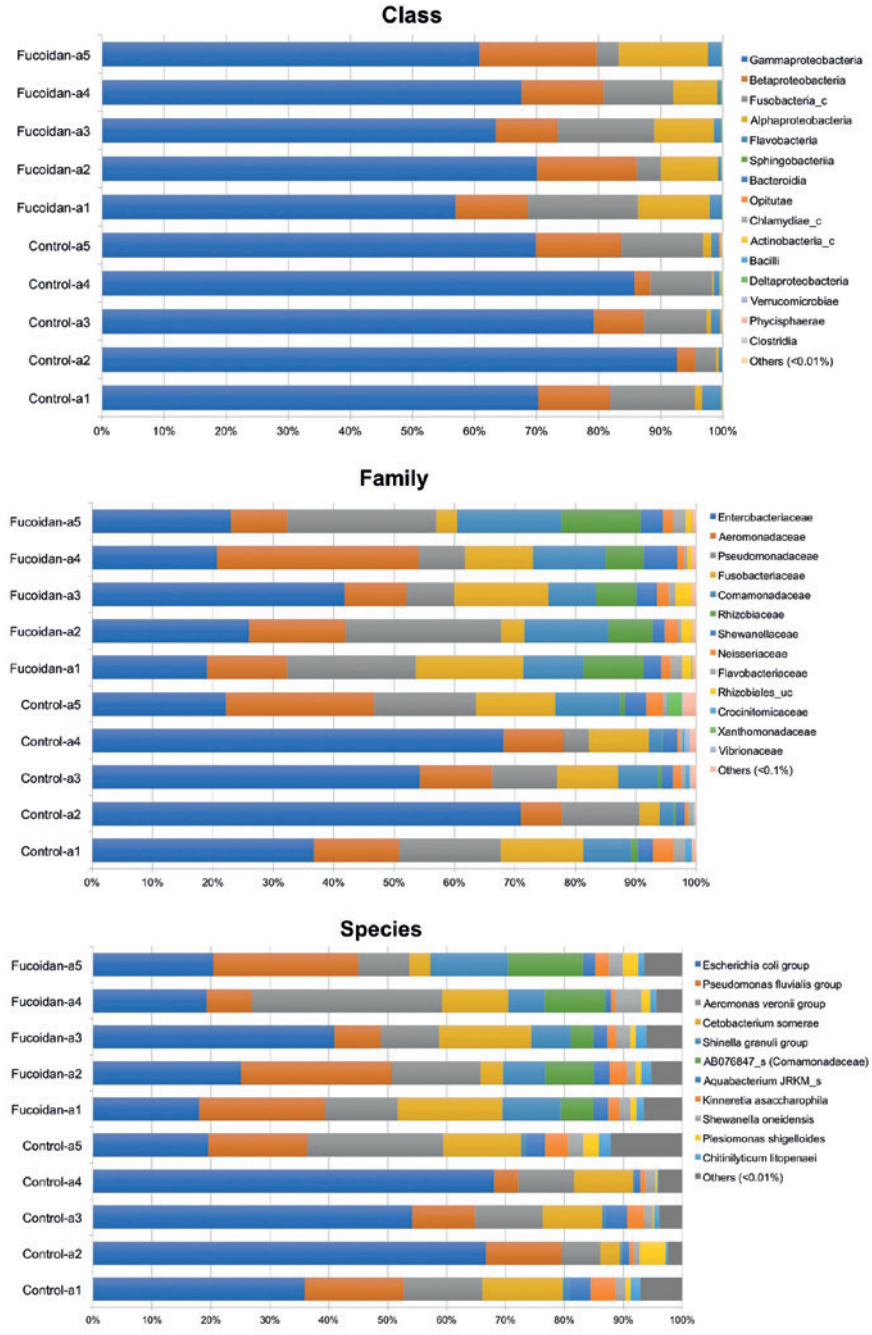


Figure S3: Continued.

Table S1 Forward (FW) and reverse (RV) sequences of the primers employed for the Real Time qPCR.

Gene	FW primer	RV primer
elfa1	5′-CTGGAGGCCAGCTCAAACAT-3′	5′-ATCAAGAAGAGTAGTAGTACCG-3′
il1b	5′-TGCGGGCAATATGAAGTCA-3′	5′-TTCGCCATGAGCATGTCC-3′
il10	5′-AGGGCTTTCCTTTAAGACTG-3′	5′-ATATCCCGCTTGAGTTCC-3′
cxcl8a	5′-TGTTTTCCTGGCATTTCTGACC-3′	5′-TTTACAGTGTGGGCTTGGAGGG-3′
tnfa	5′-CAGGGCAATCAACAAGA-3′	5′-CCTGGTCCTGGTCATCT-3′
tnfb	5′-AAACAACAAATCACCACACC-3′	5′-ACACAAAGTAAAGACCATCC-3′
mmp9	5′-ACGGCATTGCTGACAT-3′	5′-TAGCGGGTTTGAATGG-3′
il17(f2)	5′-AACCGGTTGTGTGATACTG-3′	5′-CTGGGCTTCAAAGATGAC-3′
il22	5′-GGAGGGTCTGCACAGAG-3′	5′-GTCTCCCCGATTGCTT-3′

Table S2 Summary of Illumina Miseq sequencing reads e Real Time qPCR.

Sample ID	Fish	Treatment	Total number of reads	N. valid reads for OTU clustering	N. species found (EZBioCloud)
WO1	Larvae	Control	34,295	30,752	64
WO2	Larvae	Control	35,664	31,940	56
WO3	Larvae	Control	15,484	6,788	124
WO4	Larvae	Control	31,317	28,119	58
WO5	Larvae	Fucoidan (100µg/ml)	35,533	31,659	72
WO6	Larvae	Fucoidan (100µg/ml)	23,856	19,935	59
WO7	Larvae	Fucoidan (100µg/ml)	25,129	21,708	54
WO8	Larvae	Fucoidan (100µg/ml)	27,998	25,187	54
WO9	Larvae	Fucoidan (500µg/ml)	41,070	36,810	72
WO10	Larvae	Fucoidan (500µg/ml)	10,952	8,754	68
WO11	Larvae	Fucoidan (500µg/ml)	33,106	29,792	42
WO12	Larvae	Fucoidan (500µg/ml)	39,512	34,300	75
WO13	Adult	Control (intestine)	36,761	31,419	74
WO14	Adult	Control (intestine)	36,982	32,874	65
WO15	Adult	Control (intestine)	35,574	30,818	116
WO16	Adult	Control (intestine)	29,552	25,857	79
WO17	Adult	Control (intestine)	33,927	29,362	102
WO18	Adult	Fucoidan (intestine)	42,029	36,201	76
WO19	Adult	Fucoidan (intestine)	33,696	28,920	87
WO20	Adult	Fucoidan (intestine)	39,691	33,779	84
WO21	Adult	Fucoidan (intestine)	34,574	29,631	104
WO22	Adult	Fucoidan (intestine)	36,515	31,132	104

Table S3 The most abundant (>0.1 % relative abundance in total 16S rRNA gene reads obtained in this study) bacterial operational taxonomical units (OTUs) identified from larval zebrafish and from the gut of adult zebrafish.

OTU N.	Closest GebBank affiliation[a]		Relative abundance (% of all 16S rRNA gene reads)[b]				
			Larvae[c]			Adult[d]	
	Taxonomy	%	Control	Fuc100[e]	Fuc500[e]	Control	Fuc[f]
OTU 1	Escherichia/Shigella (Enterobacteriaceae)	100	**64.67**[g]	**66.92**	**67.45**	**35.11**	**18.00**
OTU 2	Escherichia/Shigella (Enterobacteriaceae)	100	**17.65**	**17.63**	**17.88**	**9.33**	4.83
OTU 3	Shinella (Rhizobiaceae)	100	0.00	0.00	0.01	**12.44**	**17.43**
OTU 4	Aeromonas (Aeromonadaceae)	100	0.00	0.01	0.01	**11.87**	**14.12**
OTU 5	Escherichia/Shigella (Enterobacteriaceae)	99	**8.37**	**8.22**	**8.16**	4.39	2.24
OTU 6	Cetobacterium (Fusobacteriaceae)	100	0.00	0.00	0.01	**7.76**	**9.36**
OTU 7	Shinella (Rhizobiaceae)	100	0.00	0.00	0.00	0.55	**8.78**
OTU 8	Acidovorax (Comamonadaceae)	100	0.00	0.00	0.00	0.04	**7.29**
OTU 9	Shewanella (Shewanellaceae)	100	0.00	0.00	0.00	2.15	3.25
OTU 10	Aquabacterium (Comamonadaceae)	100	0.00	0.05	0.00	2.51	2.06
OTU 11	Cetobacterium (Fusobacteriaceae)	99	0.00	0.00	0.00	2.34	1.70
OTU 12	Uncultured Burkholderiales bacterium	98	0.00	0.00	0.06	2.19	1.61
OTU 13	Plesiomonas (Enterobacteriaceae)	100	0.00	0.00	0.00	1.34	1.04
OTU 14	Aeromonas (Aeromonadaceae)	100	**1.45**	0.00	0.00	0.58	0.68
OTU 15	Chitinilyticum (Chromobacteriaceae)	100	0.00	0.00	0.00	0.91	1.20
OTU 16	Aeromonas (Aeromonadaceae)	100	0.00	0.00	0.00	0.76	0.99
OTU 17	Unclassified Rhizobiales bacterium	100	0.01	0.00	0.00	0.05	**1.48**
OTU 18	Cloacibacterium (Flavobacteriaceae)	100	0.00	0.00	0.00	0.40	1.15
OTU 19	Pseudomonas (Pseudomonadaceae)	100	**0.65**	**0.91**	0.48	0.00	0.00
OTU 20	Ralstonia (Burkholderiaceae)	100	0.55	**0.38**	**0.81**	0.00	0.00
OTU 21	Vogesella (Chromobacteriaceae)	99	0.00	0.00	0.00	0.55	0.19

OTU 22	Curvibacter (Comamonadaceae)	99	0.00	0.12	**0.56**	0.00	0.00
OTU 23	Fluviicola (Crocinitomicaceae)	100	0.00	0.00	0.00	0.48	0.01
OTU 24	Escherichia/Shigella (Enterobacteriaceae)	100	0.05	0.07	0.04	0.18	0.15
OTU 25	Comamonas (Comamonadaceae)	100	0.00	0.00	0.00	0.00	0.38
Relative abundance of 25 OTUs in the total reads (%)			93.40	94.31	95.47	95.93	97.94

[a] BLASTIN search performed in May 2019
[b] Average% of 16S rRNA gene reads (QC passed) obtained from all samples from all experimental conditions
[c] Bacterial 16S rRNA sequences from whole larvae
[d] Bacterial 16S rRNA sequences from the gut of adult zebrafish
[e] Larval fish treated (immersed) with 100µg/ml (Fuc100) or 500µg/ml (Fuc500)
[f] Adult fish fed with OM fucoidan
[g] The five most abundant OTUs in each sample set are shown in bold

References

Abràmoff MD, Magalhães PJ, Ram SJ. Image processing with ImageJ. *Biophotonics Int.* (2004) 11:36–42.

Albanese D, Fontana P, de Filippo C, Cavalieri D, Donati C. MICCA: a complete and accurate software for taxonomic profiling of metagenomic data. *Sci Rep.* (2015) 5:9743. doi: 10.1038/srep09743

Ale MT, Meyer AS. Fucoidans from brown seaweeds: an update on structures, extraction techniques and use of enzymes as tools for structural elucidation. *RSC Adv.* (2013) 3:8131–41. doi: 10.1039/C3RA23373A

Araya N, Takahashi K, Sato T, Nakamura T, Sawa C, Hasegawa D, et al. Fucoidan therapy decreases the proviral load in patients with human t-lymphotropic virus type-1-associated neurological disease. *Antivir Ther.* (2011) 16:89–98. doi: 10.3851/IMP1699

Bernut A, Herrmann JL, Kissa K, Dubremetz JF, Gaillard JL, Lutfalla G, et al. *Mycobacterium* abscessus cording prevents phagocytosis and promotes abscess formation. *Proc Natl Acad Sci USA*. (2014) 111:E943–52. doi: 10.1073/pnas.1321390111

Brugman S, Ikeda-Ohtsubo W, Braber S, Folkerts G, Pieterse CMJ, Bakker PAHM. A comparative review on microbiota manipulation: lessons from fish, plants, livestock and human research. *Front Nutr.* (2018) 5:80. doi: 10.3389/fnut.2018.00080

Brugman S. The zebrafish as a model to study intestinal inflammation. *Dev Comp Immunol.* (2016) 64:82–92. doi: 10.1016/j.dci.2016.02.020

Burns AR, Miller E, Agarwal M, Rolig AS, Milligan-Myhre K, Seredick S, et al. Interhost dispersal alters microbiome assembly and can overwhelm host innate immunity in an experimental zebrafish model. *Proc Natl Acad Sci.* (2017) 114:11181–6. doi: 10.1073/pnas.1702511114

Chen Q, Liu M, Zhang P, Fan S, Huang J, Yu S, et al. Fucoidan and galacto-oligosaccharides ameliorate high-fat diet-induced dyslipidemia in rats by modulating the gut microbiota and bile acid metabolism. *Nutrition.* (2019) 65:50–9. doi: 10.1016/j.nut.2019.03.001

Cheng Y, Luthuli S, Hou LF, Jiang HJ, Chen PC, Zhang X, et al. *Sargassum fusiforme* fucoidan modifies the gut microbiota during alleviation of streptozotocin-induced hyperglycemia in mice. *Int J Biol Macromol.* (2019) 131:1162–70. doi: 10.1016/j.ijbiomac.2019.04.040

Chollet L, Sabboural P, Chauvierre C, Villemin JN, Letourneur D, Chaubet F. Fucoidans in nanomedicine. *Mar Drugs.* (2016) 14:145. doi: 10.3390/md14080145

Chu L, Wang J. Denitrification performance and biofilm characteristics using biodegradable polymers PCL as carriers and carbon source. *Chemosphere.* (2013) 91:1310–6. doi: 10.1016/j.chemosphere.2013.02.064

de Jesus Raposo MF, de Morais AMMB, de Morais RMSC. Emergent sources of prebiotics: seaweeds and microalgae. *Mar Drugs.* (2016) 14:27. doi: 10.3390/md14020027

Dhariwal A, Chong J, Habib S, King IL, Agellon LB, Xia J. MicrobiomeAnalyst: a web-based tool for comprehensive statistical, visual and meta-analysis of microbiome data. *Nucleic Acids Res.* (2017) 45:W180–8. doi: 10.1093/nar/gkx295

Dib AL, Agabou A, Chahed A, Kurekci C, Moreno E, Espigares M, et al. Isolation, molecular characterization and antimicrobial resistance of enterobacteriaceae isolated from fish and seafood. *Food Control.* (2018) 88:54–60. doi: 10.1016/j.foodcont.2018.01.005

Elizondo-Gonzalez R, Cruz-Suarez LE, Ricque-Marie D, Mendoza-Gamboa E, Rodriguez-Padilla C, Trejo-Avila LM. *In vitro* characterization of the antiviral activity of fucoidan from Cladosiphon okamuranus against newcastle disease virus. *Virol J.* (2012) 9:307. doi: 10.1186/1743-422X-9-307

Fitton JH, Stringer DS, Park AY, Karpiniec SN. Therapies from Fucoidan: new developments. *Mar Drugs.* (2019) 17:571. doi: 10.3390/md17100571

Forlenza M, Kaiser T, Savelkoul HF, Wiegertjes GF. The use of real-time quantitative PCR for the analysis of cytokine mRNA levels. *Methods Mol Biol.* (2012) 820:7–23. doi: 10.1007/978-1-61779-439-1_2

Haneji K, Matsuda T, Tomita M, Kawakami H, Ohshiro K, Uchihara JN, et al. Fucoidan extracted from *Cladosiphon okamuranus* tokida induces apoptosis of human T-cell leukemia virus type 1-infected T-cell lines and primary adult T-cell leukemia cells. *Nutr Cancer.* (2005) 52:189–201. doi: 10.1207/s15327914nc5202_9

Hidari KI, Takahashi N, Arihara M, Nagaoka M, Morita K, Suzuki T. Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga. *Biochem Biophys Res Commun.* (2008) 376:91–5. doi: 10.1016/j.bbrc.2008.08.100

Hiraishi A, Khan ST. Application of polyhydroxyalkanoates for denitrification in water and wastewater treatment. *Appl Microbiol Biotechnol.* (2003) 61:103–9. doi: 10.1007/s00253-002-1198-y

Hooper LV, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science*. (2012) 336:1268–73. doi: 10.1126/science.1223490

Hu S, Wang J, Wang J, Yang H, Yan X, Su L. Fucoidan from *Acaudina molpadioides* improves insulin resistance by altering gut microbiota dysfunction. *J Funct Foods*. (2019) 57:59–67. doi: 10.1016/j.jff.2019.03.033

Ikeda-Ohtsubo W, Brugman S, Warden CH, Rebel JMJ, Folkerts G, Pieterse CMJ. How can we define "optimal microbiota?": a comparative review of structure and functions of microbiota of animals, fish, and plants in agriculture. *Front Nutr*. (2018) 5:90. doi: 10.3389/fnut.2018.00113

Jeong JW, Hwang SJ, Han MH, Lee DS, Yoo JS, Choi IW, et al. Fucoidan inhibits lipopolysaccharide-induced inflammatory responses in RAW 264.7 macrophages and zebrafish larvae. *Mol Cell Toxicol*. (2017) 13:405–17. doi: 10.1007/s13273-017-0045-2

Khan ST, Horiba Y, Yamamoto M, Hiraishi A. Members of the family Comamonadaceae as primary poly (3-hydroxybutyrate-co-3-hydroxyvalerate) -degrading denitrifiers in activated sludge as revealed by a polyphasic approach. *Appl Environ Microbiol*. (2002) 68:3206–14. doi: 10.1128/AEM.68.7.3206-3214.2002

Kim MH, Joo HG. Immunostimulatory effects of fucoidan on bone marrow-derived dendritic cells. *Immunol Lett*. (2008) 115:138–43. doi: 10.1016/j.imlet.2007.10.016

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res*. (2013) 41:e1. doi: 10.1093/nar/gks808

Lange KW, Hauser J, Nakamura Y, Kanaya S. Dietary seaweeds and obesity. *Food Sci Hum Wellness*. (2015) 4:87–96. doi: 10.1016/j.fshw.2015.08.001

Lee SH, Ko CI, Jee Y, Jeong Y, Kim M, Kim JS. Anti-inflammatory effect of fucoidan extracted from Ecklonia cava in zebrafish model. *Carbohydr Polym*. (2013) 92:84–9. doi: 10.1016/j.carbpol.2012.09.066

López Nadal A, Ikeda-Ohtsubo W, Sipkema D, Peggs D, Mcgurk C, Forlenza M, et al. Feed, microbiota and gut immunity: using the zebrafish model to understand fish health. *Front Immunol*. (2020) 11:114. doi: 10.3389/fimmu.2020.00114

López Nadal A, Peggs D, Wiegertjes GF, Brugman S. Exposure to antibiotics affects saponin immersion-induced immune stimulation and shift in microbial composition in zebrafish larvae. *Front Microbiol*. (2018) 9:2588. doi: 10.3389/fmicb.2018.02588

Luo GZ, Hou ZW, Tian LQ, Tan HX. Comparison of nitrate removal efficiency and bacterial properties using PCL and PHBV polymers as a carbon source to treat aquaculture water. *Aquaculture Fish*. (2020) 5:92-8. doi: 10.1016/j.aaf.2019.04.002

Ma N, Guo P, Zhang J, He T, Kim SW, Zhang G, et al. Nutrients mediate intestinal bacteria–mucosal immune crosstalk. *Front Immunol*. (2018) 9:5. doi: 10.3389/fimmu.2018.00005

Makki K, Deehan EC, Walter J, Bäckhed F. The impact of dietary fiber on gut microbiota in host health and disease. *Cell Host Microbe*. (2018) 23:705–15. doi: 10.1016/j.chom.2018.05.012

Michel C, Lahaye M, Bonnet C, Mabeau S, Barry JL. *In vitro* fermentation by human faecal bacteria of total and purified dietary fibers from brown seaweeds. *Br J Nutr*. (1996) 75:263–80. doi: 10.1079/BJN19960129

Mori N, Nakasone K, Tomimori K, Ishikawa C. Beneficial effects of fucoidan in patients with chronic hepatitis c virus infection. *World J Gastroenterol*. (2012) 18:2225–30. doi: 10.3748/wjg.v18.i18.2225

Nag D, Breen P, Raychaudhuri S, Withey JH. Glucose metabolism by *Escherichia coli* inhibits *Vibrio cholerae* intestinal colonization of zebrafish. *Infect Immun*. (2018) 86:e00486–18. doi: 10.1128/IAI.00486-18

Nagamine T, Hayakawa K, Kusakabe T, Takada H, Nakazato K, Hisanaga E, et al. Inhibitory effect of fucoidan on Huh7 hepatoma cells through downregulation of CXCL12. *Nutr Cancer*. (2009) 61:340–7. doi: 10.1080/01635580802567133

Nagamine T, Kadena K, Tomori M, Nakajima K, Iha M. Activation of NK cells in male cancer survivors by fucoidan extracted from *Cladosiphon okamuranus*. *Mol Clin Oncol*. (2020) 12:81–8. doi: 10.3892/mco.2019.1943

Nagaoka M, Shibata H, Kimura-Tagaki I, Hashimoto S, Kimura K, Makino T, et al. Structural study of fucoidan from *Cladosiphon okamuranus* TOKIDA. *Glycoconj J*. (1999) 45:325–36. doi: 10.3177/jnsv.45.325

Novoa B, Figueras A. Zebrafish: model for the study of inflammation and the innate immune response to infectious diseases. *Adv Exp Med Biol*. (2012) 946:253–75. doi: 10.1007/978-1-4614-0106-3_15

Ntougias S, Melidis P, Navrozidou E, Tzegkas F. Diversity and efficiency of anthracene-degrading bacteria isolated from a denitrifying activated sludge system treating municipal wastewater. *Int Biodeterior Biodegradation*. (2015) 97:151–8. doi: 10.1016/j.ibiod.2014.11.009

Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* (2001) 29:e45. doi: 10.1093/nar/29.9.e45

Pressley M, Phelan P, Witten P, Mellon M, Kim C. Pathogenesis and inflammatory response to *Edwardsiella tarda* infection in the zebrafish. *Dev Comp Immunol.* (2005) 29:501–13. doi: 10.1016/j.dci.2004.10.007

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* (2013) 41:D590–6. doi: 10.1093/nar/gks1219

R Core Team. R: a language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna. (2019) Available online at: https://www.R-project.org/ (accessed May 22, 2020).

Rawls JF, Mahowald MA, Ley RE, Gordon JI. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell.* (2006) 127:423–33. doi: 10.1016/j.cell.2006.08.043

Renshaw SA, Loynes CA, Trushell DM, Elworthy S, Ingham PW, Whyte MK. A transgenic zebrafish model of neutrophilic inflammation. *Blood.* (2006) 108:3976–8. doi: 10.1182/blood-2006-05-024075

Ringø E, Olsen RE, Gifstad TO, Dalmo RA, Amlund H, Hemre GI, et al. Prebiotics in aquaculture: a review. *Aquacult Nutr.* (2010) 169:117–36. doi: 10.1111/j.1365-2095.2009.00731.x

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol.* (2011) 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shang Q, Jiang H, Cai C, Hao J, Li G, Yu G. Gut microbiota fermentation of marine polysaccharides and its effects on intestinal ecology: an overview. *Carbohydr Polym.* (2018) 179:173–85. doi: 10.1016/j.carbpol.2017.09.059

Spence R, Gerlach G, Lawrence C, Smith C. The behaviour and ecology of the zebrafish, *Danio rerio. Biol Rev Camb Philos Soc.* (2008) 83:13–34. doi: 10.1111/j.1469-185X.2007.00030.x

Stagaman K, Burns AR, Guillemin K, Bohannan BJ. The role of adaptive immunity as an ecological filter on the gut microbiota in zebrafish. *ISME J.* (2017) 11:1630–9. doi: 10.1038/ismej.2017.28

Swain P, Nayak SK, Nanda PK, Dash S. Biological effects of bacterial lipopolysaccharide (endotoxin) in fish: a review. *Fish Shellfish Immunol.* (2008) 25:191–201. doi: 10.1016/j.fsi.2008.04.009

Takeda K, Tomimori K, Kimura R, Ishikawa C, Nowling TK, Mori N, et al. Anti-tumor activity of fucoidan is mediated by nitric oxide released from macrophages. *Int J Oncol.* (2012) 40:251–60. doi: 10.3892/ijo.2011.1168

Trejo-Avila LM, Elizondo-Gonzalez R, Rodriguez-Santillan P, Aguilar-Briseno JA, Ricque-Marie D, Rodriguez-Padilla C, et al. Innocuity and anti-Newcastle-virus-activity of *Cladosiphon okamuranus* fucoidan in chicken embryos. *Poult Sci.* (2016) 95:2795–802. doi: 10.3382/ps/pew201

Trejo-Avila LM, Morales-Martínez ML, Ricque-Marie D, Cruz-Suarez LE, Zapata-Benavides P, Morán-Santibañez K, et al. *In vitro* anti-canine distemper virus activity of fucoidan extracted from the brown alga *Cladosiphon okamuranus. Virus Dis.* (2014) 25:474–80. doi: 10.1007/s13337-014-0228-6

Vo TS, Kim SK. Fucoidans as a natural bioactive ingredient for functional foods. *J Funct Foods.* (2012) 5:16–27. doi: 10.1016/j.jff.2012.08.007

Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol.* (2007) 73:5261–7. doi: 10.1128/AEM.00062-07

Willcox BJ, Willcox DC, Suzuki M. Demographic, phenotypic, and genetic characteristics of centenarians in Okinawa and Japan: part 1- centenarians in Okinawa. *Mech Ageing Dev.* (2017) 165:75–9. doi: 10.1016/j.mad.2016.11.001

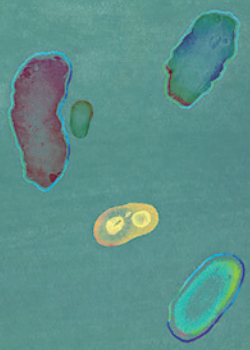
Wu H, Shen J, Wu R, Sun X, Li J, Han W, et al. Biodegradation mechanism of 1H-1,2,4-triazole by a newly isolated strain *Shinella* sp. NJUST26. *Sci Rep.* (2016) 6:29675. doi: 10.1038/srep29675

Yokota T, Nomura K, Nagashima M, Kamimura N. Fucoidan alleviates high-fat diet-induced dyslipidemia and atherosclerosis in ApoE ^{shl} mice deficient in apolipoprotein E expression. *J Nutr Biochem.* (2016) 32:46–54. doi: 10.1016/j.jnutbio.2016.01.011

Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, et al. Introducing EzBioCloud: a taxonomically united database of 16S rRNA and whole genome assemblies. *Int J Syst Evol Microbiol.* (2017) 67:1613–7. doi: 10.1099/ijsem.0.001755

Chapter 7

Omics and imaging combinatorial approach reveals butyrate-induced inflammatory effects in the zebrafish gut



Adrià López Nadal, Jos Boekhorst, Carolien Lute, Frank van den Berg, Michelle A. Schorn, Tommy Bergen Eriksen, David Peggs, Charles McGurk, Detmer Sipkema, Michiel Kleerebezem, Geert F. Wiegertjes, Sylvia Brugman

In press

Abstract

Background: Prebiotic feed additives aim to improve gut health by influencing the microbiota and the gut barrier. Most studies on feed additives concentrate on one or two (monodisciplinary) outcome parameters, such as immunity, growth, microbiota or intestinal architecture. A combinatorial and comprehensive approach to disclose the complex and multifaceted effects of feed additives is needed to understand their underlying mechanisms before making health benefit claims. Here, we used juvenile zebrafish as a model species to study effects of feed additives by integrating gut microbiota composition data and host gut transcriptomics with high-throughput quantitative histological analysis. Zebrafish received either control, sodium butyrate or saponin-supplemented feed. Butyrate-derived components such as butyric acid or sodium butyrate have been widely used in animal feeds due to their immunostimulant properties, thereby supporting intestinal health. Soy saponin is an antinutritional factor from soybean meal that promotes inflammation due to its amphipathic nature.

Results: We observed distinct microbial profiles associated with each diet, discovering that butyrate (and saponin to a lesser extent) affected gut microbial composition by reducing the degree of community-structure (co-occurrence network analysis) compared to controls. Analogously, butyrate and saponin supplementation impacted the transcription of numerous canonical pathways compared to control-fed fish. For example, both butyrate and saponin increased the expression of genes associated with immune response and inflammatory response, as well as oxidoreductase activity, compared to controls. Furthermore, butyrate decreased the expression of genes associated with histone modification, mitotic processes and G-coupled receptor activity. High-throughput quantitative histological analysis depicted an increase of eosinophils and rodlet cells in the gut tissue of fish receiving butyrate after one week of feeding and a depletion of mucus-producing cells after 3 weeks of feeding this diet. Combination of all datasets indicated that in juvenile zebrafish, butyrate supplementation increases the immune and the inflammatory response to a greater extent than the established inflammation-inducing anti-nutritional factor saponin. Such comprehensive analysis was supplemented by *in vivo* imaging of neutrophil and macrophage transgenic reporter zebrafish (mpeg1:mCherry / mpx:eGFPi^{114}) larvae. Upon exposure to butyrate and saponin, these larvae displayed a dose-dependent increase of neutrophils and macrophages in the gut area.

Conclusion: The omics and imaging combinatorial approach provided an integrated evaluation of the effect of butyrate on fish gut health and unraveled inflammatory-like features not previously reported that question the usage of butyrate supplementation to enhance fish gut health under basal conditions. The zebrafish animal model due to its unique advantages proves informative in effects of commonly used feed additives in the context of gut health.

Background

In the last decades, the implications of the microbiome in human and animal health have gained interest among and beyond the scientific community. As a consequence, food ingredients able to modulate the microbiome, such as prebiotics, became increasingly popular and accepted among the general public and have been utilized in human dietary supplements as well as in animal feed (Kothari, Patel and Goyal, 2014; Cunningham *et al.*, 2021). A prebiotic is a substrate that is selectively utilized by host microorganisms and thereby proposed to confer a health benefit on the host (reviewed in Gibson *et al.*, 2017). Butyrate is a short-chain fatty acid (SCFA) derived from fiber fermentation by the gut bacteria that exhibits some prebiotic properties, playing a role in the interaction between bacterial population dynamics and host gut homeostasis (Laserna-Mendieta *et al.*, 2018). Butyrate has a direct impact on the immune system via signaling G-protein coupled receptors (GPCR) on epithelial and immune cells and also induces epigenetic changes via regulation of histone acetylase and histone deacetylase enzymes (reviewed in van der Hee and Wells, 2021). In the last years, butyrate has been extensively used in animal feed, including its supplementation to several fish diets in the form of butyric acid or sodium butyrate due to its growth-promoting, immuno-stimulating and antioxidative properties (reviewed in Abdel-Latif *et al.*, 2020) and to mitigate detrimental effects of sub-optimal plant-containing diets (Gao *et al.*, 2011; Rimoldi *et al.*, 2016; Liu *et al.*, 2019; Volatiana *et al.*, 2020).

Plant-based protein ingredients have been replacing fish meal in feed due to their more favorable price and availability. However, several anti-nutritional components derived from plant-based protein sources were reported to be detrimental for fish health (reviewed in Sales, 2009). For instance, soy saponin is an anti-nutritional component of soybean meal that interacts with cell membranes and promotes pore formation, vesiculation and membrane domain disruption (Augustin *et al.*, 2011). Various studies linked the presence of soy saponin to inflammatory responses in the intestinal mucosa, enteritis as well as microbiota modulation in several fish species (Chikwati *et al.*, 2012; Costas *et al.*, 2014; Krogdahl *et al.*, 2015), including zebrafish (López Nadal *et al.*, 2018). In zebrafish larvae, the number of neutrophils increased in the gut after soybean meal feeding (Hedrera *et al.*, 2013) or exposure to soy saponin in solution (López Nadal *et al.*, 2018). After assessing the inflammatory effect of soy saponin, soy-containing diets have been employed as a model for feed-induced inflammation to decipher the underlying diet-microbe-host interactions in the zebrafish gut and to assess feed compounds that can potentially protect the gut from becoming inflamed (Solis *et al.*, 2020).

Experimental designs of fish feed studies are often based on specific outcome parameters, including fish growth, expression of a limited set of genes, plasma levels of

antioxidants, semi-quantitative scoring of histological parameters, profiling of the gut bacteria composition or pathogenic challenges. Habitually, only end-point analysis have been performed, ignoring the kinetics of the responses. Most of these studies base eventual gut health claims of dietary treatments on one or two of these parameters. For instance, the effect of microalgae on neutrophil quantification in the zebrafish gut larvae (Bravo-Tello *et al.*, 2017) or the expression of a small set of genes were proposed to support immune-boosting, anti-inflammatory and antioxidative stress properties of phytates after soybean-meal feeding in zebrafish larvae (Santos *et al.*, 2019). Although quantitative assessment of health associated phenotypes is critical to support health claims, basing these on the determination of a single or only few parameters may lead to overstated conclusions. To appropriately assess and understand the complex and multifaceted effects of feed supplements on (fish) gut health more integrated and holistic approaches are warranted (as reviewed in López Nadal *et al.*, 2020).

Zebrafish have been traditionally used as an animal model to study developmental biology (Meyer, Biermann and Orti, 1993) and immunology (Yoder *et al.*, 2002). Zebrafish became established as a popular vertebrate model organism for various research domains because of their small size, rapid development, fast growth and the high presence (~70%) of human orthologous genes in its genome (Howe *et al.*, 2013). Additionally, the well-annotated genome of zebrafish facilitates the performance of various omics techniques to unravel complicated host-immune interactions (Yoder *et al.*, 2002). Many gut functions and immune genes are conserved between mammals and zebrafish. Moreover, zebrafish larvae are optically transparent and together with the development of several transgenic fish lines that express fluorescent proteins in specific cell-lineages facilitates *in vivo* tracking of certain immune cells, which empowered the use of the zebrafish model to examine intestinal inflammation (reviewed in Brugman, 2016) as well as a model organism to evaluate novel feeds for farmed fish (Ulloa, Medrano and Feijo, 2014). However, the zebrafish gastrointestinal tract presents several particularities. For example, zebrafish lack a stomach and instead employ the anterior gut segment, named intestinal bulb, as a reservoir for feed. Although this intestinal bulb lacks gastric glands, it produces digestive enzymes and mimics what may occur in the stomach (Nalbant *et al.*, 1999; Flores *et al.*, 2020). The zebrafish gut epithelial layer also lacks intestinal crypts that are typically found in other fish species or in mammals and rather forms protrusions called folds that decrease in size from anterior to posterior gut segments (Wallace and Pack, 2003). Nevertheless, the canonical intestinal epithelial cells (IECs) such as enterocytes, mucin-producing goblet cells and enteroendocrine cells are present in the zebrafish gut. Moreover, zebrafish gut segments presented analogous expression to their mammalian counterparts (Wang *et al.*, 2010) and when transcriptomics where performed on IECs from zebrafish, stickleback, mouse and human a highly conserved expression was found between zebrafish and

mammals (Lickwar *et al*., 2017). Like in many animal models for inflammation, mucus-producing cells (goblet cells) decrease and granulocytes (mainly neutrophils and eosinophils), macrophages and lymphocytes increase upon inflammation in the zebrafish gut (Brugman *et al*., 2009; López Nadal *et al*., 2018). Due to its shared expression and functionality the zebrafish is an excellent model to understand host-microbe-immune interactions as long as their particular anatomical features are taken into account when translating the research outcomes to other fish species or mammals.

In the present study we used zebrafish to perform a combined comprehensive approach of feed additives on gut health by i) using novel sodium butyrate (0.01 inclusion level) and soy saponin (0.33 inclusion level) supplemented diets; ii) sampling environmental (water) samples and fish guts at two timepoints, after 1 week of feeding (acute response) and 3 weeks of feeding (prolonged exposure); iii) collecting multivariate datasets from fish gut samples including microbiome composition and genome-wide transcriptomes in combination with (iv) high-throughput quantitative histology and v) quantification of neutrophil and macrophage recruitment to the gut area by *in vivo* fluorescent imaging of transgenic zebrafish larvae.

Material and methods

Ethics Statement

The present study was approved by the Dutch Committee on Animal Welfare (2017.W-0034) and the Animal Welfare Body (IvD) of the Wageningen University (The Netherlands). Furthermore, we adhered to standard biosecurity and institutional safety procedures at Wageningen University and Research.

Zebrafish and diets

Adult double transgenic (mpeg1:mCherry / mpx:eGFPi^{114}) expressing mCherry under the macrophage-specific mpeg1 promotor and GFP under the neutrophil-specific mpx promotor fish were housed and fed as previously described (López Nadal et al., 2018). Embryos were obtained by natural spawning. Fish were fed as follows: weeks 1 and 2 with rotifers (x4/day from 5 days post fertilization -dpf-), week 3 with rotifers and Artemia Nauplii 230.000 npg (Nauplii per gram) (Ocean Nutrition Europe, Essen, Belgium) (x2/day), week 4 with Artemia (x2/day) and until 40 dpf Artemia and Tetramin Flakes (Tetra, Melle, Germany) (x2/day). When fish reached the juvenile stage, at 40 dpf (Singleman and Holtzman, 2014), fish were randomly distributed into 6 tanks (2 per each diet) for a blind feeding trial in which fish were fed until slightly before satiation twice a day. Each tank received one of the following: a control diet, a saponin-supplemented diet or a butyrate-supplemented diet. Full diet composition is listed in **Table 1**.

Experimental design

Water quality was set to standard values by replacing half of the water in the zebrafish system before the start of the experiment and monitored twice a week during the whole experiment (**Supplementary Figure 1**). A pH meter (Hanna Instruments, Nieuwegein, The Netherlands) was used to measure the pH and the water conductivity. Kits to measure ammonium, nitrite and nitrate (Merck KGaA, Darmstadt, Germany) were used according to manufacturer's instructions. Additionally, nitrite, nitrate, general hardness, carbonate hardness, pH and chlorine were (re)measured by using Tetra Test 6in1 (Tetra, Melle, Germany) according to manufacturer's instructions. Fish survival and standard length -from the tip of the head until the bifurcation of the caudal fin- were assessed by using a digital caliper (Sylvac, Yverdon, Switzerland) during the experiment (**Supplementary Figure 2**). The dietary intervention consisted of three diets identical in composition except the supplementation with 1g/kg feed of sodium butyrate in the butyrate diet and 3.3g/kg of 95% ultrapure soy saponin in the saponin diet (**Table 1**). We sampled fish guts after 1 week (54 dpf, 1st timepoint) and after 3 weeks (68dpf, 2nd timepoint) after the start of the dietary intervention. Fish were fed twice daily until satiation and the amount of feed provided was quantified with a micro-spoon, feeding 15.1mg of feed per tank per day (averaging to 0.46mg of feed per day per fish). A summary of the experiment design is depicted in **Figure 1**.

Single gut and water samples RNA extraction

Guts were extracted from juvenile zebrafish, rinsed in sterile PBS, snap frozen in liquid nitrogen and preserved at -80°C for total gut RNA extraction. RNA extraction was performed from single intestines as previously described (Kang et al., 2009). Water samples were obtained by filtering 2L of water from each fish tank using Nalgene™ Rapid-Flow™ Sterile Disposable Bottle Top Filters with PES Membrane 0.45µm (ThermoFisher Scientific MA, USA). Aliquots of total RNA were used for cDNA synthesis with the Maxima H minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, MA, USA), following the standard protocol using random hexamer primers to create cDNA. This cDNA was used for 16S rRNA gene profiling of the bacterial communities and the extracted RNA was used for metatranscriptomic analysis. The quantity, quality and purity of total RNA was determined using the Qsep100^{TM} Bio-Fragment Analyzer (Bioptic inc., New Taipei City, Taiwan) and the Qubit^{TM} RNA BR Assay Kit (ThermoFisher Scientific, MA, USA). A schematic pipeline of the whole process from sample collection to results analyses is depicted in **Figure 2**.

Table 1 Formulation of experimental diets.

The three diets are similar in composition (dry-matter, protein, fat and ash). 95% ultrapure soy saponin was kindly provided by Trond Kortner NMBU Oslo Norway, origin: Organic Technologies, Coshocton, OH, (Krogdahl *et al.*, 2015).

	Control diet (%)	Butyrate diet (%)	Saponin diet (%)
Wheat	7.00	6.99	6.67
Wheat gluten	16.00	16.00	16.00
Sunflower meal	1.68	1.68	1.68
Soy protein concentrate	15.16	15.16	15.16
Fish meal	52.00	52.00	52.00
Fish oil	4.40	4.40	4.40
Rapeseed oil	2.00	2.00	2.00
Vitamin mix	0.35	0.35	0.35
Mineral mix	1.92	1.92	1.92
Butyrate	0.00	0.01	0.00
Saponin	0.00	0.00	0.33
[VOLUME]	100.0	100.0	100.0
Dry matter	92.2	92.0	92.0
Crude protein	56.0	57.4	57.4
Crude fat	13.5	13.8	13.8
Ash	8.9	8.9	8.9

Microbiome: 16S rRNA Profiling and Sequencing Data Analysis

Amplicon libraries of the V4 region of the 16S rRNA were generated from the cDNA synthetized from single gut and water samples, using barcoded and modified F515-806R primers (Walters *et al.*, 2016). The PCRs were performed in triplicate, purified, and quantified as previously described (Hartinger *et al.*, 2019). Purified PCR amplicons were pooled in an equimolar mix and sent for library preparation and sequencing using the Illumina NovaSeq 6000 S2 PE150 XP technology at Eurofins Genomics Germany GmbH (Eurofins Genomic, Ebersberg, Germany). Raw paired-end reads were analyzed using the standard parameters of NG-Tax 2.0 (Poncheewin *et al.*, 2020), with the exception of using 100 bp as the forward and reverse read length, as implemented in Galaxy (Afgan *et al.*, 2018), to obtain Amplicon Sequence Variants (ASVs). Taxonomy was assigned to ASVs using the Silva_132 database (Quast *et al.*, 2013). Two synthetic "mock communities" with known compositions were amplified and sequenced as positive controls and a no-template control was also included as a negative control (Ramiro-Garcia *et al.*, 2018). The distribution of reads per sample and the variance in ASVs were assessed and Alpha- and Beta-diversity measurements were

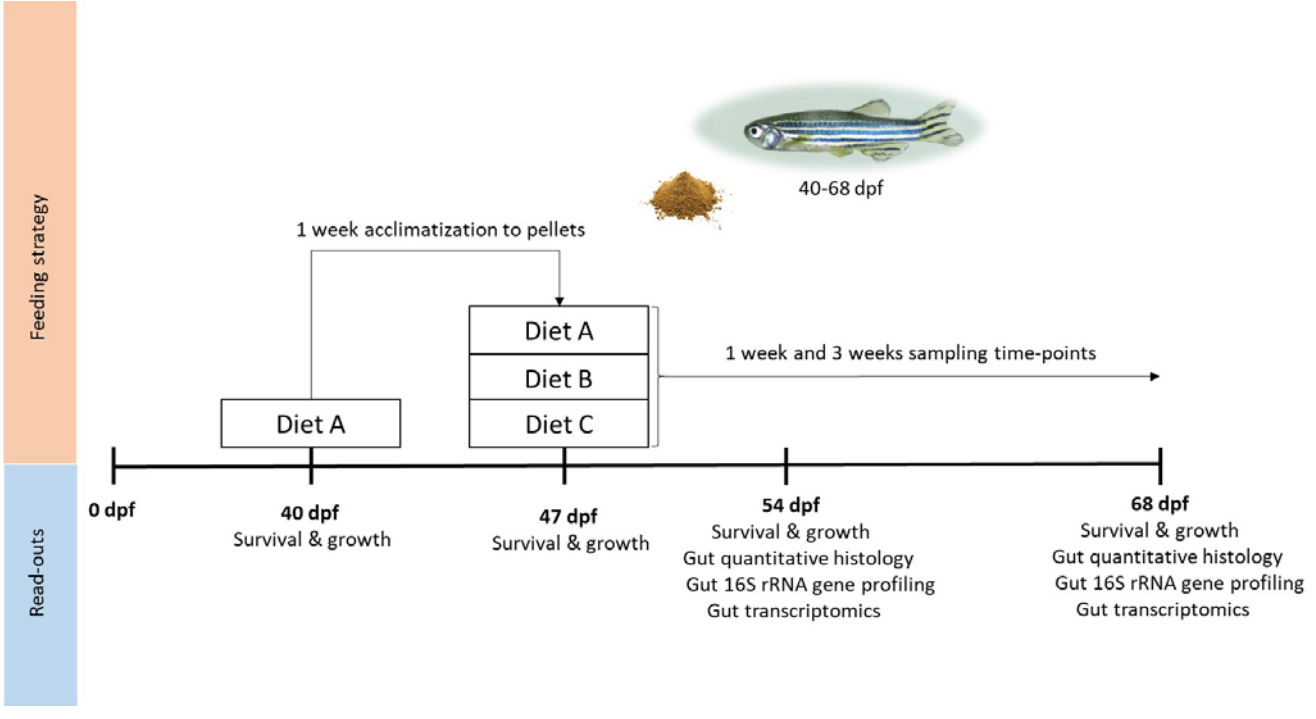


Figure 1: Experimental design. Fish were bred and raised as described in the section 'Zebrafish and diets'. At 40 dpf (juvenile stage) they were fed diet A for 1 week for acclimatisation to dry feed pellets. At 47 dpf, fish were randomly distributed into tanks and fed one of the diets (A, B or C) for 3 weeks. Survival and growth were measured before and during the whole experiment. After 1 week (54 dpf) and after 3 weeks (68 dpf) of feeding the fish. Gut samples were collected for histological, metatranscriptomic and microbiome analyses.

performed using R v4.1.2 and RStudio (Lahti and Shetty, 2019), using packages ggplot2, (Wickham, 2009), ape, (Paradis et al., 2004), plyr, (Wickham and Wickham, 2020), vegan, (Oksanen *et al.*, 2013), RColorBrewer, (Neuwirth and Brewer, 2014), reshape2, (Wickham, 2012), scales (Wickham, 2016), data.table, (Dowle *et al.*, 2019), microbiome, (Lahti *et al.*, 2017), dplyr, (Wickham *et al.*, 2017), phyloseq, (McMurdie and Holmes, 2013), ggdendro, (de Vries and Ripley, 2013) and DT (Xie, et al. 2018). The analysis yielded 17,203,234 high-quality reads. We excluded one sample (54 dpf butyrate diet) because it had 2 reads only and we kept all the other samples (>30.000 reads). Rarefaction curves for all samples reached a plateau, indicating that sufficient sequencing depths was achieved (data not shown). For the calculation of alpha-diversity indices, data was rarefied against the sample containing the lowest number of reads (31,814 reads). Redundancy analysis (RDA) and principal component analysis (PCA) were performed with Canoco v5.15 (Braak & Smilauer, 2012) using analysis type "constrained" or "unconstrained", respectively. Response variables were log-transformed with the formula log(10000*relative_abundance+1). RDA p-values were determined through permutation testing (500 permutations). Boxplots were generated using Prism v.9.0.0 (GraphPad Software, San Diego, California USA). Cytoscape v3.9.1 (Shannon *et al.*, 2003) was used to visualize the diet-specific co-occurrence of ASVs based on their relative abundances. Additional data handling and format conversions were done in Python (https://www.python.org/).

Zebrafish gut transcriptome analyses

Total RNA (n=5 diet/timepoint) was sent to Novogene (Cambridge, UK), where quality control was done, rRNA was depleted and the metatranscriptome libraries were prepared. Paired-end reads were generated by NovaSeq 6000 PE150. For the host reads we used nf-core/rnaseq Nextflow pipeline (Ewels *et al.*, 2020) and the zebrafish (*Danio rerio*) genome assembly GRCz11 (NCBI) and according to the MultiQC reports generated, the quality check parameters were satisfactory for all samples. "Salmon" was used to quantify the expression of the transcripts (Patro *et al.*, 2017) and DEseq2 (Love, Huber and Anders, 2014), ggplot2, (Wickham, 2009), scales (Wickham, 2016), viridis (Garnier *et al.*, 2021) in RStudio to investigate the differentially expressed genes (DEG) in our diet treatments and timepoints. PCA analyses were performed in the Canoco v5.15 software suite (v5.02, Braak & Smilauer, 2012). Gene Score Resampling (GSR) analyses was performed ErmineJ (v3.1.2) (Gillis, Mistry and Pavlidis, 2010) with the annotation file of zebrafish (*Danio rerio*; genome assembly GRCz11) generated by Gemma (Zoubarev *et al.*, 2012). GSR used DEG scores from DEseq2 from all genes in the dataset and calculated a p-value for each Gene Ontology (GO) term. The fold-change of each GO term across dietary interventions was calculated by collapsing individual transcripts per million (tpm) of each gene to the belonging GO term(s). Differentially expressed GO terms were visualized as a network by using Cytoscape v3.9.1

(Shannon *et al.*, 2003): the nodes contained the fill depicting the log2 fold change (FC) of the control vs butyrate at T2 and the border depicting the log2 FC of the control vs saponin at T2. The nodes with an absolute FC≥0.5 and p value≤0.1 between dietary interventions where taken into account. The edges connected the relevant nodes if the GO term contains at least 10 genes and shared at least half of them with the connecting GO term(s) with FC≥0.2 and p value≤0.05 between dietary interventions. All data and files used to generate these visualisations can be found in **Supplementary folder 1**.

High-throughput Quantitative Histology

At 54 dpf and 68 dpf zebrafish were euthanized in buffered MS222 overdose (Westerfield, 2007) 250mg/L Tricaine (Sigma-Aldrich, DL, United States). Intestines were removed, rinsed in PBS, placed in 4% paraformaldehyde overnight and transferred to 70% ethanol on the next day. After subsequent dehydration steps, total intestines were embedded in paraffin blocks. Five-micrometer sections were stained with one of the following: hematoxylin and eosin (H&E) or Alcian blue periodic acid-Schiff (ABPAS) as previously described in (Brugman *et al.*, 2009) or by immunohistochemistry (IHC). For the latter, antigen retrieval was performed using the PT Link automatic antigen retrieval machine (Dako Agilent, CA, USA): samples were placed into citrate buffer pH 6.1 (Dako Agilent, CA, USA) at 60 °C, heated to 97 °C in 20 minutes, kept at 97 °C for 20 minutes, and cooled down to 60 in 20 minutes. Samples were stained using an automated staining machine (Autostainer Link 48, Dako Agilent, CA, USA) with anti-proliferating cell nuclear antigen (PCNA mouse mAb Clone PC10, M0879, Dako A/S, Denmark, diluted 1:10.000) or with anti-Zeta chain of T cell receptor associated protein kinase 70 (ZAP70 Rabbit mAb 99F2, Cell Signaling Technology USA, diluted 1:300) antibodies. Proliferating cells were stained to understand the intestinal epithelial renewal and to show effects of butyrate on proliferating cells and T cells were stained to show effects of supplements on the adaptive immune system. Samples were scanned at 20x magnification using Pannoramic SCAN II (3DHISTECH, Budapest, Hungary) to produce digital whole slide images and analyzed using Visiopharm v. 2019.07 image analysis software (Visiopharm, Hoersholm, Denmark). Specialized automated image analysis protocols were developed for each staining type. Before employing the quantitative histology, tissue regions were manually defined on the images to select representative tissue (avoiding artefacts). The automated analysis was then preformed only within those regions. Making an automated protocol involves selecting pre-processing steps, such as median filters to reduce noise and enhance structures, training the Bayesian classifier algorithm by annotating examples of the image background, tissue, and target cells, utilizing post-processing steps based on shape, size and pixel colour to enhance the final image segmentation, and define calculations to give the output data (area, counts, and perimeters). This method for image analysis allowed us to perform quantitative

histology which differs from the commonly used semi-quantitative scoring. The latter involves a pathologist ascribing a subjective scoring with ordinal data, which is strongly operator-biased and time consuming. Quantitative histology is automated, more detailed, thorough, and consistent, producing numerical data that can detect subtle differences between states. The cell types imaged were mucus (goblet) cells, PAS+ cells (granulocytes), rodlet cells; PCNA+ cells (proliferative cells) and Zap-70 cells (NK-like and T lymphocytes) (Moore *et al.*, 2016). The histological parameters quantified were as follows. Absorptive capacity (AC): which was formulated as (interface length between mucosa and lumen / interface length between serosa and exterior of the gut). Cell area fraction (%) (tissue area made up of cells) formula: area cell type A / total tissue area *100. Cell density: cell number / total tissue area. Cell size: area of cells / cell number. Cell distance: the distance of the cells from the outer serosal layer, where a higher distance would indicate a cell migrating towards the mucosal fold (villus) end towards the lumen. The imaging and the histological quantification were performed at the facilities of Skretting Aquaculture Innovation (Skretting, Stavanger, Norway). Further downstream processing of the multivariate analysis was performed by using Canoco v.5.12 (v5.02, Braak & Smilauer, 2012) using principal coordinate (PCoA) and Redundancy (RDA) analyses, performing analysis type "unconstrained" and "constrained", respectively. Response variables (histological parameters) were scaled (0-1) and biplots were generated. RDA p-values were determined through permutation testing (500 permutations). Boxplots were generated using Prism v.9.0.0 (GraphPad Software, San Diego, California USA).

Fluorescent *in vivo* imaging experiment

Adult Tg (mpeg1:mCherry / mpx:eGFPi[114]) were housed and fed as previously described (López Nadal et al., 2018) and embryos obtained by natural spawning and raised with E3 water (0.10 mM NaCl in demineralized water, pH 7.6) in petri dishes at 28°C (12/12-hour light/dark cycle) (Westerfield, 2007). Larvae were randomly distributed in 6 well plates (n = 20 fish/well) and exposed to different concentrations [0.005, 0.01 mg/ml] of butyrate and [0.5, 0.7 mg/ml] saponin dissolved in E3 water (10 ml solution/well) from 3–6 dpf. Larvae were anaesthetized and *in vivo* imaged as previously described in (López Nadal *et al.*, 2018). Pictures were analyzed with ImageJ® software (United States National Institutes of Health, Bethesda, United States): the intestinal area was selected manually for each fish from the bright field and copied to the other channels and fluorescent cells quantified and boxplots were generated using Prism v.9.0.0 (GraphPad Prism Software, San Diego, California, USA).

Complete pipeline of the combinatorial approach: omics and quantitative histology

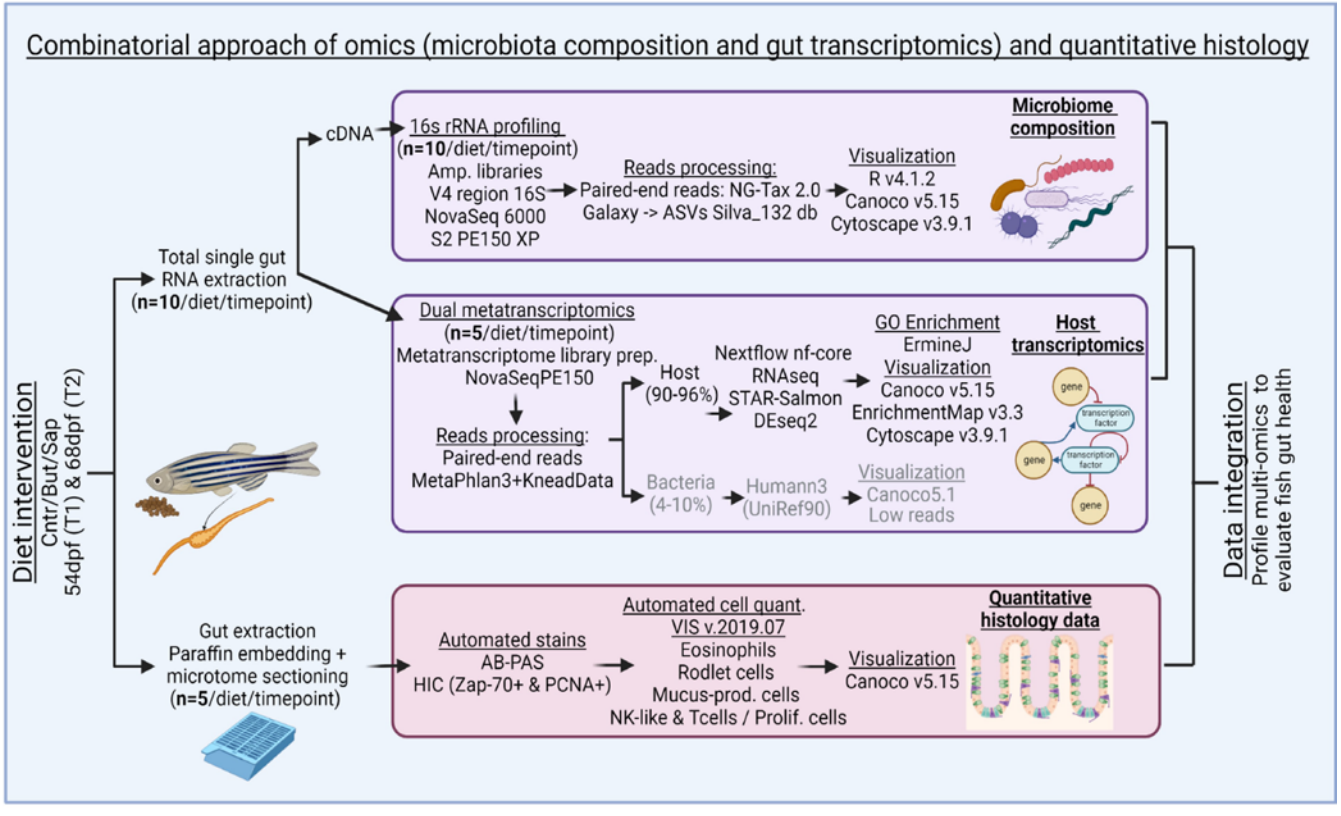


Figure 2: Combinatorial approach employed: total RNA was extracted from single zebrafish gut fed on different diets for both timepoints. Aliquots of total RNA were used for cDNA. For the 16S rRNA gene profiling, amplicon libraries of the V4 region of the 16S RNA gene were generated from the cDNA synthetized. NG-Tax 2.0 Galaxy was sued to obtain the ASVs. Several packages of R v4.1.2., Canoco v5.15 and Cytoscape v3.9.1 were used for results visualization. For transcriptomics, the cDNA libraries were sent to NovaSeq 6000 PE150 for sequencing. MetaPhlAn 3.0 (Beghini et al., 2021) and KneadData were used to trim the overrepresented sequences. Nf-core/rnaseq Nextflow pipeline was used for processing of the reads with the GRCz11 genome assembly. The results were visualized by R v.4.1.2, Canoco v5.15 and ErmineJ was used for the GO Enrichment analysis. The histological samples were extracted and embedded in paraffin and sectioned using a microtome. AB-PAS and HIC stains were automated. Samples were digitally scanned and an automated quantification of the histological parameters was performed using VIS v.2019.07 and Canoco v5.15 and GraphPad Prism v9.0.0 to visualize the results. The data integration was performed using heatmaps of normalized relevant parameters from all datasets, both timepoints and all diets.

Results

Butyrate and saponin diets did not affect survival nor fish growth

All of the fish survived the dietary intervention and fish growth was comparable regardless of the diet provided (**Supplementary Figure 1**). The water quality indicators measured: water pH, water conductivity (µS/m), nitrite (NO_2^- in mM), ammonia (NH_4^+ in mM), nitrate (NO_3^- in mM), chlorine (Cl_2^- in mM), general hardness (Ca^{2+} and Mg^{2+} per volume of water) and carbonate hardness ($CaCO_3$ and $MgCO_3$ per volume of water) were consistently within the recommended range (**Supplementary Figure 2**). Moreover, water quality indicators remained constant during the whole experiment, indicating that the diet-related changes described below result from the dietary intervention and not from differences in fish growth rates or fluctuations in water quality.

Butyrate- and saponin-supplemented diets altered gut microbiota composition over time

Ten gut samples per diet per timepoint were used to determine prokaryotic community composition based on amplicon sequencing of 16S rRNA. The samples yielded 17,203,234 high-quality reads, with an average of 286,720 reads per sample, ranging from 31,814 to 577,719. The reads resulted in 579 amplicon sequence variants (ASVs) which were reduced to 204 ASVs after filtering out the ones occurring in ≤2 counts. Alpha-diversity indexes for richness (observed ASVs and Chao1) and diversity (Shannon, Inverse Simpson, Fisher and Phylogenetic Diversity) within the samples did not reveal any significant differences between diets and timepoints for all samples. Only the Phylogenetic Diversity slightly increased for butyrate fed fish overtime (**Figure 3**).

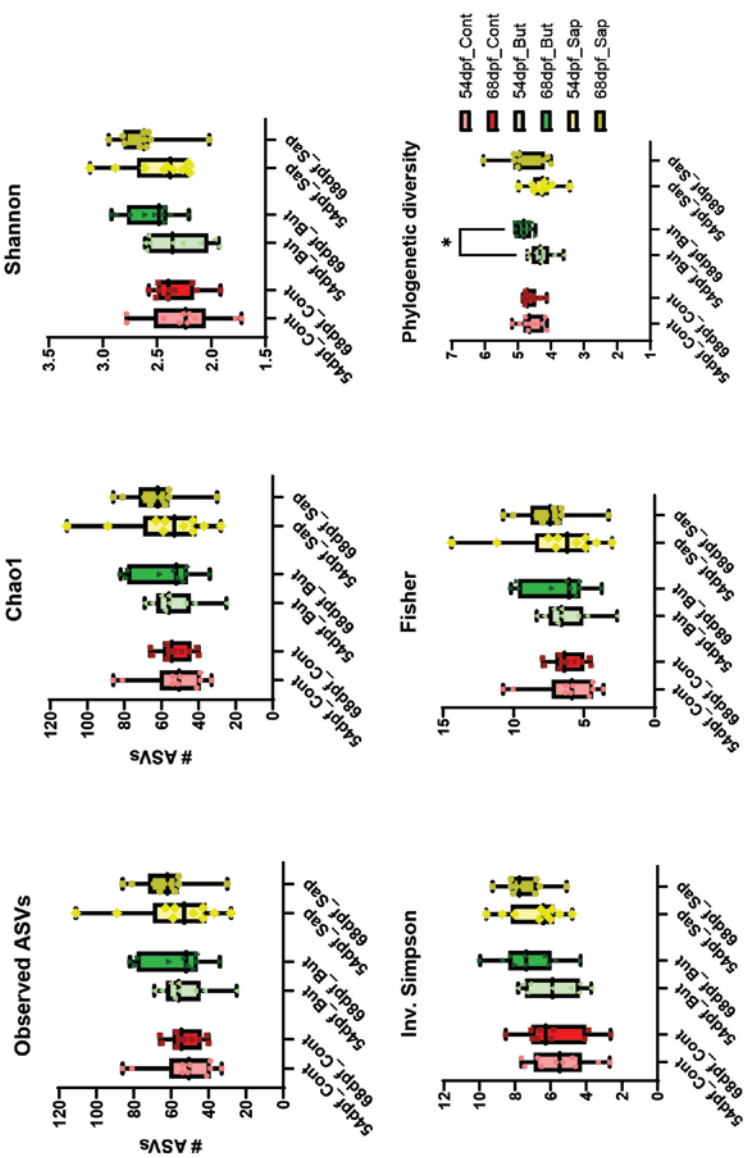


Figure 3: Alpha-diversity indexes for richness (observed ASVs and Chao1) and for diversity (Shannon, Inverse Simpson, Fisher and Phylogenetic Diversity). *p≤0.05, Ordinary one-way ANOVA after confirming normally distributed data by Shapiro-Wilk test. Whiskers: min. to max. shall all points with median.

A principal component analysis (PCA) analysis shows that time (from 54 to 68 dpf) explains ~17% of the variation observed in the microbial communities (x-axis **Figure 4A**). To analyze the effect of diet on the microbial communities, we performed redundancy analysis (RDA) separately for each timepoint. After one week on the different diets (54 dpf), the gut microbiota composition was not significantly different between the different diet groups ($p=0.24$) (**Supplementary Figure 3**), whereas, after prolongation of the diet intervention (3 weeks, 68 dpf) a significant association between the diet and the gut microbiota was detected ($p=0.018$). The top-15 most discriminant genera associated with the diet induced microbiota difference were further investigated (**Figure 4B**), revealing that these genera were absent in all fish after one week on the distinctive diets (**Supplementary Figure 4**). This finding implies that short term diet exposure (1 week) is insufficient to elicit the diet induced microbiota changes. The relative abundances of the most discriminating genera of the gut samples were consistently zero or extreme low except for *Rhodobacter* and *Pseudomonas* (**Supplementary Figure 4**), indicating that microbiota fluctuations in the zebrafish gut were not influenced to a larger extend by the surrounding water microbiota composition. RDA of the genera composition at 68 dpf associated *ZOR006* and unclassified Desulfovibrionaceae with fish fed a control diet, whereas associated *Mycobacterium*, *Vibrio*, *Aeromonas* and *Methylobacterium* with fish fed a saponin-supplemented diet and associated *Flavobacterium*, unclassified Sutterellaceae, *Bacteroides*, *Pandoraea*, *Rhodobacter*, unclassified Barnesiellaceae and *Plesiomonas* with fish fed butyrate-supplemented diet. The relative abundances of the most discriminative genera detected by the RDA (**Figure 4B**, subset of boxplots around the RDA) together with the heatmap of the relative abundances of most important taxa (**Supplementary Figure 5**) demonstrated distinct microbial profiles associated with butyrate and saponin-supplemented diets.

Butyrate reduced taxa connectivity in the zebrafish gut

After assessing distinct microbiota composition due to diets after 3 weeks of feeding (68 dpf), taxa connectivity was analyzed by network analyses of co- and anti-occurrence of each pair of taxa at 68 dpf, based on the relative abundances (**Figure 5**). The gut microbiota in fish fed the control diet presented a higher degree of taxa connectivity when compared to the gut microbiota of fish that were fed either the butyrate- or saponin-supplemented diet. Quantification of pairs of taxa with significant connectivity were compared using the cumulative frequency histogram ($p<0.05$; $p.log<1.30$, **Supplementary Figure 6A**), showing an increase of connecting pairs of taxa in the control fed fish compared to saponin and butyrate fed fish at 68 dpf. These differences in taxa connectivity were not present after 1 week of feeding (54 dpf) and occurred exclusively after 3 weeks of feeding (68 dpf) where only control fed fish increased taxa connectivity from 54 dpf to 68 dpf and not saponin and butyrate fed fish (**Supplementary Figure 6B**).

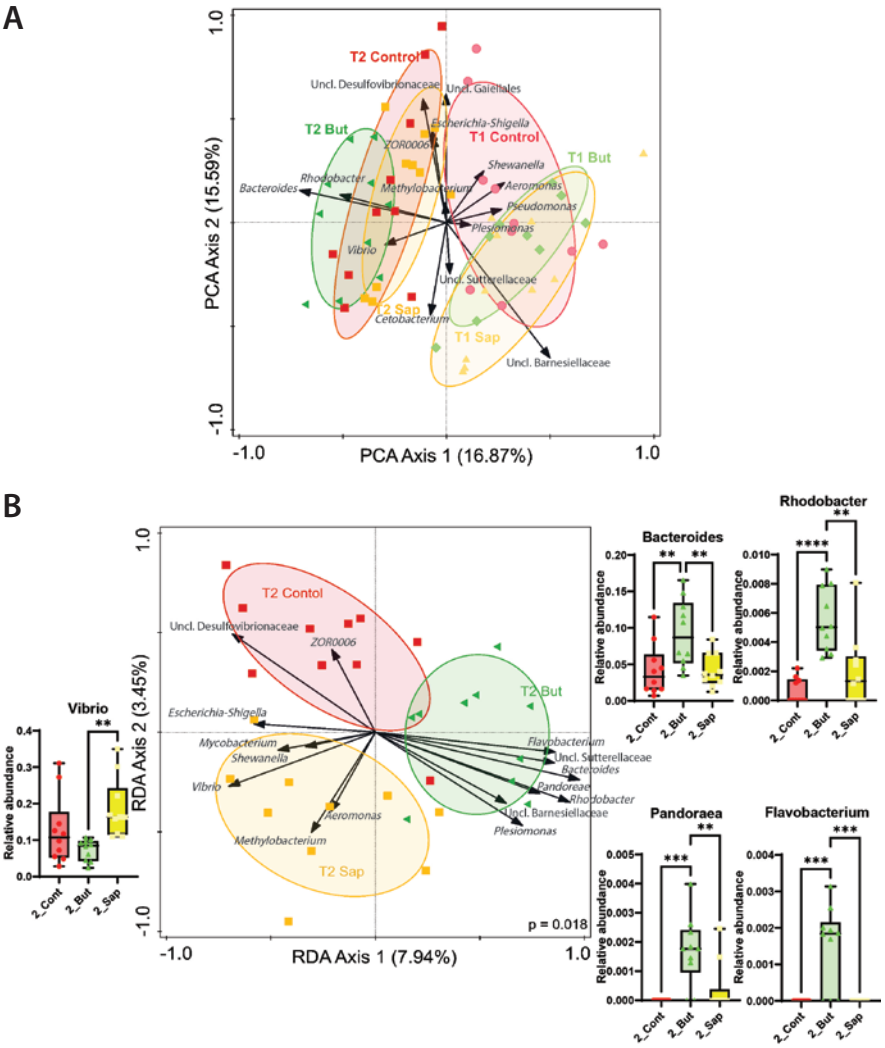


Figure 4: **(A)** Principal component analysis exploring the interaction of diet and time. The x axis depicts the effect of time, separating the samples after 1 week feeding (54 dpf) from samples after 3 weeks feeding (68 dpf) and explains 16.87% of the variation observed. **(B)** Redundancy analysis of samples after 3 weeks of feeding (68 dpf), the x axis separates saponin from butyrate fed fish and explains 7.94% of the microbial differences observed and the y axis separates the control from the saponin fed fish and explains 3.45% of the microbial differences observed. The microbial communities changed significantly due to diets (p=0.018). The relative abundance of the most discriminative genera are depicted with boxplots around the RDA. In both analyses, the top 15 most distinctive genera are represented with black arrows. The direction of the arrows correlated with the dietary treatments and the timepoints and their length correlate with the strength of the correlation. Whiskers: min. to max. shall all points with median.

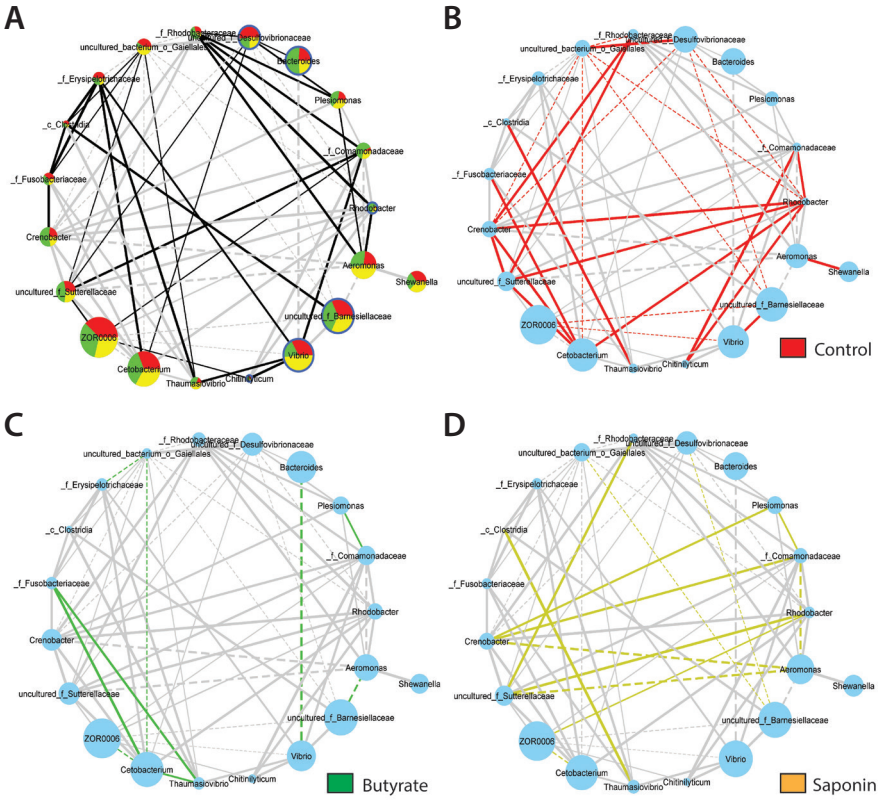


Figure 5: Taxa connectivity: taxa included when prevalence is ≥ in 3/10 samples, abundance is ≥ 10 counts in 1M and significance ≤0.1. The lines inform about the nature of the taxa interaction: the thickness of the lines represents the strength of the correlation (r-score value) and the shape of the lines represents the direction of the correlation, straight lines mean positive correlation (co-occurrence) whereas dashed lines mean negative correlation of the pairs of taxa (anti-occurrence). **(A)** Pairs of taxa co- and anti-occurring for all the diets: in black the interactions occurring in all three diets whereas in grey the interactions not occurring in all diets. Node size corresponds to average abundance of taxa for all diets at 68 dpf. **(B)** In red the interactions occurring in the control fed fish and not in the other two diets. Node size corresponds to average abundance of taxa for control diet at 68 dpf. **(C)** In green the interactions occurring in the butyrate fed fish and not in the other two diets. Node size corresponds to average abundance of taxa for butyrate diet at 68 dpf. **(D)** In yellow the interactions occurring in the saponin fed fish and not in the other two diets. Node size corresponds to average abundance of taxa for saponin diet at 68 dpf.

Gut transcriptome analysis reveals unique and shared effects of butyrate and saponin

After observing substantial dietary induced differences in bacterial composition and taxa connectivity, transcriptome profiles of the same zebrafish gut samples were analyzed. This analysis (pipeline described in **Figure 2**) resulted in a total of 47,046 genes expressed in transcripts per million (tpm). Within-group transcriptome differences across diets and timepoints revealed a significant difference of dissimilarity of the transcriptomic samples after 3 weeks of feeding (68 dpf) and not after 1 week of feeding (54 dpf). At 68 dpf fish fed the butyrate diet presented more significantly homogeneous gut transcriptomic profile than fish fed the control and the saponin diets (**Figure 6A**).

Since the transcriptomic profiles were most dissimilar after 3 weeks of feeding the unique and shared effects of butyrate and saponin on the host gut were examined by creating a transcriptome network analysis (**Figure 6B,** all raw data in **Supplementary**

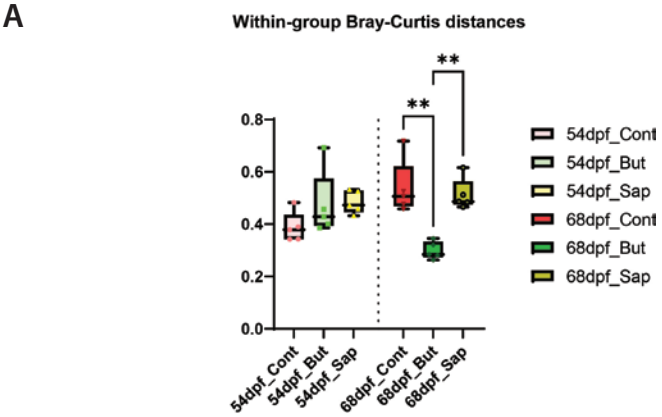


Figure 6: Effects of butyrate and saponin on the host gut transcriptome. **(A)** Bray-Curtis distances to examine the dissimilarity of the host transcriptome across diets and timepoints. ** p≤0.01, Kruskal-Wallis test after testing for non-normally distributed data by Shapiro-Wilk test. Whiskers: min. to max. shall all points with median. **(B)** Network depicting transcriptomic regulation of butyrate and saponin supplemented diets vs control diet at 68 dpf. Each node is a GO term and the node border represent the log2 fold-change of the control diet vs the saponin supplemented diet and the node fill represent the log2 fold-change of the control diet vs the butyrate supplemented diet. The edges connect nodes containing at least 10 genes and sharing 50% of the contained genes. Related GO terms are encircled encompassing canonical pathways. Shared effects on the gut transcriptome can be observed when edge and fill of a node have the same color in the network: up-regulation -in red- and down-regulation -in blue- compared to the control feed. **(C)** Immune response-associated GO terms and particularly inflammatory response analysed in fish fed a control, butyrate and saponin diet. Genes are expressed in tpm and scaled colored per individual gene value. The heatmap contained genes color-scaled per individual gene that reflect the individual within group fish-to-fish variation.

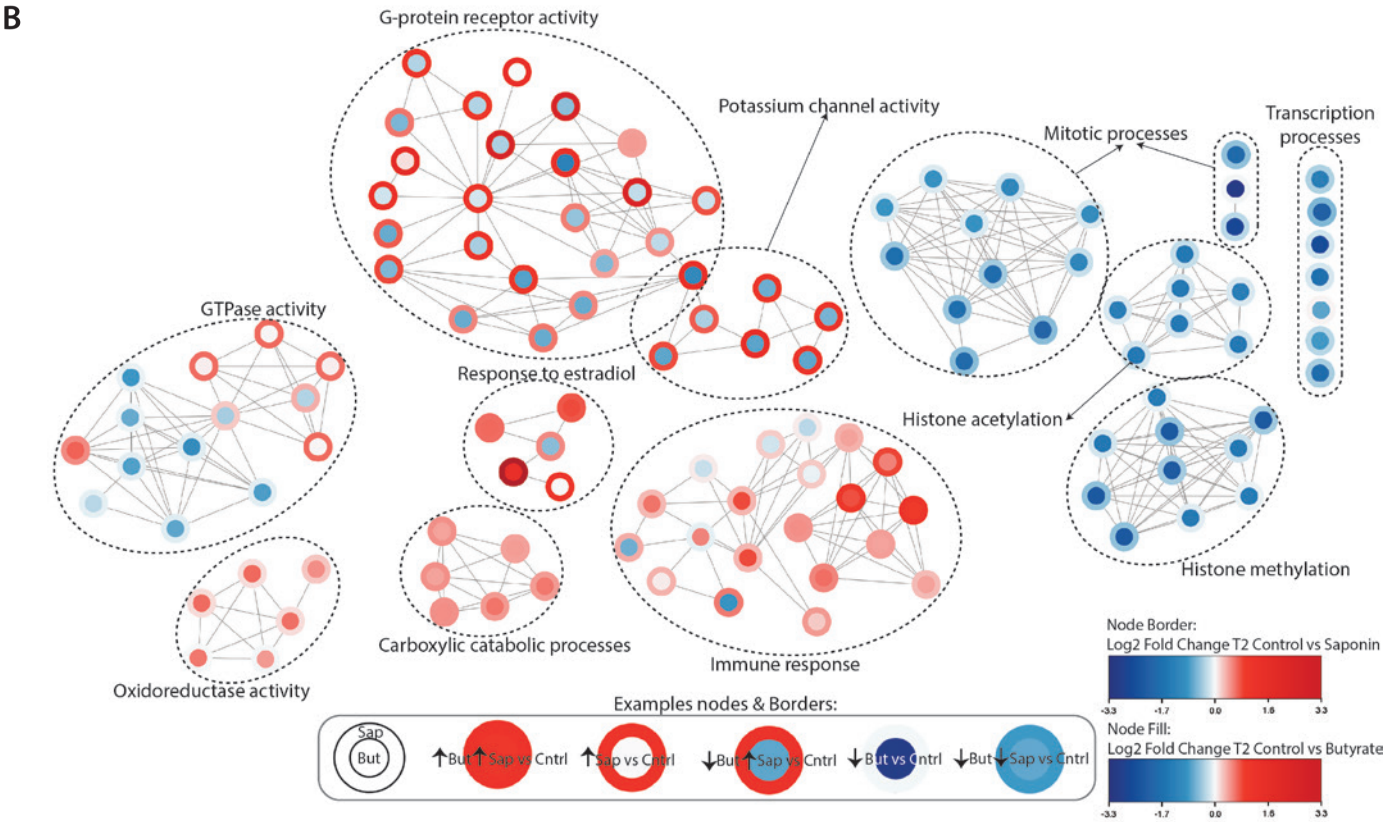


Figure 6: Continued.

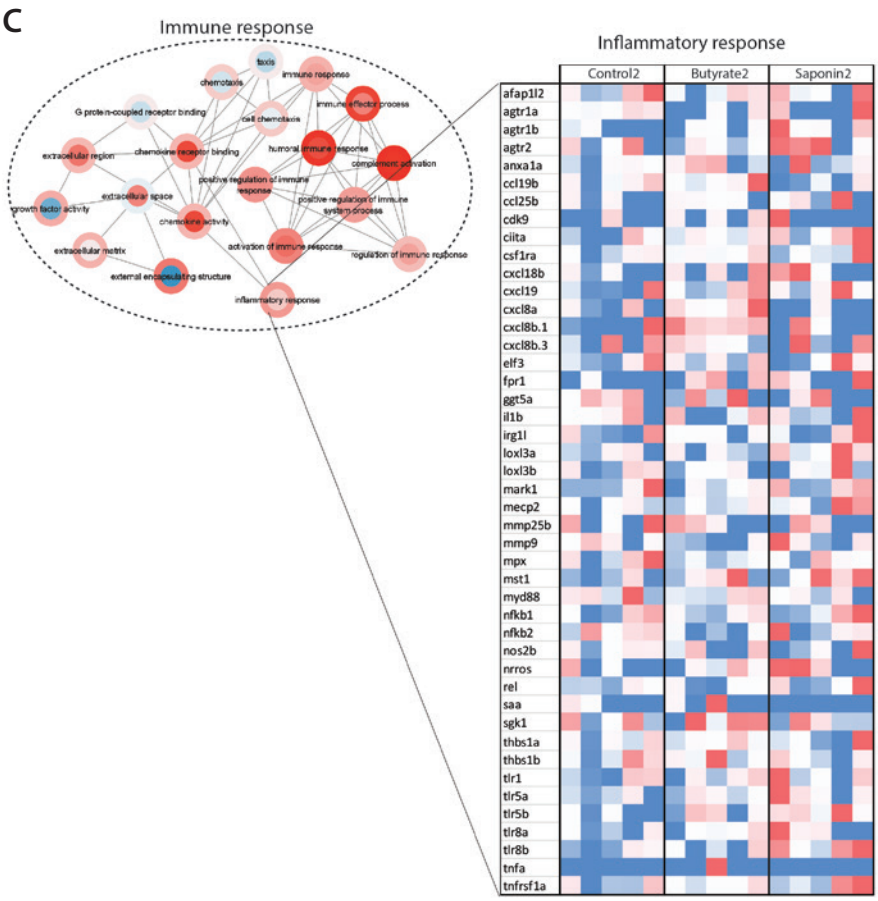


Figure 6: Continued.

folder 1). Compared to control fed fish, butyrate and saponin significantly down-regulated 893 GO terms while significantly up-regulated 40 GO terms out of a total of 6111 GO terms (**Supplementary folder 1**, SharedEffects.xlsx). The transcriptomic network depicts a shared down-regulation of the transcription and mitotic processes as well as histone acetylation and histone methylation, that is most prominently observed in butyrate fed fish (**Figure 6B**). Compared to control fed fish, both butyrate and saponin up-regulated the carboxylic catabolic processes, the oxidoreductase activity, response to estradiol and the immune response although some specific GO terms within these processes present differential modulation (**Figure 6B**).

Compared to control fed fish, saponin up-regulated 37 GO terms that were down-regulated in butyrate fed fish while butyrate up-regulated 79 GO terms that were

down-regulated in saponin fed fish (**Supplementary folder 1**, DifferentialEffects.xlsx). Saponin up-regulated genes associated to GTPase activity, potassium channel activity and G-protein receptor activity which were down-regulated in butyrate fed fish (**Figure 6B**). G-protein receptor activity is the GO term category that shows the strongest opposite regulation between saponin (up-regulated) and butyrate (down-regulated) and encompassed GO terms associated to photoreceptor activity, serotonin receptors activity as well as synaptic signaling (**Figure 6B**).

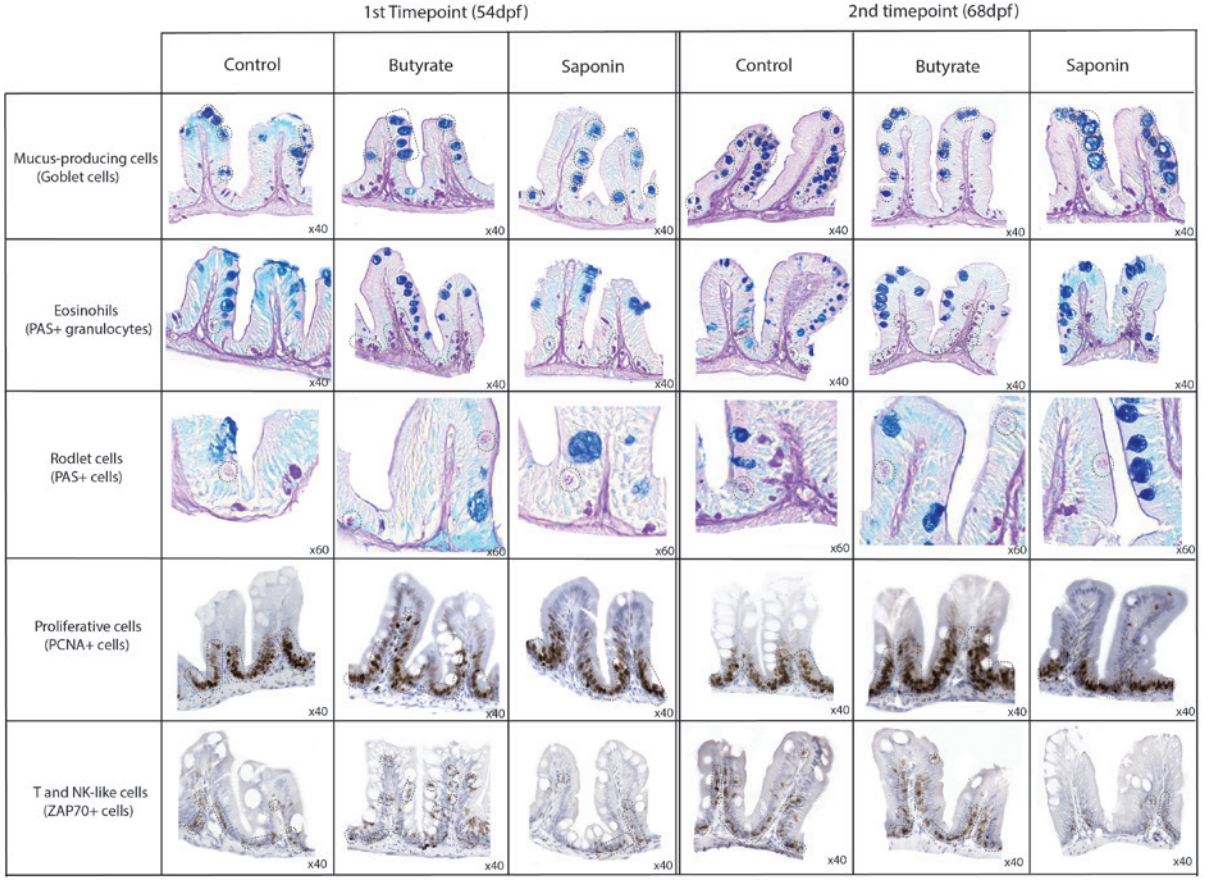
To explore the immune-related effects of butyrate and saponin, the immune response of the transcriptome network was zoomed in on (**Figure 6C**). In particular, the GO term "inflammatory response" was examined for butyrate and saponin fed fish. Compared to controls, saponin and butyrate up-regulated genes involved in the "inflammatory response" associated to chemokine activity as well as leukocyte and innate cell recruitment (*ccl19b, ccl25b, csfr1ra, cxcl18b. cxcl19, cxcl8a, cxcl8b.1, cxcl8b.3, fpr1, mpx, mst1* and *tlr*-family*)* (**Figure 6C**).

Gut quantitative histological analysis depicted distinct gut architectural profiles for butyrate and saponin

The zebrafish gut samples collected were analyzed using high-throughput quantitative histological analysis. While microbiota and transcriptomic data showed differential as well as similar effects of the butyrate and the saponin supplementation, the tissue make-up and topography provided further insight on whether changes in gene expression and microbiota also coincide with morphological indications of disturbed intestinal host gut health. Whole images were obtained from scanned slides and quantification was automated for several parameters: the AC and the cell area fraction, cell density, cell size, and the distance of each individual cell to the outer serosal layer for cell lineages of particular interest, including mucus cells (goblet cells), eosinophils (PAS+ granulocytes), rodlet cells (PAS+), proliferative cells (PCNA+ cells) and T and NK-like cells (Zap70+ cells). Representative pictures of all cell-types and time-points are shown in **Figure 7A**.

The effect of time did not correlate to any of the histological parameters analyzed (black arrows in the RDA graph, **Figure 7B**, p. value = 0.066) except for the increase of the PCNA area over time, indicative that the relative number of proliferative cells increased during fish development. Significant differences on the histological gut parameters were found due to the dietary interventions (p = 0.036) (**Figure 7C**). The absorptive capacity of the fish gut was decreased for the butyrate fed fish compared to saponin and control fed fish at 54 dpf, although displayed similar values at 68 dpf (boxplots around **Figure 7C**). The area of the eosinophils and rodlet cells increased in butyrate fed fish compared to saponin and control fed fish after 1 week of feeding (54 dpf), suggesting an inflammatory condition which was partly alleviated but not fully resolved at 68 dpf. In addition, compared to

A


1st Timepoint (54dpf)
2nd timepoint (68dpf)
Control
Butyrate
Saponin
Control
Butyrate
Saponin
Mucus-producing cells (Goblet cells)
Eosinohils (PAS+ granulocytes)
Rodlet cells (PAS+ cells)
Proliferative cells (PCNA+ cells)
T and NK-like cells (ZAP70+ cells)
x40
x60

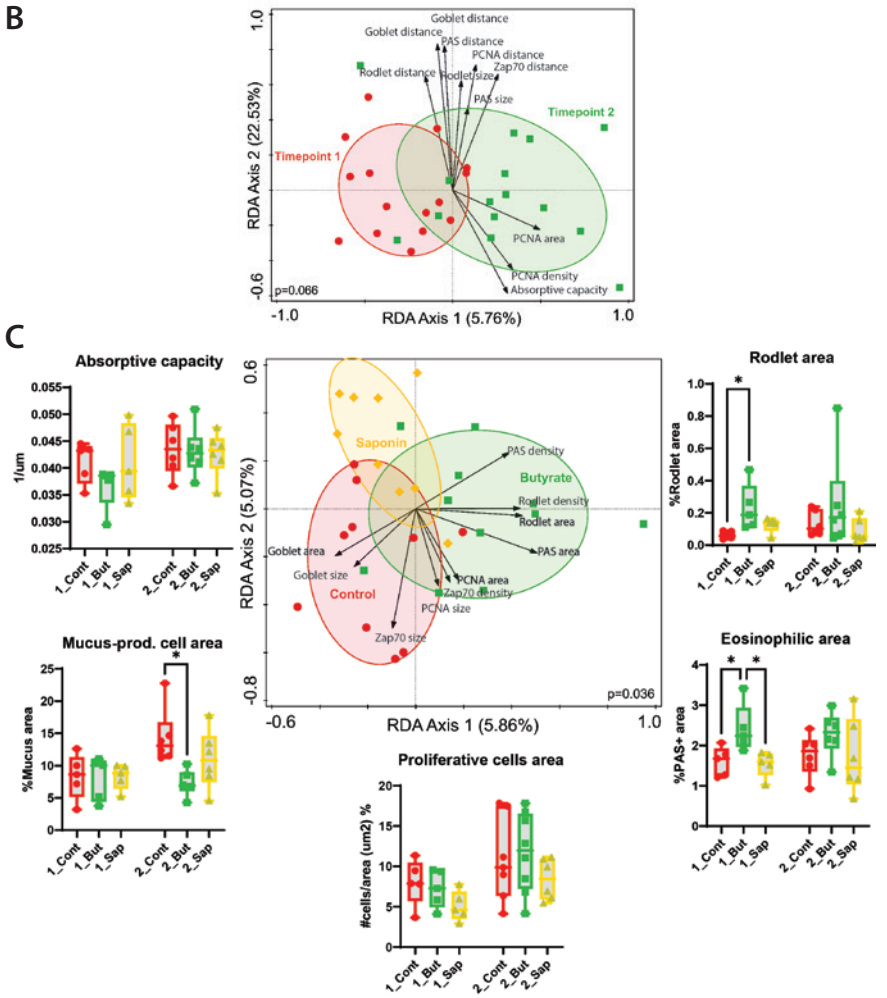


Figure 7: High-throughput quantitative histological analysis. **(A)** Representative pictures of all cell-types analyzed for all the diets and timepoints with cells of interest in dashed black lines per each group. **(B)** Redundancy analysis to examine the effect of time on the histological parameters analyzed. The x axis separated the samples by timepoints and explained 5.76% of the variation observed. The link of time and variation of the histological parameters was not significant (p=0.066) **(C)** Redundancy analysis to examine the effect of diet on the histological parameters analyzed. The x axis separated the samples of butyrate fed fish from saponin and control fed fish and explained 5.86% of the variation explained. The y axis separated saponin fed fish from control fed fish and explained 5.07% of the variation observed. The link of time and variation of the histological parameters was significant (p=0.036). The top 10 most distinctive histological parameters are depicted in black arrows. The direction of the arrows correlate with the dietary intervention and the length of the arrows represents the strength of the correlation. The boxplots around the RDA depicted the absorptive capacity and the percentage area of cells of interest compared to the total gut area per diet and timepoint. Whiskers: min. to max. shall all points with median.

controls, fish fed the butyrate diet showed a clear mucus-producing cell depletion after 3 weeks of feeding (68 dpf). Saponin fed fish presented a reduced proliferative cell area compared to butyrate and control fed fish. To illustrate these differences in histological parameters, accepting the biological fish to fish variation within each group, a heatmap of each individual fish and all the histological parameters per each RDA axis was generated (**Supplementary Figure 7**). The combination of these observations suggested an acute inflammatory response (after one week of exposure) of the fish fed the butyrate diet by increased eosinophils, rodlet cells and a decrease of the AC. The inflammatory condition remain unresolved after 3 weeks of feeding (68 dpf) as the fish fed the butyrate diet still presented increased eosinophils and rodlet cells and a depletion of mucus cells compared to saponin and control fed fish (black arrows and boxplots **Figure 7C**, representative pictures **Figure 7A**).

Combinatorial approach reveals distinct profiles for saponin and butyrate fed fish

In order to define robust and multi-parameter supported effects of butyrate and saponin supplementation, the key findings of the different datasets were integrated in a heatmap (**Figure 8**). Control fed fish did not present extreme microbiota fluctuations over time. Butyrate fed fish presented the most divergent microbiota composition (with increased relative abundance of *Bacteroides, Rhodobacter, Pandoraea* and *Flavobacterium*) and the lowest taxa connectivity compared to the other diets, which might be indicative of disturbed ecosystem stability. Saponin fed fish presented an increased number of *Vibrio* contrasting with butyrate fed fish. Compared to control fish, butyrate and saponin shared an increased expression of genes associated to immune responses, inflammatory responses and oxidoreductase activity. Besides, butyrate fed fish presented down-regulated genes in GO terms associated with histone acetylation, histone methylation, mitotic processes and G-protein coupled receptor activity. These differential gene expressions patterns were stronger after 3 weeks of feeding (68 dpf) compared to 1 week of feeding (54 dpf). In terms of histology, after 1 week of feeding butyrate, fish already showed increased area of eosinophils and rodlet cells compared to saponin or control fed fish, which is consistent after 3 weeks of feeding. Butyrate fed fish at 68 dpf showed decreased area of mucus cells compared to control fed fish. The histological parameters for saponin fed fish appeared to be less pronounced than those of butyrate fed fish. Collectively, these observations, showed fish fed a butyrate supplemented diet elicited a stronger response in terms of changes in the microbial composition, expression of genes associated to immune activation processes as well as the presence of (pro)inflammatory-like cells such as eosinophils and rodlet cells and depletion of mucus cells.

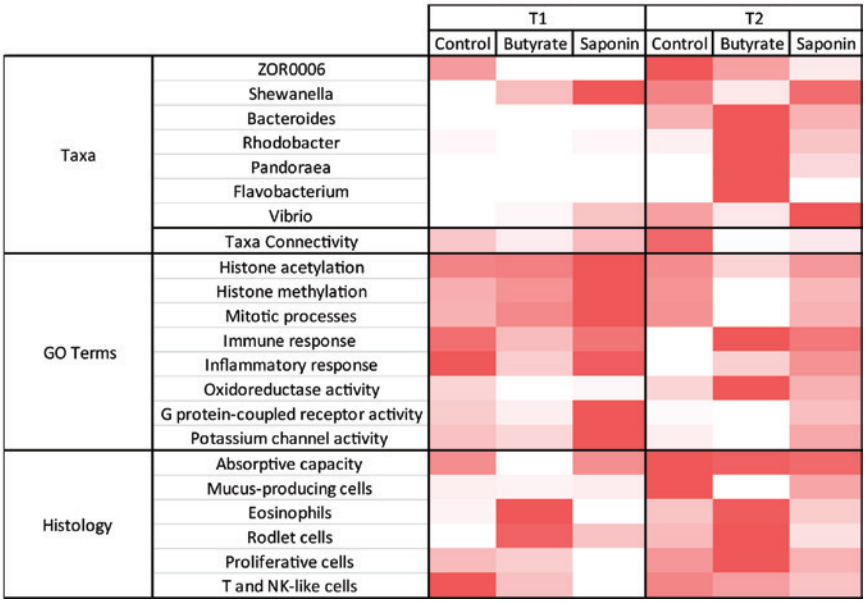


Figure 8: The heatmap brings together the main observations of each analysis and compare them per diet and timepoint. The more representative genera are illustrated with the average relative abundance per timepoint and diet. The taxa connectivity contained the amount of pairs of taxa that correlate to each other in a significant fashion ($p \leq 0.05$). The GO terms contain the transcripts per million (tpm) of all genes expressed in the dataset that collapsed under that GO term. All histological parameters are normalized and scaled (from 0 to 1). Each individual feature within the heatmap is normalized and colored from red (more present) to white (absent).

Butyrate and saponin increased neutrophil and macrophage recruitment in the gut of zebrafish larvae

Since the data clearly indicated an unexpected induction of immunity related functions upon butyrate addition to the feed (**Figure 6B**), the advantages of the zebrafish model system were used to validate the results by *in vivo* imaging of fluorescently labeled neutrophils and macrophages upon butyrate and saponin exposure in zebrafish larvae. Double Tg(mpeg1:mCherry / mpx:eGFPi[114]) zebrafish larvae were exposed to butyrate and saponin in different doses for 3 days (3-6dpf) and were (*in vivo*) imaged at 6dpf. Fish treated with butyrate as well as saponin presented a dose-dependent increase of neutrophils and macrophages in the intestinal area (**Figure 9A**). The quantification of the cells present in the gut area showed that butyrate as well as saponin significantly increased neutrophils and macrophages in the gut of zebrafish larvae (**Figure 9B**).

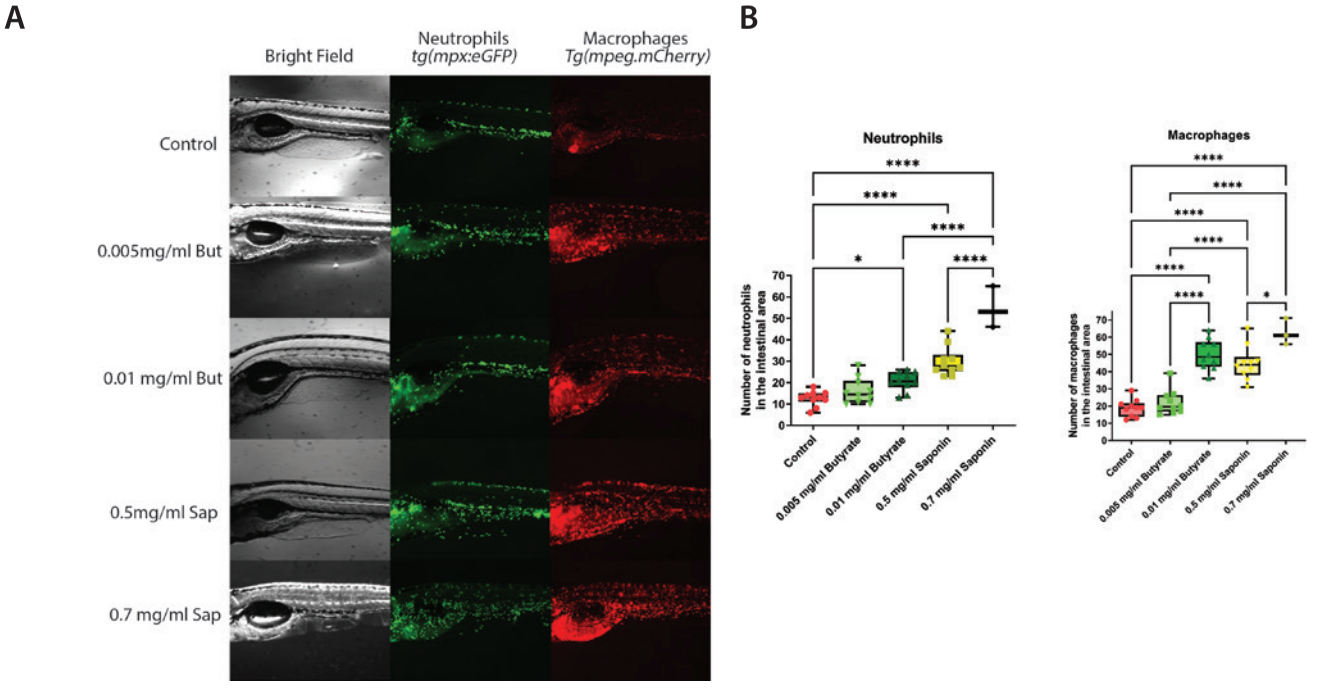


Figure 9: **(A)** Representative pictures of the fluorescent in vivo imaging of the gut area of Tg(mpeg1:mCherry / mpx:eGFPi114) larvae exposed to control media, 0.005mg/ml and 0.01 mg/ml butyrate and 0.5mg/ml and 0.7mg/ml saponin. **(B)** Quantification of neutrophils and macrophages in the gut area of the zebrafish larvae (n=10 in all groups except 0.7mg/ml saponin where n=3). *p≤0.5, **** p≤0.0001 Kruskal-Wallis test after testing for non-normally distributed data by Shapiro-Wilk test. Whiskers: min. to max. shall all points with median.

Discussion

In the present study the effects of butyrate and saponin-supplemented feed in the zebrafish gut were assessed following a combinatorial approach by integrating several datasets and validating the results by *in vivo* imaging. Juvenile zebrafish fed a butyrate-supplemented feed for 3 weeks presented a modulated microbial composition and low taxa connectivity, increased expression of genes associated with immune response together with an increased eosinophil and rodlet cell presence and mucus-producing cell depletion in the gut tissue. Moreover, butyrate increased the neutrophil and macrophage *in vivo* recruitment to the gut area in transgenic zebrafish larvae. Zebrafish fed a saponin-supplemented diet showed differentially modulated microbial composition from butyrate and low taxa connectivity as well as increased expression of immune response while the histological parameters comparable to control fed fish. The combinatorial approach of bacterial microbiome profiling, host gut transcriptomics, automated high-throughput quantitative histology (novel in zebrafish research) together with *in vivo* innate cell recruitment in the gut area in zebrafish larvae revealed evidence of the pro-inflammatory effects exerted by butyrate supplementation which were partly shared with the well- establish pro-inflammatory saponin supplementation, indicating detrimental effects of butyrate in the zebrafish intestinal milieu.

While saponin and soybean meal have been consistently associated with gut inflammation in several fish species, including carp (P. A. Urán *et al.*, 2008; Zheng *et al.*, 2012), salmon (Bakke-McKellep *et al.*, 2000; Knudsen *et al.*, 2007; P A Urán *et al.*, 2008; Krogdahl *et al.*, 2015; Hu *et al.*, 2016) and zebrafish (Hedrera *et al.*, 2013; López Nadal *et al.*, 2018), butyrate has been reported to convey beneficial effects when supplemented to fish feed. Reports include effects associated with acting intestinal growth enhancement (Robles *et al.*, 2013), and as an immunostimulant and antioxidant (Liu, *et al.*, 2014; Estensoro *et al.*, 2016), (reviewed in Abdel-Latif *et al.*, 2020). However, addition of (sodium) butyrate, either as a separate supplement or added to the feed, has not consistently been reported to confer beneficial health effects in fish. Divergent results depended on fish species, co-treatment(s) employed and duration of the feeding intervention. For instance, 0.8% inclusion of sodium butyrate in a low percentage plant-containing diet in gilthead sea bream for 10 weeks resulted in a mild inflammatory reaction whereas in the same study, 0.4% inclusion of sodium butyrate in high percentage plant-based diet for a longer period protected the host during a bacterial challenge (Piazzon *et al.*, 2017). In the present study 0.01% inclusion of butyrate induced pleiotropic damaging response captured in the combinatorial approach taken for the study comparable to the well-establish pro-inflammatory anti-nutritional factor soy saponin in a context of healthy growing juvenile fish.

In mammals, colonocytes located along the gut crypts take up the butyrate produced by the microbiota, preventing high concentrations of this SCFA to reach the proliferating stem cells at the bottom of the crypts. In fact, high concentrations (1.5-2mM) of butyrate were shown to be toxic to mouse pluripotent stem cells *in vitro* (Liang *et al.*, 2010). This is especially relevant in cryptless organisms such as fish (LØkka *et al.*, 2013)(Verdile *et al.*, 2020) (Aghaallaei *et al.*, 2016), where higher concentrations of butyrate can reach the stem cells localized between the intestinal folds (villi). Mechanistic studies in mouse and zebrafish larvae suggested that butyrate at high concentrations inhibits stem cell proliferation via FoxO3 in cryptless organisms at (such as zebrafish) (Kaiko *et al.,* 2016). Taking this observations together with our combinatorial and multifaceted approach which revealed that zebrafish fed a butyrate-supplemented diet showed a compromised intestinal epithelial barrier function, coinciding with a modulated microbiota composition that displayed decreased taxa connectivity, and increased expression levels of genes associated with inflammatory and immune responses that was confirmed by enhanced (innate) immune cell recruitment *in* vivo to the zebrafish gut. Our data warrants that further research should investigate the long term effects of butyrate-supplemented feed and susceptibility towards infectious or inflammatory challenges which were not investigated here. Potentially, butyrate-associated immuno-stimulation early in life, could boost immunity and strengthen disease resistance in later life stages (trained immunity) (Petit and Wiegertjes, 2016).

Disruption of the gut microbiota homeostasis, often caused by an imbalance in the bacterial population (or dysbiosis), is commonly associated with inflammatory conditions in the zebrafish gut (Bates *et al.*, 2007; Brugman *et al.*, 2009) also (reviewed in Brugman, 2016; López Nadal *et al.*, 2020). In inflammatory bowel disease patients, topological properties of the co-occurring bacterial networks identified anti- and pro-inflammatory key organisms that defined the degree of structure of the ecosystem (Baldassano and Bassett, 2016). In fish, recent studies validated the usage of co-occurrence and anti-occurrence taxa networks to identify the core gut European seabass microbiota and their relationship with the other gut microbes (Kokou *et al.*, 2019) or the effects of prebiotics and probiotics on fish gut microbiome that revealed co- and anti-occurring interactions among the main phyla Proteobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Maas *et al.*, 2021). In the present study, zebrafish fed 3 weeks a butyrate-supplanted feed presented altered the microbiota composition as well as reduced taxa connectivity (co- and anti-occurrence) compared to control (and to a lesser extent to saponin)-fed fish (**Figure 4, 5 and Suppl. Figure 6**). Butyrate increased the relative abundance of the genera *Rhodobacter, Flavobacterium* and *Bacteroides* that were previously associated with gut inflammation in fish (Lorenzen, *et* al., 1997; Tran *et al.*, 2018; Xia *et al.*, 2018) whereas saponin increased the relative abundance of the *Vibrio* genus, which contains several

pathobiont species which might become pathogenic upon challenge of the gut barrier integrity (reviewed in Colwell and Grimes, 1984). In mammals, butyrate is produced by fermenting bacteria in the intestinal tract and until now scientists were not able to measure any naturally occurring concentrations of butyrate in the zebrafish gut (Cholan *et al.*, 2020). Since it is not certain whether fish gut may produce butyrate, exogeneous butyrate supplementation may disrupt the growth of bacteria since they may not be used to metabolize such substrate. In butyrate-fed fish increased abundance of *Bacteroides* correlated with lower abundance of *Vibrio*. Interestingly, *in vitro* studies have revealed that butyrate exposure can negatively impact the colonization of specific *Vibrio campbellii* PUGSK8 by its effect on biofilm formation capacity in these bacteria. Taken together, these findings warrant further studies to understand the mechanisms by which butyrate influences microbial ecosystems.

Inflammatory-associated taxa in butyrate-fed fish matched with an increased expression of genes belonging to inflammatory and immune responses (**Figure 8**). While targeted gene expression is commonly used in (fish) nutrition studies, this approach is often hypothesis-driven and the discovery risk of novel premises is relatively low compared to more comprehensive transcriptome analyses. In the present study, butyrate down-regulated genes associated with mitotic and transcription processes which is in line with the inhibition of stem cell proliferation previously reported (Kaiko *et al.,* 2016), although proliferative cells (PCNA+) were not decreased in butyrate-fed fish as shown by the histological dataset. Butyrate down-regulated genes associated with histone modifications (acetylation and methylation) in line with previously described epigenetic effects of butyrate in mammals (reviewed in van der Hee and Wells, 2021). Further research may elucidate whether there is an effect of butyrate supplemented feed on epigenetic markers and in the affirmative case whether such epigenetic modifications can be passed on the fish offspring.

A clear subset of chemokines within the inflammatory response appeared to be up-regulated after butyrate-supplemented feeding (**Figure 6C**) among which *cxcl8a, cxcl8b.1* and *cxcl8b.3. Cxcl8* (or *il8*) is known as one of the most potent chemoattractant molecules for recruiting neutrophils (expressing CXCR1/2 receptors for *Cxcl8*) and other leukocytes upon inflammation (de Oliveira *et al.*, 2013). Although IL8 did not affect human eosinophils *in vitro* (Petering *et al.*, 1999), eosinophils are able (via granule proteins) to stimulate neutrophils that produce IL8 and superoxide contributing to gastrointestinal pathologies (Rosenberg, Dyer and Foster, 2013). However, eosinophil research in the context of gastrointestinal health is limited in humans and mice (Jacobsen *et al.*, 2021) as well as (zebra)fish (Balla *et al.*, 2010). Butyrate increased eosinophil and rodlet cell area even after 1 week of feeding, while reduced the presence of mucus cells overtime (**Figure 7**), features associated with (chemically-induced) intestinal inflammation in zebrafish

(Brugman *et al.*, 2009). Rodlet cells were first reported to act against fish parasites and later studies disclosed their granulocyte nature and include them as part of the innate fish immune system, increasing in number when exogeneous stressors were present (Iger and Abraham, 1997; Manera and Dezfuli, 2004; Reite and Evensen, 2006; Dezfuli *et al.*, 2022). More research into this well-known but often forgotten cell type may elucidate its role in (zebra)fish mucosal immunology.

Furthermore, using the advantages of the zebrafish larvae model it was shown that butyrate and saponin increased neutrophils and macrophages recruitment to the gut by quantification of fluorescent *in vivo* imaging. The fact that saponin induced a stronger cell recruitment than butyrate could be explained by the fact that lower concentrations of butyrate were used (mimicking the ones employed in the diets) (**Figure 9**). Other studies showed decreased neutrophil recruitment after tail wounding when zebrafish larvae were immersed to butyrate (Cholan *et al.*, 2020). However, such studies briefly immersed zebrafish larvae to extremely high concentrations of sodium butyrate (30 mM = 3303 mg/ml) and such study design may greatly differ from the naturally occurring physiological situation in the zebrafish gut. We hypothesize that the increased chemokine expression in butyrate fed fish might be the driving force for the increased leucocyte recruitment in the gut and further research may disclose specific butyrate modes of action in the (zebra) fish gut.

In the present study butyrate-supplemented feed appeared to modulate the microbial composition as indicated by low taxa connectivity, increased expression of gene associated to inflammatory processes as well as increased presence of rodlet cells, and eosinophils while decreasing Goblet cells. Moreover, we supplemented this data with *in vivo* observations of the increased recruitment of the neutrophil and macrophage population in the gut upon butyrate and saponin exposure. The combination of these datasets indicate that butyrate promotes some fish-specific effects on the gut homeostasis that differ from the mammalian counterparts (Guilloteau *et al.*, 2010). The particular fish gut structure, lacking intestinal crypts could play an important role on the absorption and the effect of the butyrate on the epithelial lining where chemokines might orchestrate the inflammatory-like response. While more mechanistic studies are needed to shed light on the specific modes of action of butyrate on the fish gut health, the present combined study (omics, histology and imaging) provides evidence to support non-beneficial effect of butyrate-supplemented feed on growing juvenile zebrafish. Integration of multi-layered high-throughput studies still remain a challenge in scientific studies because of various reasons. On the one hand, there are difficulties to fully comprehend the connections between the complex layers of data deriving from high-throughput methods and to select the most relevant outcomes (biomarkers). On the other hand, scientist may not have yet the technology to adequately obtain multi-omics data with sufficient resolution

(lack of noise) and reproducibility that facilitates omics datasets combination. In the present study, the detrimental effects of butyrate towards the zebrafish gut were congruent throughout all the datasets in our combinatorial approach strengthening the biologically relevant observation that butyrate appears detrimental to the zebrafish gut. Steps towards observational scientific studies with an integrative view, combining high-throughput datasets with imaging techniques in relevant model organisms to understand complex multifactorial biological processes such as (fish) gut health may contribute to expand our knowledge on gut health further.

Declarations

Ethics approval and Consent to participate

The present study was approved by the Dutch Committee on Animal Welfare (2017.W-0034) and the Animal Welfare Body (IvD) of the Wageningen University (The Netherlands). Furthermore, we adhered to standard biosecurity and institutional safety procedures at Wageningen University and Research.

Consent for publication

All authors revised and agreed on the submitted version of this manuscript.

Availability of data and materials

Raw data of the transcriptomic analyses can be found in Supplementary folder 1. All raw data will be uploaded to an open access repository.

Competing interests

The authors declare to have no competing interests.

Funding

This study was funded by NWO-TTW Applied and Engineering Sciences (project number 15566).

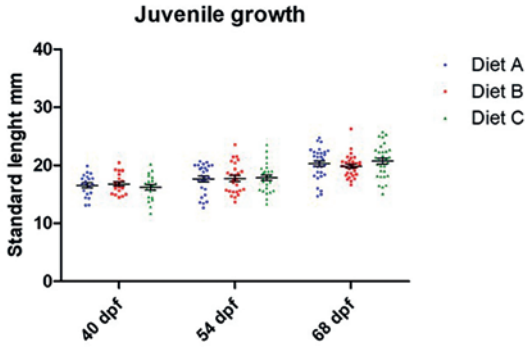
Author's contributions

ALN performed research, analyzed and integrated all data, drafted manuscript and figures. JB analyzed and integrated all data, drafted manuscript and figures. CL and FvdB and TBE collected and analyzed histological data. MAS analyzed 16S and transcriptomics data and drafted manuscript. DP and CMcG drafted and edited manuscript and developed and provided feeds. DS MK drafted and edited manuscript. GW and SB drafted and edited manuscript and provided the funding. All authors review the manuscript.

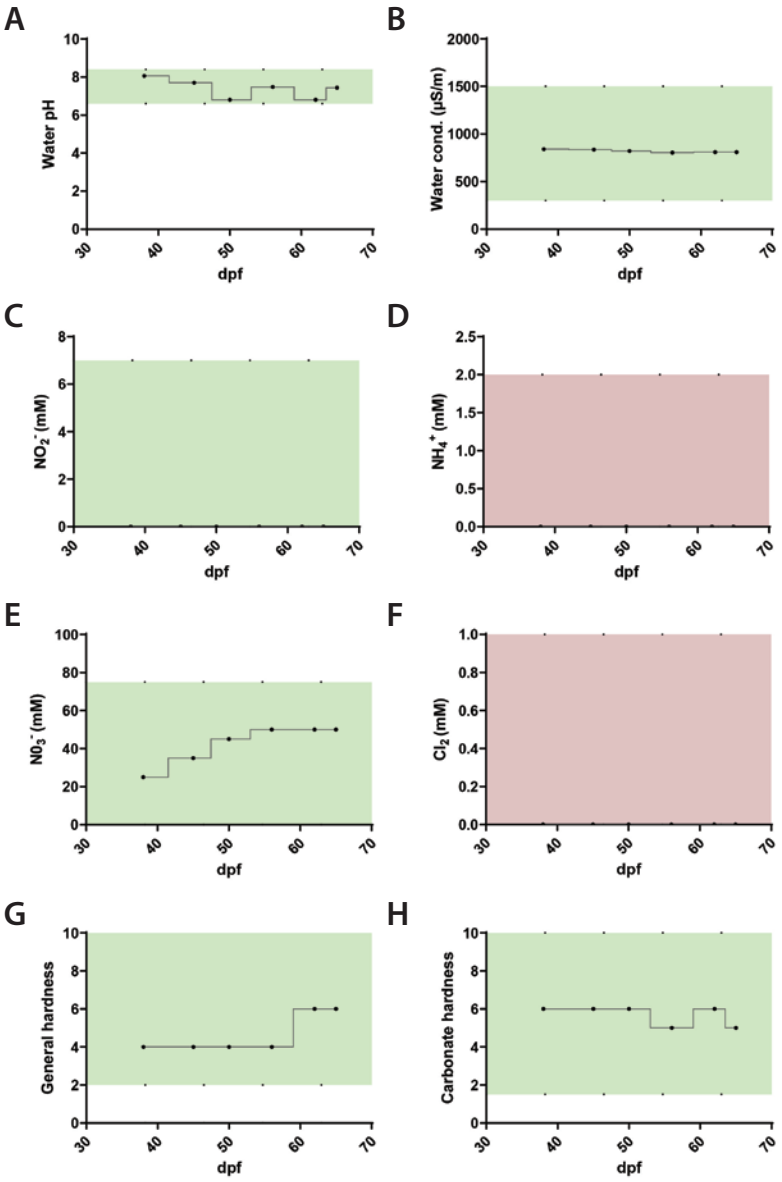
Acknowledgements

The authors would like to thank Steven Aalvink and Ineke Heikamp-de Jong for extracting RNA and the PCRs for the molecular analyses and Trond Kortner to supply to us the 95% ultra-pure saponin.

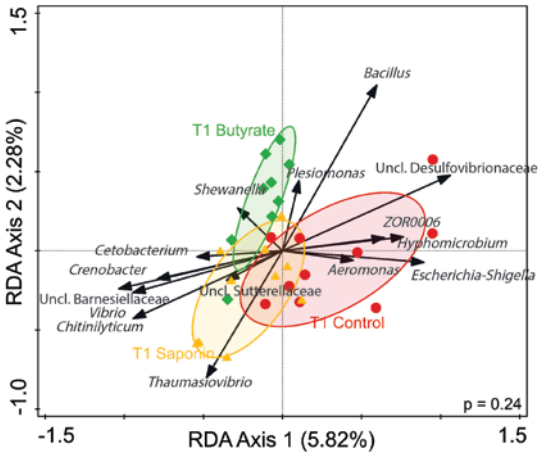
Supplementary Figures



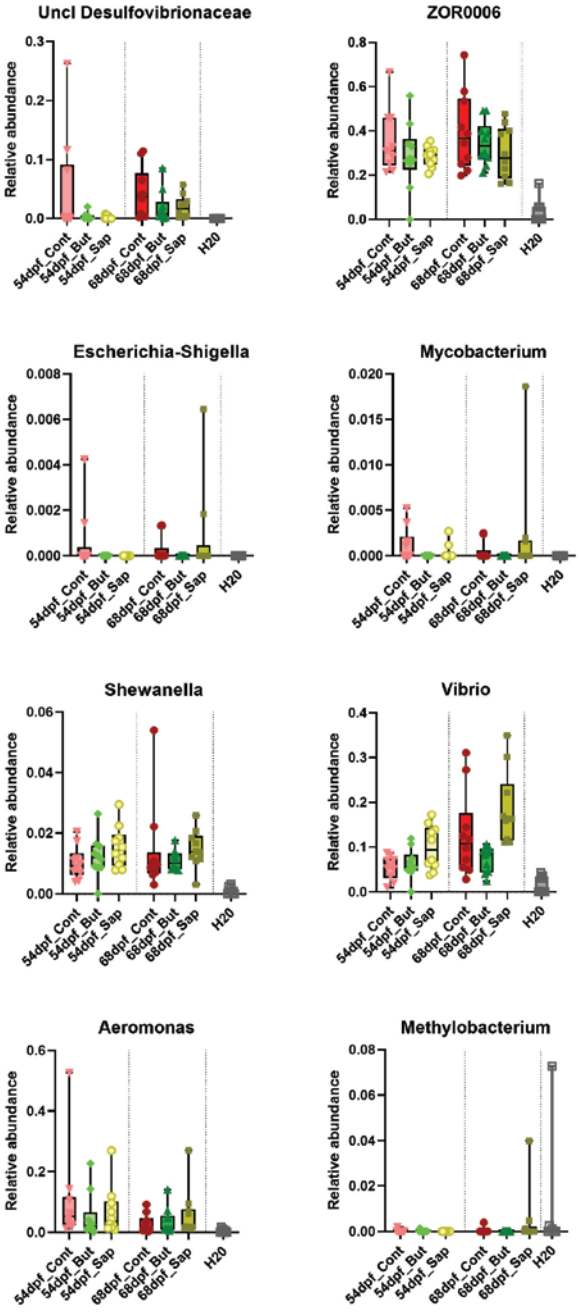
Supplementary Figure 1: Standard length (mm) was measured at 40, 54 and 68 dpf for the 3 diets by using a digital calliper.



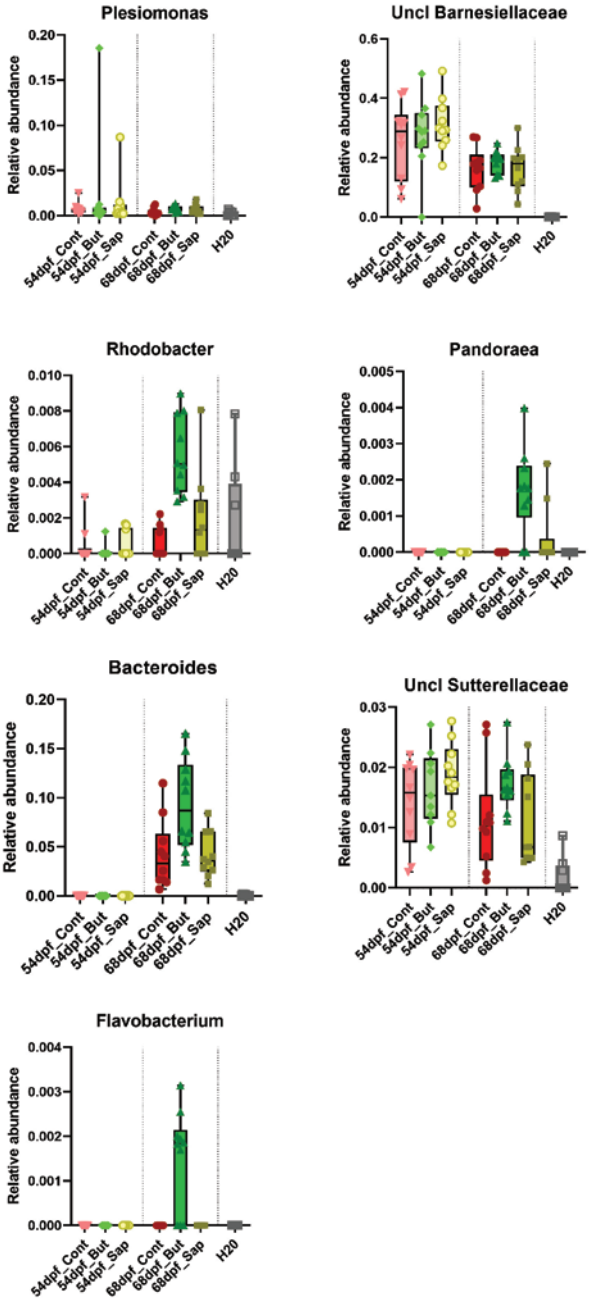
Supplementary Figure 2: Water quality values just before and during the experiment at 38, 45, 50, 56, 62 and 65 dpf. In green the range of preferable values for the measurements and in red the values above which the water quality is considered to be detrimental for the fish according to manufacturer's instructions: **(A)** pH (accepted range 6.6-8.4), **(B)** Water conductivity (accepted range 300-1500 µS/m), **(C)** Nitrite (accepted range 0-7 mM), **(D)** Ammonium (only 0 mM accepted), **(E)** Nitrate (accepted range 0-70 mM), **(F)** Chlorine (only 0 mM accepted), **(G)** General hardness (accepted range 2-16) and **(H)** Carbonate hardness (accepted range 1.5-10).



Supplementary Figure 3: Redundancy Analysis (RDA) at the 1st timepoint to examine the effect of the diets on the gut microbiota. The x axis separates saponin from control fed fish and explains 5.82% of the microbial differences observed and the y axis separates the butyrate from the saponin fed fish and explains 2.28% of the microbial differences observed. The top 15 most distinctive genera are depicted as supplementary variables in black arrows, p=0.24.



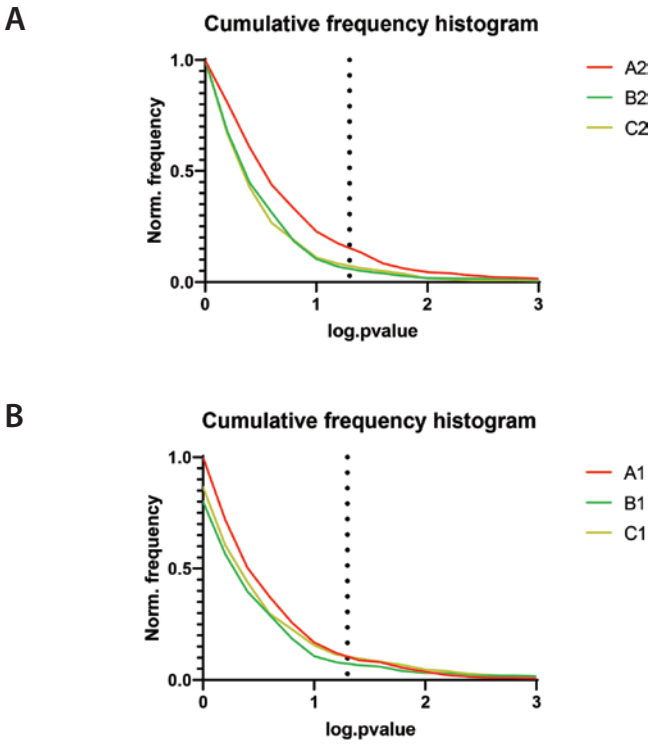
Supplementary Figure 4: relative abundances of the top 15 most distinctive genera for all diets at both timepoints, including water samples from all fish tanks at both timepoints.



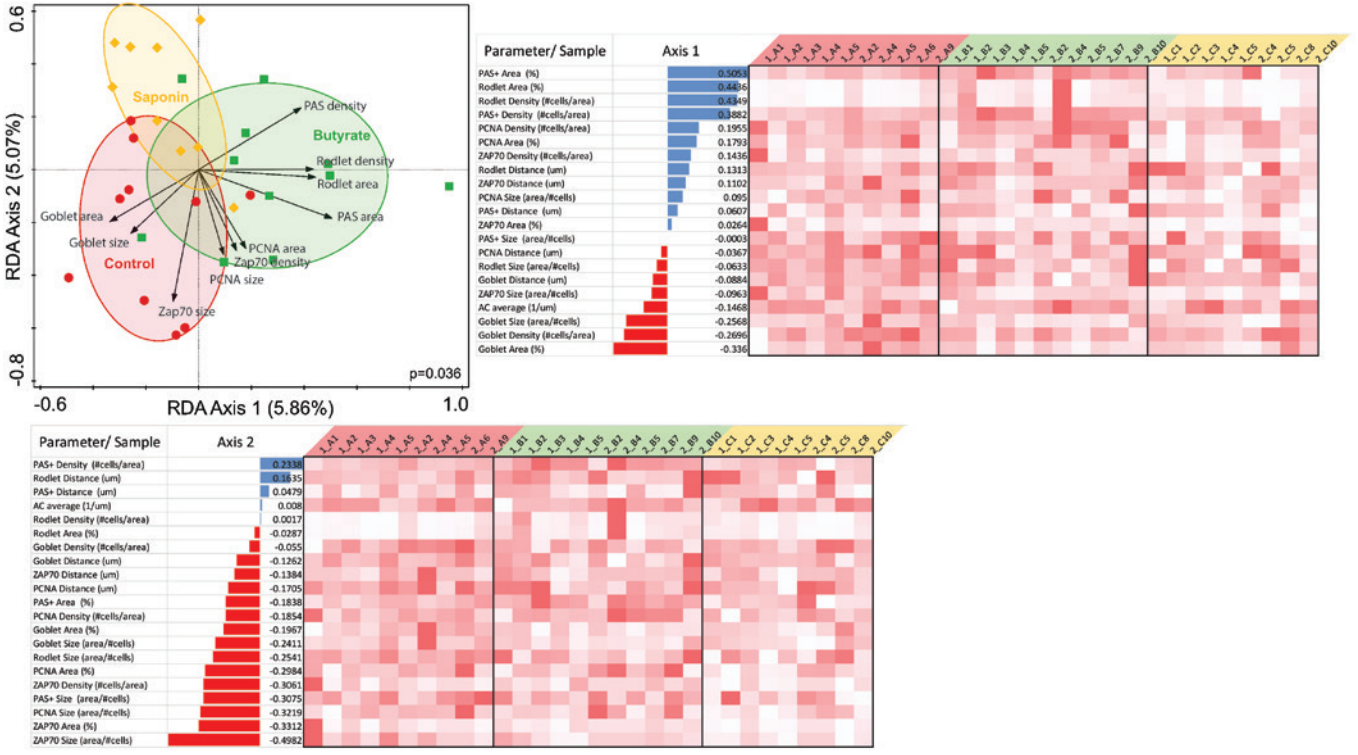
Supplementary Figure 4: Continued.

Taxa		A1_2	A10_2	A2_2	A3_2	A4_2	A5_2	A6_2	A7_2	A8_2	A9_2	B1_2	B10_2	B2_2	B3_2	B4_2	B5_2	B6_2	B7_2	B8_2	B9_2	C1_2	C10_2	C2_2	C3_2	C4_2	C5_2	C6_2	C7_2	C8_2	C9_2	rel. abundance	
	importance*	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B	B	B	C	C	C	C	C	C	C	C	C	C	min	max
Crenobacter	0.788067313																															0	0.036541
Bacteroides	0.766758508																															0.0068954	0.165341
Rhodobacter	0.758821751																															0	0.008978
uncultured_Sutterellaceae	0.662579354																															0.0012641	0.027453
Flavobacterium	0.659822287																															0	0.003138
uncultured_Desulfovibrionaceae	0.651540398																															0	0.114263
Plesiomonas	0.651524405																															0	0.018023
Pandoraea	0.64667665																															0	0.003986
Vibrio	0.574617342																															0.0237343	0.349666
uncultured_Barnesiellaceae	0.55408512																															0.0280948	0.299927
uncultured_bacterium_Gaiellales	0.546021694																															0	0.053184
uncultured_Gemmataceae	0.493911935																															0	0.013243
Methylobacterium-Methylorubrum	0.46160351																															0	0.039805
Escherichia-Shigella	0.444003705																															0	0.00645
Bacillus	0.4129927																															0	0.026393
ZOR0006	0.364882461																															0.1608421	0.742786
Mycobacterium	0.361167579																															0	0.018633
Aeromonas	0.35308533																															0.002278	0.270884
Chitinilyticum	0.318967663																															0	0.003234
Cetobacterium	0.301202424																															0.0258898	0.321319
Shewanella	0.260644202																															0.0030388	0.053974
Cellvibrio	0.256785085																															0	0.044386
Thaumasiovibrio	0.222726402																															0	0.013828
Pseudomonas	0.182835445																															0	0.001239

Supplementary Figure 5: Heatmap of the relative abundance (relative to 1) of the most distinctive and important taxa for all diets at the 2nd timepoint. Importance was calculated as (sqrt(CorS1^2+CorS2^2)), i.e., the length of the arrows in **Figure 4B**.



Supplementary Figure 6: Normalized cumulative frequency histogram depicting the amount of significant pairs of taxa correlations per each diet **(A)** at 68 dpf, **(B)** at 54 dpf; (dotted line represents logarithmic p value =1.30 and p =0.05).



Supplementary Figure 7: Heatmaps of each individual gut fish sample (n=5 diet / timepoint) for both axis of the redundancy analysis. Despite of the fish to fish variation present dietary effects are visible for both timepoints. Values are normalized and scaled from 0-1.

References

Abdel-Latif, H. M. R. *et al.* (2020) 'Benefits of Dietary Butyric Acid, Sodium Butyrate, and Their Protected Forms in Aquafeeds: A Review', *Reviews in Fisheries Science & Aquaculture*. Taylor & Francis, 28(4), pp. 421–448. doi: 10.1080/23308249.2020.1758899.

Afgan, E. *et al.* (2018) 'The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update', *Nucleic Acids Research*, 46(W1), pp. W537–W544. doi: 10.1093/nar/gky379.

Aghaallaei, N. *et al.* (2016) 'Identification, visualization and clonal analysis of intestinal stem cells in fish', *Development*. The Company of Biologists Ltd, 143(19), pp. 3470–3480.

Augustin, J. M. *et al.* (2011) 'Molecular activities, biosynthesis and evolution of triterpenoid saponins', *Phytochemistry*. Elsevier, 72(6), pp. 435–457.

Bakke-McKellep, A. M. *et al.* (2000) 'Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, Salmo salar L., with soybean meal-induced enteritis', *Journal of Fish Diseases*, 23(2), pp. 115–127. doi: 10.1046/j.1365-2761.2000.00218.x.

Baldassano, S. N. and Bassett, D. S. (2016) 'Topological distortion and reorganized modular structure of gut microbial co-occurrence networks in inflammatory bowel disease', *Scientific reports*. Nature Publishing Group, 6(1), pp. 1–14.

Balla, K. M. *et al.* (2010) 'Eosinophils in the zebrafish: Prospective isolation, characterization, and eosinophilia induction by helminth determinants', *Blood*, 116(19), pp. 3944–3954. doi: 10.1182/blood-2010-03-267419.

Bates, J. M. *et al.* (2007) 'Intestinal Alkaline Phosphatase Detoxifies Lipopolysaccharide and Prevents Inflammation in Zebrafish in Response to the Gut Microbiota', *Cell Host and Microbe*, 2(6), pp. 371–382. doi: 10.1016/j.chom.2007.10.010.

Braak, C. J. F. ter and Smilauer, P. (2012) *Canoco reference manual and user's guide: software for ordination, version 5.0*. 1813, , : Microcomputer Power.

Bravo-Tello, K. *et al.* (2017) 'Effect of microalgae on intestinal inflammation triggered by soybean meal and bacterial infection in zebrafish', *PLoS ONE*, 12(11), pp. 1–13. doi: 10.1371/journal.pone.0187696.

Brugman, S. *et al.* (2009) 'Oxazolone-Induced Enterocolitis in Zebrafish Depends on the Composition of the Intestinal Microbiota', *Gastroenterology*. Elsevier Inc., 137(5), pp. 1757-1767.e1. doi: 10.1053/j.gastro.2009.07.069.

Brugman, S. (2016) 'The zebrafish as a model to study intestinal inflammation', *Developmental and Comparative Immunology*, 64, pp. 82–92. doi: 10.1016/j.dci.2016.02.020.

Chikwati, E. M. *et al.* (2012) 'Interaction of soyasaponins with plant ingredients in diets for Atlantic salmon, Salmo salar L.', *British Journal of Nutrition*. Cambridge University Press, 107(11), pp. 1570–1590.

Cholan, P. M. *et al.* (2020) 'Conserved anti-inflammatory effects and sensing of butyrate in zebrafish', *Gut microbes*. Taylor & Francis, 12(1), p. 1824563.

Colwell, R. R. and Grimes, D. J. (1984) 'Vibrio diseases of marine fish populations', *Helgoländer Meeresuntersuchungen*. BioMed Central, 37(1), pp. 265–287.

Costas, B. *et al.* (2014) 'Gilthead seabream (Sparus aurata) immune responses are modulated after feeding with purified antinutrients', *Fish & Shellfish Immunology*. Elsevier, 41(1), pp. 70–79.

Cunningham, M. *et al.* (2021) 'Shaping the future of probiotics and prebiotics', *Trends in Microbiology*. Elsevier, 29(8), pp. 667–685.

Dezfuli, B. S. *et al.* (2022) 'Rodlet cells, fish immune cells and a sentinel of parasitic harm in teleost organs', *Fish & Shellfish Immunology*. Elsevier.

Dowle, M. *et al.* (2019) 'Package "data. table"', *Extension of 'data. frame.*

Estensoro, I. *et al.* (2016) 'Dietary butyrate helps to restore the intestinal status of a marine teleost (Sparus aurata) fed extreme diets low in fish meal and fish oil', *PLoS One*. Public Library of Science San Francisco, CA USA, 11(11), p. e0166564.

Ewels, P. A. *et al.* (2020) 'The nf-core framework for community-curated bioinformatics pipelines', *Nature Biotechnology*, 38(3), pp. 276–278. doi: 10.1038/s41587-020-0439-x.

Flores, E. M. *et al.* (2020) 'The zebrafish as a model for gastrointestinal tract–microbe interactions', *Cellular microbiology*. Wiley Online Library, 22(3), p. e13152.

Gao, Y. *et al.* (2011) 'Supplementation of fishmeal and plant protein-based diets for rainbow trout with a mixture of sodium formate and butyrate', *Aquaculture*. Elsevier, 311(1–4), pp. 233–240.

Garnier, S. *et al.* (2021) 'Viridis—Colorblind-Friendly Color Maps for R', *R package version 0.6*, 2.

Gerard E. Kaiko, Stacy H. Ryu, Olivia I. Koues, Patrick L. Collins, Lilianna Solnica- Krezel, Edward J. Pearce, Erika L. Pearce, Eugene M. Oltz, and T. S. S. (2016) 'The colonic crypt protects stem cells from microbiota-derived metabolites', *Cell*, 165(7), pp. 1708–1720. doi: doi:10.1016/j.cell.2016.05.018. The.

Gibson, G. R. *et al.* (2017) 'Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics', *Nature reviews Gastroenterology & hepatology*. Nature Publishing Group, 14(8), pp. 491–502.

Gillis, J., Mistry, M. and Pavlidis, P. (2010) 'Gene function analysis in complex data sets using ErmineJ', *Nature protocols*. Nature Publishing Group, 5(6), pp. 1148–1159.

Guilloteau, P. *et al.* (2010) 'From the gut to the peripheral tissues: the multiple effects of butyrate', *Nutrition research reviews*. Cambridge University Press, 23(2), pp. 366–384.

Hartinger, T. *et al.* (2019) 'Differently Pre-treated Alfalfa Silages Affect the in vitro Ruminal Microbiota Composition.', *Frontiers in microbiology*, 10, p. 2761. doi: 10.3389/fmicb.2019.02761.

Hedrera, M. I. *et al.* (2013) 'Soybean Meal Induces Intestinal Inflammation in Zebrafish Larvae', *PLoS ONE*, 8(7), pp. 1–10. doi: 10.1371/journal.pone.0069983.

van der Hee, B. and Wells, J. M. (2021) 'Microbial regulation of host physiology by short-chain fatty acids', *Trends in Microbiology*. Elsevier, 29(8), pp. 700–712.

Howe, K. *et al.* (2013) 'The zebrafish reference genome sequence and its relationship to the human genome', *Nature*, 496(7446), pp. 498–503. doi: 10.1038/nature12111.

Hu, H. *et al.* (2016) 'Intestinal fluid permeability in Atlantic salmon (Salmo salar L.) is affected by dietary protein source', *PLoS ONE*, 11(12), pp. 1–18. doi: 10.1371/journal.pone.0167515.

Iger, Y. and Abraham, M. (1997) 'Rodlet cells in the epidermis of fish exposed to stressors', *Tissue and Cell*, 29(4), pp. 431–438. doi: https://doi.org/10.1016/S0040-8166(97)80029-8.

Jacobsen, E. A. *et al.* (2021) 'Eosinophil knockout humans: uncovering the role of eosinophils through eosinophil-directed biological therapies', *Annu Rev Immunol*, 39(1), pp. 719–757.

Kang, S. *et al.* (2009) 'An Efficient RNA Extraction Method for Estimating Gut Microbial Diversity by Polymerase Chain Reaction', *Current Microbiology*, 58(5), p. 464. doi: 10.1007/s00284-008-9345-z.

Kiran, G. S. *et al.* (2016) 'Degradation intermediates of polyhydroxy butyrate inhibits phenotypic expression of virulence factors and biofilm formation in luminescent Vibrio sp. PUGSK8', *NPJ biofilms and microbiomes*. Nature Publishing Group, 2, p. 16002.

Knudsen, D. *et al.* (2007) 'Saponin-containing subfractions of soybean molasses induce enteritis in the distal intestine of Atlantic salmon', *Journal of Agricultural and Food Chemistry*, 55(6), pp. 2261–2267. doi: 10.1021/jf0626967.

Kokou, F. *et al.* (2019) 'Core gut microbial communities are maintained by beneficial interactions and strain variability in fish', *Nature microbiology*. Nature Publishing Group, 4(12), pp. 2456–2465.

Kothari, D., Patel, S. and Goyal, A. (2014) 'Therapeutic spectrum of nondigestible oligosaccharides: overview of current state and prospect', *Journal of food science*. Wiley Online Library, 79(8), pp. R1491–R1498.

Krogdahl, Å. *et al.* (2015) 'Soya Saponins Induce Enteritis in Atlantic Salmon (Salmo salar L.)', *Journal of Agricultural and Food Chemistry*, 63(15), pp. 3887–3902. doi: 10.1021/jf506242t.

LØ kka, G. *et al.* (2013) 'Intestinal morphology of the wild Atlantic salmon (Salmo salar)', *Journal of morphology*. Wiley Online Library, 274(8), pp. 859–876.

Lahti, L. *et al.* (2017) 'microbiome R package', *Tools Microbiome Anal R*.

Lahti, L. and Shetty, S. (2019) 'Introduction to the microbiome R package'. Available at: https://microbiome.github.io/tutorials/.

Laserna-Mendieta, E. J. *et al.* (2018) 'Determinants of reduced genetic capacity for butyrate synthesis by the gut microbiome in Crohn's disease and ulcerative colitis', *Journal of Crohn's and Colitis*. Oxford University Press UK, 12(2), pp. 204–216.

Liang, G. *et al.* (2010) 'Butyrate promotes induced pluripotent stem cell generation', *Journal of Biological Chemistry*. ASBMB, 285(33), pp. 25516–25521.

Lickwar, C. R. *et al.* (2017) *Genomic dissection of conserved transcriptional regulation in intestinal epithelial cells*, *PLOS Biology*. doi: 10.1371/journal.pbio.2002054.

Liu, W. *et al.* (2014) 'Effects of dietary microencapsulated sodium butyrate on growth, intestinal mucosal morphology, immune response and adhesive bacteria in juvenile common carp (Cyprinus carpio) pre-fed with or without oxidised oil', *British Journal of Nutrition*. Cambridge University Press, 112(1), pp. 15–29.

Liu, Y. *et al.* (2019) 'Sodium butyrate supplementation in high-soybean meal diets for turbot (Scophthalmus maximus L.): effects on inflammatory status, mucosal barriers and microbiota in the intestine', *Fish & shellfish immunology*. Elsevier, 88, pp. 65–75.

López Nadal, A. *et al.* (2018) 'Exposure to Antibiotics Affects Saponin Immersion-Induced Immune Stimulation and Shift in Microbial Composition in Zebrafish Larvae', *Frontiers in Microbiology*, 9(October), pp. 1–16. doi: 10.3389/fmicb.2018.02588.

López Nadal, A. *et al.* (2020) 'Feed, Microbiota, and Gut Immunity: Using the Zebrafish Model to Understand Fish Health ', *Frontiers in Immunology* . Available at: https://www.frontiersin.org/article/10.3389/fimmu.2020.00114.

Lorenzen, E., Dalsgaard, I. and Bernardet, J.-F. (1997) 'Characterization of isolates of Flavobacterium psychrophilum associated with coldwater disease or rainbow trout fry syndrome I: phenotypic and genomic studies', *Diseases of aquatic organisms*, 31(3), pp. 197–208.

Love, M. I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biology*, 15(12), p. 550. doi: 10.1186/s13059-014-0550-8.

Maas, R. M. *et al.* (2021) 'Exogenous enzymes and probiotics alter digestion kinetics, volatile fatty acid content and microbial interactions in the gut of Nile tilapia', *Scientific Reports*, 11(1), p. 8221. doi: 10.1038/s41598-021-87408-3.

Manera, M. and Dezfuli, B. S. (2004) 'Rodlet cells in teleosts: a new insight into their nature and functions', *Journal of fish biology*. Wiley Online Library, 65(3), pp. 597–619.

McMurdie, P. J. and Holmes, S. (2013) 'phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data', *PloS one*. Public Library of Science, 8(4).

Meyer, A., Biermann, C. H. and Orti, G. (1993) 'The phylogenetic position of the zebrafish (Danio rerio), a model system in developmental biology: an invitation to the comparative method', *Proceedings of the Royal Society of London. Series B: Biological Sciences*. The Royal Society London, 252(1335), pp. 231–236.

Moore, J. C. *et al.* (2016) 'T Cell Immune Deficiency in zap70 Mutant Zebrafish.', *Molecular and cellular biology*, 36(23), pp. 2868–2876. doi: 10.1128/MCB.00281-16.

Nalbant, P. *et al.* (1999) 'Functional characterization of a Na+–phosphate cotransporter (NaPi-II) from zebrafish and identification of related transcripts', *The Journal of physiology*. Wiley Online Library, 520(1), pp. 79–89.

Neuwirth, E. and Brewer, R. C. (2014) 'ColorBrewer palettes', *R package version*, p. 1.

Oksanen, J. *et al.* (2013) 'Package "vegan"', *Community ecology package, version*, 2(9), pp. 1–295.

de Oliveira, S. *et al.* (2013) 'Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response.', *Journal of immunology (Baltimore, Md. : 1950)*, 190(8), pp. 4349–4359. doi: 10.4049/jimmunol.1203266.

Paradis, E., Claude, J. and Strimmer, K. (2004) 'APE: analyses of phylogenetics and evolution in R language', *Bioinformatics*. Oxford University Press, 20(2), pp. 289–290.

Patro, R. *et al.* (2017) 'Salmon provides fast and bias-aware quantification of transcript expression', *Nature Methods*, 14(4), pp. 417–419. doi: 10.1038/nmeth.4197.

Petering, H. *et al.* (1999) 'The biologic role of interleukin-8: functional analysis and expression of CXCR1 and CXCR2 on human eosinophils', *Blood, The Journal of the American Society of Hematology*. American Society of Hematology Washington, DC, 93(2), pp. 694–702.

Petit, J. and Wiegertjes, G. F. (2016) 'Long-lived effects of administering β-glucans: Indications for trained immunity in fish', *Developmental and Comparative Immunology*. Elsevier Ltd, 64, pp. 93–102. doi: 10.1016/j.dci.2016.03.003.

Piazzon, M. C. *et al.* (2017) 'Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets', *Microbiome*. Microbiome, 5(1), p. 164. doi: 10.1186/s40168-017-0390-3.

Poncheewin, W. *et al.* (2020) 'NG-Tax 2.0: A Semantic Framework for High-Throughput Amplicon Analysis', *Frontiers in Genetics*, 10(January), pp. 1–12. doi: 10.3389/fgene.2019.01366.

Quast, C. *et al.* (2013) 'The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools', *Nucleic Acids Research*, 41(D1), pp. 590–596. doi: 10.1093/nar/gks1219.

Ramiro-Garcia, J. *et al.* (2018) 'NG-Tax, a highly accurate and validated pipeline for analysis of 16S rRNA amplicons from complex biomes [version 2; referees: 2 approved, 1 approved with reservations, 1 not approved]', *F1000Research*, 5. doi: 10.12688/f1000research.9227.2.

Reite, O. B. and Evensen, Ø. (2006) 'Inflammatory cells of teleostean fish: A review focusing on mast cells/eosinophilic granule cells and rodlet cells', *Fish & Shellfish Immunology*, 20(2), pp. 192–208. doi: https://doi.org/10.1016/j.fsi.2005.01.012.

Rimoldi, S. *et al.* (2016) 'Butyrate and taurine exert a mitigating effect on the inflamed distal intestine of European sea bass fed with a high percentage of soybean meal', *Fisheries and Aquatic Sciences*. BioMed Central, 19(1), pp. 1–14.

Robles, R. *et al.* (2013) 'Effect of partially protected butyrate used as feed additive on growth and intestinal metabolism in sea bream (Sparus aurata)', *Fish Physiology and Biochemistry*. Springer, 39(6), pp. 1567–1580.

Rosenberg, H. F., Dyer, K. D. and Foster, P. S. (2013) 'Eosinophils: changing perspectives in health and disease', *Nature Reviews Immunology*. Nature Publishing Group, 13(1), pp. 9–22.

Sales, J. (2009) 'The effect of fish meal replacement by soyabean products on fish growth: a meta-analysis', *British Journal of Nutrition*. Cambridge University Press, 102(12), pp. 1709–1722.

Santos, K. O. *et al.* (2019) 'Probiotic expressing heterologous phytase improves the immune system and attenuates inflammatory response in zebrafish fed with a diet rich in soybean meal', *Fish & Shellfish Immunology*, 93, pp. 652–658. doi: https://doi.org/10.1016/j.fsi.2019.08.030.

Shannon, P. *et al.* (2003) 'Cytoscape: a software environment for integrated models of biomolecular interaction networks.', *Genome research*, 13(11), pp. 2498–2504. doi: 10.1101/gr.1239303.

Singleman, C. and Holtzman, N. G. (2014) ' Growth and Maturation in the Zebrafish, Danio Rerio : A Staging Tool for Teaching and Research ', *Zebrafish*, 11(4), pp. 396–406. doi: 10.1089/zeb.2014.0976.

Solis, C. J. *et al.* (2020) 'Intestinal Inflammation Induced by Soybean Meal Ingestion Increases Intestinal Permeability and Neutrophil Turnover Independently of Microbiota in Zebrafish ', *Frontiers in Immunology* . Available at: https://www.frontiersin.org/article/10.3389/fimmu.2020.01330.

Tran, N. T. *et al.* (2018) 'Altered gut microbiota associated with intestinal disease in grass carp (Ctenopharyngodon idellus)', *World Journal of Microbiology and Biotechnology*. Springer, 34(6), pp. 1–9.

Ulloa, P. E., Medrano, J. F. and Feijo, C. G. (2014) 'Zebrafish as animal model for aquaculture nutrition research', *Frontiers in Genetics*, 5(SEP), pp. 1–6. doi: 10.3389/fgene.2014.00313.

Urán, P A *et al.* (2008) 'Soybean meal-induced enteritis in Atlantic salmon (Salmo salar L.) at different temperatures', *Aquaculture Nutrition*. Wiley Online Library, 14(4), pp. 324–330.

Urán, P. A. *et al.* (2008) 'Soybean meal induces intestinal inflammation in common carp (Cyprinus carpio L.)', *Fish and Shellfish Immunology*, 25(6), pp. 751–760. doi: 10.1016/j.fsi.2008.02.013.

Verdile, N. *et al.* (2020) 'The 3d pattern of the rainbow trout (Oncorhynchus mykiss) enterocytes and intestinal stem cells', *International Journal of Molecular Sciences*. Multidisciplinary Digital Publishing Institute, 21(23), p. 9192.

Volatiana, J. A. *et al.* (2020) 'Effects of butyrate glycerides supplementation in high soybean meal diet on growth performance, intestinal morphology and antioxidative status of juvenile black sea bream, Acanthopagrus schlegelii', *Aquaculture Nutrition*. Wiley Online Library, 26(1), pp. 15–25.

de Vries, A. and Ripley, B. D. (2013) 'ggdendro: Tools for extracting dendrogram and tree diagram plot data for use with ggplot. R package version 0.1–14'.

Wallace, K. N. and Pack, M. (2003) 'Unique and conserved aspects of gut development in zebrafish', *Developmental Biology*, 255(1), pp. 12–29. doi: 10.1016/S0012-1606(02)00034-9.

Walters, W. *et al.* (2016) 'Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys.', *mSystems*, 1(1). doi: 10.1128/mSystems.00009-15.

Wang, Z. *et al.* (2010) 'Morphological and molecular evidence for functional organization along the rostrocaudal axis of the adult zebrafish intestine', *BMC Genomics*, 11(1). doi: 10.1186/1471-2164-11-392.

Westerfield, M. (2007) *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio).* 5th edn. Eugene: University of Oregon Press, Eugene.

Wickham, H. (2009) 'Elegant graphics for data analysis (ggplot2)'. New York, NY: Springer-Verlag.

Wickham, H. (2012) 'reshape2: Flexibly reshape data: a reboot of the reshape package', *R package version*, 1(2).

Wickham, H. (2016) 'scales: Scale Functions for Visualization. R package version 0.4. 0'.

Wickham, H. *et al.* (2017) 'dplyr: a grammar of data manipulation, 2013', *URL https://github. com/hadley/dplyr. version 0.1.[p 1]*.

Wickham, H. and Wickham, M. H. (2020) 'Package "plyr"', *Obtenido de https://cran. rproject. org/web/packages/dplyr/dplyr. pdf*.

Xia, J. *et al.* (2018) 'Effects of short term lead exposure on gut microbiota and hepatic metabolism in adult zebrafish', *Comparative Biochemistry and Physiology Part - C: Toxicology and Pharmacology*, 209(February), pp. 1–8. doi: 10.1016/j.cbpc.2018.03.007.

Xie, Y., Cheng, J. and Tan, X. (2018) 'DT: A Wrapper of the JavaScript Library "DataTables"', *R package version 0.4*.

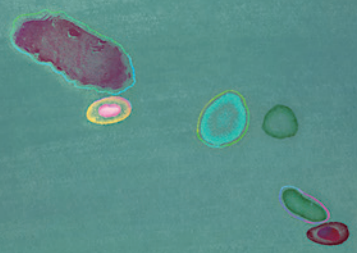
Yoder, J. A. *et al.* (2002) 'Zebrafish as an immunological model system.', *Microbes and infection*, 4(14), pp. 1469–78. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12475637.

Zheng, Q. *et al.* (2012) 'Effect of replacing soybean meal with cottonseed meal on growth, hematology, antioxidant enzymes activity and expression for juvenile grass carp, Ctenopharyngodon idellus', *Fish Physiology and Biochemistry*, 38(4), pp. 1059–1069. doi: 10.1007/s10695-011-9590-0.

Zoubarev, A. *et al.* (2012) 'Gemma: a resource for the reuse, sharing and meta-analysis of expression profiling data.', *Bioinformatics (Oxford, England)*, 28(17), pp. 2272–2273. doi: 10.1093/bioinformatics/bts430.

Chapter 8

General Discussion



In this final chapter (**chapter 8**) the two main goals of this thesis (described in **chapter 1**) are discussed:

1) to set-up a fish *in vivo* model to study host-microbe-immune interactions in the context of nutrition

2) to create a toolbox of readouts for feed-derived inflammation.

In this thesis we employed soy saponin as a known inducer of inflammatory responses (**chapter 2**). By using transgenic zebrafish we visualized *in vivo* the recruitment of neutrophils and macrophages into the gut area in response to soy saponin. In **chapter 3** we studied how altered compositions of intestinal microbiomes affect the innate immune response upon saponin-induced inflammation. We further characterized saponin-induced responses and describe novel inflammatory-associated behavior in zebrafish (**chapter 4**). In a review of existing literature on zebrafish we highlight that the establishment of a robust and consistent animal model of feed-derived inflammation is a crucial step for subsequent assessments aiming to evaluate the effect of novel feeds on fish gut health (**chapter 5**). We first showed the potential of the zebrafish model to evaluate the effects of novel feed supplements by evaluating effects of fucoidans derived from brown algae (**chapter 6**) and then expanded on the concept of zebrafish to evaluate the effects of novel feed supplements in **chapter 7**. To this end, we applied different high-throughput techniques encompassing a novel quantitative histology pipeline, characterization of the gut bacterial communities and their taxa connectivity as well as host gut transcriptomics for juvenile zebrafish fed either a butyrate- or a saponin-supplemented diet. Here, in **chapter 8** I critically discuss the value of the zebrafish animal model as a robust and consistent animal model of feed-derived inflammation building on *in vivo* visualization (due to larval transparency) and effects on several biological processes like epithelial gut barrier integrity. I describe and discuss hallmarks of zebrafish gut inflammation in the context of nutrition and the molecular mechanisms of host-microbe-immune interactions.

What to feed farmed fish is becoming a pressing issue in the aquaculture sector due to the increasing prices and the scarcity of raw materials to produce fishmeal (derived from unsold catch fish and fish offal), all in an attempt to design a more sustainable aquaculture sector which can contribute to nourish the ever-growing world population (Froehlich *et al.*, 2018). In this context, aquafeed companies developed new feeds from alternative protein sources such as meals containing soybean (Baeverfjord and Krogdahl, 1996), faba bean (Azaza *et al.*, 2009), rapeseed (Enami, 2011), peas (Øverland *et al.*, 2009) insects (Nogales-Mérida *et al.*, 2019) and algae (Norambuena *et al.*, 2015). More recently, suggestions are to utilize crop "waste" to improve aquafeed digestibility and nutrient uptake with the aim to contribute to a circular bio-economy (FAO, 2020). However, to

secure the viability of novel feeds and their feed ingredients and uncover the mechanisms by which novel feed ingredients may influence fish gut inflammation and host-microbe-immune interactions, robust and consistent animal models are needed.

Currently, a robust and consistent *in vivo* animal model to screen the effects of novel dietary components on fish (gut) health, is lacking. Such an animal model could support screening effects of new components prior to extensive rounds of testing performance (i.e. digestibility, growth, etc) and health in the target fish species. Zebrafish are a promising model for fish intestinal health in the context of nutrition (reviewed in Ulloa, Medrano and Feijo, 2014). Zebrafish could serve such an animal model role because they are easy to breed in larger numbers and they are smaller than commercially relevant fish species which facilitates the use of smaller housing i.e. aquaria for experimentation. Moreover, the use of transparent zebrafish larvae of transgenic reporter fish lines allow for non-invasive *in vivo* visualisation of gut inflammation (by the number of immune cells) as well as direct assessment of the gut barrier integrity. The zebrafish model therefore could be a suitable platform to pre-screen novel aquafeed ingredients supporting balanced choices for health-promoting ingredients aiming at facilitating sustainable alternatives to fishmeal-based diets. Furthermore, common use of a zebrafish model contributes to generate more fundamental scientific knowledge on the interaction between dietary components, microbiota and gut immunology in bony species.

I divided this general discussion according to the following aims: **1)** I assess the strengths and weaknesses of zebrafish as a model to study gut health of other fish species. Then, I provide examples of how larval transparency allows *in vivo* visualisation of fish gut health. **2)** I examine the hallmarks of (gut) feed-derived inflammation from our datasets (and the existing literature) and I link them to their molecular mechanisms. Then, I analyse the biological meaning of the disturbances of microbiota composition in the context of fish gut health. Finally, I discuss the societal relevance of zebrafish as a model to pre-screen novel aquafeeds for a sustainable protein transition as well as a model to uncover complex biological processes.

1: Is the zebrafish a good animal model to study gut health of other fish species?

There are requirements for an organism to become a well-established and scientifically useful animal model, which should be able: **i)** to share the investigated cellular processes with other organisms of interest, **ii)** to be handled easily, **iii)** to be affordable and to be easily maintainable in laboratory conditions, **iv)** to allow genetic modification and **v)** to possess resources in terms of strains, transgenics and well-annotated genome (reviewed

in Ribas and Piferrer, 2014). Taking into consideration these requirements, it is clear why zebrafish became an animal model of interest for studying many complex biological processes, like intestinal inflammation and gut health, from fish to humans (reviewed in Brugman, 2016; López Nadal et al., 2020). Yet, for translations of observations in zebrafish to other fish it is important to appreciate the heterogeneity in the fish gastrointestinal tract (GIT) implying that the results obtained from using the zebrafish model cannot always be directly extrapolated to other (fish) species without thorough evaluation. After all, zebrafish are omnivores and their GIT show some particularities (discussed in **chapter 1**) not always shared with other teleost fish species and fish families. For instance, zebrafish lack a stomach (Wallace and Pack, 2003) and consequently zebrafish may not necessarily be the most suitable organism to perform digestibility studies for stomach-containing carnivorous species in aquaculture, but rather be informative on cellular processes and other fundamental biological interests. After all, gut segments of the adult zebrafish have been functionally correlated to the mammalian gut segments by transcriptomics analyses (Wang *et al*., 2010) and intestinal epithelial cells (IECs) presented a conserved transcriptomic profile across zebrafish, stickleback, mouse and humans (Lickwar *et al*., 2017). These intestinal-associated highly-conserved transcriptional similarities suggest that zebrafish can provide a valid animal model, not just as an alternative to laboratory mice for biomedical research but also for other cyprinids and possibly other fish families such as sparids and salmonids, at least when it comes to investigating well-conserved aspects of intestinal function.

Zebrafish larval transparency to visualize gut health

The transparency of zebrafish larvae is crucial for *in vivo* visualization of the fluorescently-labelled transgenic reporter cell lines, as seen for neutrophils and macrophages in **chapters 2**, **3**, **4**, **6** and **7**. Furthermore, larval transparency allows for the possibility to study *in vivo* visualization of feed uptake and feed passage through the GIT using fluorescently labeled (GFP) diets. To this end, we GFP-stained six newly-developed diets (dry oil-coated pellets) and we assessed feed uptake and how long the diets remained in the GIT (**Figure 1**). We fed the pellets to the zebrafish larvae once a day for two days. After each feeding we took *in vivo* pictures to assess the GFP signal in their guts. After two days of feeding most of the larvae engulfed all six diets (GFP^+ signal in the guts). Then, fish were fasted and the GFP signal of the soybean meal (SBM) diet and SBM+0.5%But diet were visible in the fish gut for a longer time than the other diets. A plausible explanation for the loss of GFP signal in the gut is that the GFP molecules break down due to nutrient uptake by the enterocytes. In **Figure 1** the fact that GFP^+ diet remains longer than the control diet in the gut is suggestive of certain difficulty for the host to uptake the nutrients from that feed formulation possibly resulting from gut inflammation and leading to fish

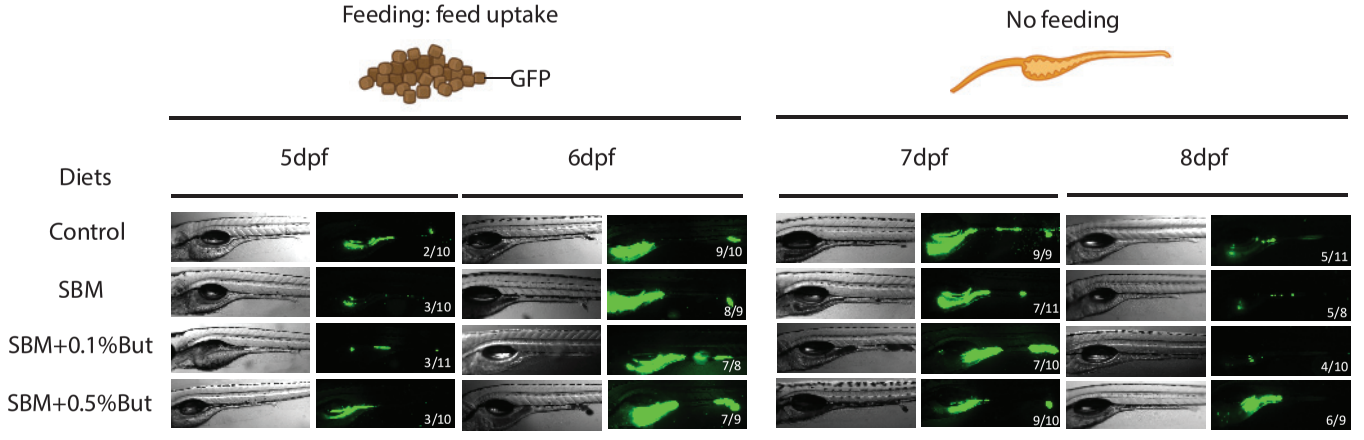


Figure 1: Feed uptake and feed processing of GFP-stained novel diets in the zebrafish larvae model. Fish were fed two consecutive days (5 and 6 dpf) and imaged 1h after the feed was administrated. Then, fish were fasted two consecutive days (7 and 8dpf) and imaged daily to visualise the processing of the fluorescent feed. Diets in which GFP+ signal stayed more time than controls of fish fed. Fish were embedded in low-melting point 1% agarose and in vivo imaged in the stereo fluorescent microscope. The diets are: Control diet (high in fish meal), SBM (high in soybean meal) and SBM+0.1%But / SBM+0.5But (high on soybean meal and 0.1% or 0.5% butyrate inclusion). In the fluorescent pictures there is indicated the numbers of fish guts showing fluorescent GFP+ signal from total number of guts analysed per diet and timepoint.

malnourishment. A transgenic line with fluorescently labelled intestinal epithelial cells (IECs) could help in the *in vivo* visualization of diet uptake in the zebrafish gut. Researchers engineered a zebrafish line in which the expression of claudin 15 (crucial for the tight junctions) was labelled by GFP: TgBAC(*cldn15la-GFP*) which combined with phalloidin (red) staining delimited the gut lumen surface and the IECs (Alvers et al., 2014, not used in this thesis). Combining our fluorescently stained diets with this transgenic zebrafish line TgBAC(*cldn15la-GFP*) and the phalloidin staining would allow us to visualize the uptake of novel feeds in the zebrafish gut. Future research may exploit the advantages of zebrafish larval transparency and transgenesis to visualize stimulation of the innate immune cells upon feed uptake of novel diets.

Another example of the advantage of zebrafish larval transparency to visualize gut health comes from the usage of Dextran-FITCs. Zebrafish larvae are used to explore gut permeability and epithelial gut barrier integrity by using the GPF-enriched Dextran-FITC (4 KD) molecules. The rationale behind that technique is that larvae engulf fluorescent molecules and these leak out of the gut when the epithelial gut barrier is structurally compromised (Philip *et al.*, 2017). Moreover, since the Dextran-FITCs labels fluorescently the region of the gut in which it is present from the anterior to the posterior region, the peristaltic movements that guide the molecules through the gut can be inlayed. In fact, we explored the epithelial barrier integrity and the peristaltic bowel movements of zebrafish previously exposed to soy saponin or butyrate. We observed that fish exposed to saponin had a non-fully fluorescent gut because the Dextran-FITC molecules could not pass through towards the mid and posterior gut, suggesting impaired peristaltic bowel movements (**Figure 2**). Zebrafish larval transparency once again is informative of complex biological processes in the context of gut health that cannot be easily visualised *in vivo* in other (fish) spices.

The importance of the route of exposure in the zebrafish model for gut health

In the experiments of this thesis, we have combined two main approaches to treat zebrafish: **i)** zebrafish larvae immersion in a solution with the compounds of interest and **ii)** juvenile and adult zebrafish feed supplementation of the compounds of interest (within oil-coated pellets or directly combined with the feed). In evaluating zebrafish as a good animal model to study gut health of other fish species, it is important to take into consideration the route of exposure especially when it comes to using transparent larvae which do not yet eat and have to be exposed by immersion to the compounds of interest. Here, zebrafish larvae were immersed for assessing the immunological response towards antinutritional factors (saponin, **chapter 2, 3** and **4**), feed supplements (fucoidan, **chapter 6**), anti-inflammatory glucocorticoids (dexamethasone, **chapter 4**) as well as environmental pollutants (antibiotics in **chapter 2**). All the observations gathered in these chapters

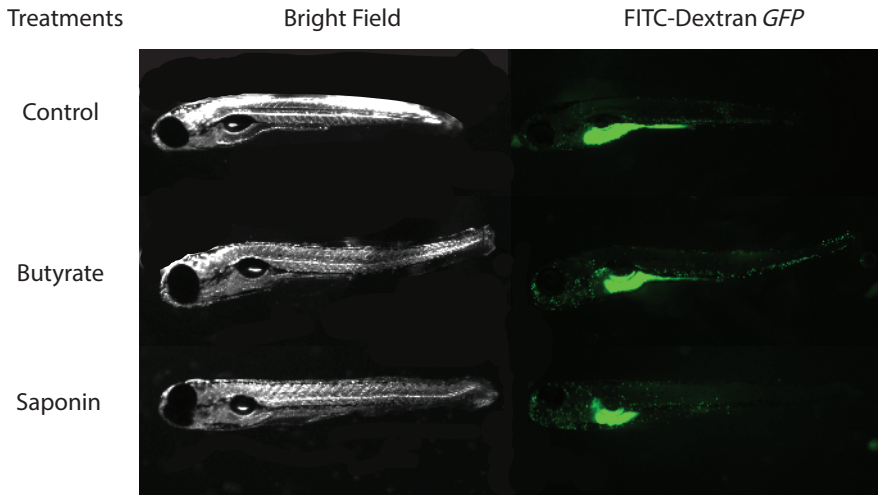


Figure 2: Zebrafish larvae exposed from 3-6 dpf to Control, Butyrate (0.05mg/ml) or Saponin (0.5mg/ml) and immersed in Dextran-FITC for 40 minutes before being embedded in low-melting point 1% agarose and *in vivo* imaged in the stereo fluorescent microscope.

support the conclusion that immersion is a valid route of exposure to assess health-related effects in the zebrafish larvae model (also previously validated for drug discovery purposes, Diekmann and Hill, 2013). Treatment compounds presumably enter the zebrafish via the gills, the skin and also the mouth. The exact amount of compound reaching the gut via immersion treatment is difficult to quantify (reviewed in López Nadal et al., 2020, **chapter 5**) and therefore, when studying intestinal health, a limitation of immersion treatments is that the effects on the gut may be partly indirect. This of course puts restrictions on the interpretations of feed components derived inflammation in the gut when compounds are applied by immersion.

Additionally, we and others used gut-specific treatments to evaluate gut health in zebrafish. For instance, researchers used microgavage to deliver Dextran-FITC directly to the lumen of the anterior intestine in zebrafish larvae and for controlling the timing of the *in vivo* imaging of the epithelial barrier integrity (Cocchiaro and Rawls, 2013). In **chapter 7** we were able to quantify the amount of saponin and butyrate engulfed by juvenile zebrafish. Butyrate and saponin-supplemented feeds within oil-coated pellets were developed to assure a gut-specific effect and preventing a loss of the bioactivity when the feed is in contact with the water. Furthermore, diets supplemented with soybean meal and at two inclusions levels of sodium butyrate were developed to evaluate feed uptake in the zebrafish larvae model (**Figure 1**).

Taking all these observations together, the biological processes in the context of gut health that can be studied and visualized *in vivo* by using transgenic zebrafish larvae are not possible nowadays by using any other (fish) species. Nonetheless, when translating the results to other fish species it is important to take into account the gastrointestinal architecture and physiology as well as the naturally occurring diet of each fish species.

2: Hallmarks of inflammation in our zebrafish model

The stages of inflammation and their hallmarks

The second aim of this thesis was to create a toolbox of readouts for feed-derived inflammation in zebrafish. In this section of the **General Discussion** I correlate the stages of inflammation (reviewed in **chapter 1**) to specific readouts obtained from our experiments (chapters of this thesis) and data from the literature. In this thesis we have mainly focused on the induction and regulation of inflammatory processes rather than on subsequent tissue repair processes and therefore our readouts for inflammation are mostly associated with: **1)** release of mediators, **2)** effect of mediators and **3)** cellular recruitment (**Table 1**).

1) Release of mediators: in **chapters 2, 3, 4** and **6** I reported that the expression of *il1b*, *tnfa,* and mmp9 is upregulated upon saponin exposure in zebrafish larvae. *Il1b* is a crucial cytokine for the development of inflammation and it is firstly expressed in macrophages and subsequently at very high levels in neutrophils in zebrafish (Nguyen-Chi *et al.*, 2014). *Tnfa* is also expressed by macrophages and is a key cytokine for the induction of "classically activated M1" macrophages with a pro-inflammatory profile (tnfa-expressing GFP transgenic zebrafish line is available but not employed in this thesis) (Nguyen-Chi *et al.*, 2015). The matrix metalloproteinase 9 (*mmp9*) is induced with cellular damage and plays an important role in the onset of the inflammatory response by mediating leukocyte recruitment via the activation of chemokines (Xu *et al.*, 2018; Silva et al., 2020). Taking these observations together we suggest the increase of expression in *il1b, tnfa* and *mmp9* as an innate immune hallmark of feed-(saponin)-derived inflammation in zebrafish larvae. Future research employing knockout zebrafish may elucidate novel functions of these candidate genes and the possible regulation between them.

2) Effect of mediators: in **chapters 2, 4** and **7** I reported increased expression of chemokines like C-X-C motif chemokine ligand 8 (*cxcl8a*), C-C motif chemokine ligand 2 (*ccl2* more recently renamed *ccl38a.5*) upon saponin exposure. *Cxcl8a* has been previously showed to recruit neutrophils (de Oliveira *et al.*, 2013) while *ccl38a.5* recruits macrophages (Xu *et al.*, 2018; Sommer *et al.*, 2021) upon inflammation in zebrafish.

Table 1 Readouts for feed-derived induced inflammation from the studies of this thesis.

Study in the thesis	Animal life stage	Treatments	Release of mediators	Effect of mediators
Chapter 2	Larvae	Saponin (SAP), ciprofloxacin and oxytetracycline (OxyT) exposure in solution	il1b, mmp9, il22 and tnfb	cxcl8a, ccl2
Chapter 3	Larvae	Adult OxyT-treated microbial content + challenge with SAP in solution	mmp9 and il22 upon saponin challenge	-
Chapter 4	Larvae	SAP and dexamethasone (DEX)	il1b, mmp9, tnfa and tnfb	cxcl8a (increased SAP, reversed DEX), ccl38
Chapter 6	Larvae and adults	Fucoidan exposure and feed supplementation	Up in larvae: il1b (down in adult guts), tnfa, mmp9	-
Chapter 7	Juveniles and larvae	Butyrate and saponin-supplemented feeds and exposure in solution to larvae	In juveniles: up genes associated with oxidoreductase activity, immune responses, inflammatory response	In juveniles: up genes associated with innate cell recruitment

Chemokines are responsible for amplification of the immune response by recruiting innate immune cells. Saponin also increased the expression of *tnfb* (**chapter 2** and **4**). Other studies showed that *tnfb* expression in macrophages is a biomarker for pro-inflammatory M1-type macrophages in zebrafish (Nguyen-Chi *et al.*, 2015) and in humans (Martinez *et al.*, 2006). Furthermore, *tnfb* activates fish thrombocytes which contribute to restore homeostatic conditions by tissue healing upon inflammation (Ferdous and Scott, 2015). Altogether *cxcl8a*, *ccl38a.5* and *tnfb* indicate an amplification of the inflammation and recruitment of immune cells to the gut area.

3) Cellular recruitment: the increased chemokine expression is a chemoattractant cue for innate immune cells, mainly neutrophils (*cxcl8a*) and macrophages (*ccl38a.5*). In this thesis the quantification of neutrophils and macrophages was possible due to the *in vivo* imaging of the double transgenic fish *Tg (mpeg1:mCherry / mpx:eGFP^{i114})* (**chapters 2, 3, 4, 6,** and **7**). I showed that saponin (and butyrate) in solution increased the amount of neutrophils and macrophages in the gut area (**chapters 2, 3, 6** and **7**)

	Cellular recruitment	Regulation of inflammation	Changes in microbial composition compared to controls	Other parameters
	Increase in neutrophils and macrophages with SAP	il10, il22, tnfb	Yes	-
	Increase macrophages with saponin and decreased in larvae with OxyT-treated parental microbiota	il22	Yes	-
	Increase neutrophils and macrophages with SAP. Neutrophil presence reversed by DEX	il10. tnfb	-	SAP increase preference for warmer waters, DEX reversed it
	-	il10 (up in larvae)	Yes (in adults more than larvae)	-
	In juveniles: increase in eosinophils and rodlet cells; decrease in goblet cells. In larvae: increase neutrophils and macrophages	-	Yes (in juveniles)	-

as well as in the overall fish (**chapter 3** and **4**). From these observations the following question arises: is the increase in innate immune cells a sign of acute inflammation or the first sign of an immune modulation that would lead to a tolerogenic response? Oral tolerance is an active process characterized by the unresponsiveness of the local (gut) and systemic immune cells towards orally ingested antigens (such as ingredients or feed). Several immune cells are implicated in such process. Foxp3^{+} regulatory T cells (Tregs) are necessary for oral tolerance in mammals because they can suppress immune responses in the gut mucosa (Mucida *et al.*, 2005). After antigen feeding, Tregs expressing TGFβ (detected by surface expression of LAP) are induced and produce IL10 (Tordesillas *et al.*, 2017). Innate lymphoid cells type 3 (ILC3) secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) that promotes Treg homeostasis (Mortha *et al.*, 2014). GM-CSF is regulated by microbial signals via a cross-talk between IL1β-producing macrophages and ILC3 in the gut mucosa and acts on dendritic cells and tissue-resident macrophages promoting their tolerogenic

phenotype (reviewed in Tordesillas and Berin, 2018). One study attempted to study the tolerogenic response in zebrafish larvae by a naive (first time) feeding and a developed (re-exposure) feeding of a control and a SBM diet (Coronado *et al.*, 2019). SBM diet and control diet induced an innate immune response (increased neutrophils and macrophages in the gut) in both naive and developed feeding. Interestingly, the control diet in the naive feeding elicit an inflammatory response from the "adaptive immune system" (increased il17a/f3 expression) without a tolerogenic response. The control diet in the developed feeding showed a tolerogenic response with increased levels of *foxp3* and *il10* transcripts (Coronado *et al.*, 2019). However, in zebrafish larvae T cell and ILCs functionality has not been proven (as reviewed in the **General Introduction**) therefore to the question of how a tolerogenic response is established in the zebrafish larval gut remains unsolved and requires future investigation.

Our observations in **chapter 3** showed that the microbiota established in the zebrafish larvae (from the exposure of the adult gut content) showed a decrease in the number of macrophages upon saponin exposure. A plausible explanation could be that the disrupted microbiota do not have the ability to interact with macrophages and stimulate *il1b* production to further favor a tolerogenic response in the gut. In fact, we see that larvae with a disrupted microbiota did not showed an increase in *il1b* expression but showed an decrease in *il22* expression and a decrease in macrophage presence upon saponin challenge. In mammals, macrophages are reported to express the IL22 receptor and induce IL22-producing group 3 innate lymphoid cells (ILC3) (Treerat, Nature 2017; Bain Immunol Rev. 2014). This mechanisms has not been proven do far to be conserved between mammals and fish. For now, to answer the biological meaning of the innate immune cell increase after our saponin challenge we supplement that observation with the increase of several pro- and anti-inflammatory cytokines indicating that an inflammatory response is developed rather the initiation of a tolerogenic response. Nonetheless, re-exposure experiments in fully immuno-competent zebrafish (with functional ILCs and T cells) are required to further investigate the gut tolerogenic environment in zebrafish.

In **chapter 7** we fed juvenile zebrafish wither a saponin or a butyrate-supplemented feed. Butyrate increased the expression of genes associated with oxidoreductase activity and with the inflammatory response. "Classically activated M1" macrophages contribute to the inflammatory process by the release of ROS which are regulated by oxidoreductase enzymes. Butyrate-supplemented feed increased the amount of eosinophils and rodlet cells as well as decreased the number of goblet cells in the gut (**chapter 7**). The reduction of goblet cells impairs the production of mucus and the maintenance of the protective mucus layer on top of the gut epithelium compromising fish gut health. The increase of

eosinophils and rodlet cells is directly link to the recognition of a foreign insult and to the development of an inflammatory reaction towards the butyrate supplementation. Our data support the previously examined parameters associated to gut inflammation (**Figure 3**, in the **General Introduction**). Since macrophages present high phenotypical plasticity (M1/M2), participate in the tolerogenic responses in the gut and its recruitment is dependent on microbiota composition (**chapter 3**) upon inflammation, we propose the quantification of neutrophils as a stronger, more robust and more consistent biomarker of zebrafish gut inflammation.

Non-canonical hallmarks of inflammation in zebrafish

In **chapter 4** I reported the observation that inflamed fish spend more time in warm waters than control fish. Saponin-exposed fish showed hallmarks of inflammation such as increased expression of pro- and anti-inflammatory cytokines and increased neutrophil and macrophage presence in the gut area. A glucocorticoid with anti-inflammatory properties reversed the number of neutrophils, the expression of *cxcl8a* and the thermal preference to control levels (so fish did no longer prefer warm waters). Other studies previously reported that since fish are ectotherms, after a bacterial or viral infection they spent more time in warm waters to accelerate the immune response and the resolution of the associated inflammation, a process called behavioural fever (Reynolds, Casterlin and Covert, 1976; Estepa *et al.*, 2013; Rey *et al.*, 2017). However, to our knowledge there are no studies that describe a fish-specific thermal preference associated to inflammation. In this thesis we showed for the first time that the inflammatory process by itself (without a pathogen) it is sufficient to change the preference of the zebrafish towards spending more time in warm waters than controls.

Previous research showed that TNF-α is a necessary cytokine to elicit a canonical fever-like response (IL1-IL6-TNF-α; COX2 and PGE2 axis, reviewed in Evans, Repasky and Fisher, 2015). Furthermore, a herpesvirus expressed a decoy TNF- α receptor to delay the onset of behavioural fever and favour its own replication (Rakus *et al.*, 2017). In **chapter 4** we did observe an increase of *il1b*, *tnfa/b* as a cytokines with pyrogenic activity. However, after co-exposing saponin with a glucocorticoid with anti-inflammatory properties the only cytokine that significantly decreased and associated with the fact that fish did not spend more time in warm anymore, was *cxcl8a*. Interestingly, IL8 or CXCL8 has been reported to contribute to the non-canonical induction of fever upon acute inflammation by neutrophil recruitment in mammals (Singh et al., 2008; Tulapurkar et al., 2012; reviewed in Evans, Repasky and Fisher, 2015). Heat-induced non-canonical expression of CXCL8 is regulated by the heat-inducible transcription factor heat shock factor protein 1 in mice (HSF1) (Rice *et al.*, 2005). In an attempt to explore the inflammation-associated thermal preference molecular mechanisms, in **chapter 4** we engineered *cxcl8a* knockout zebrafish

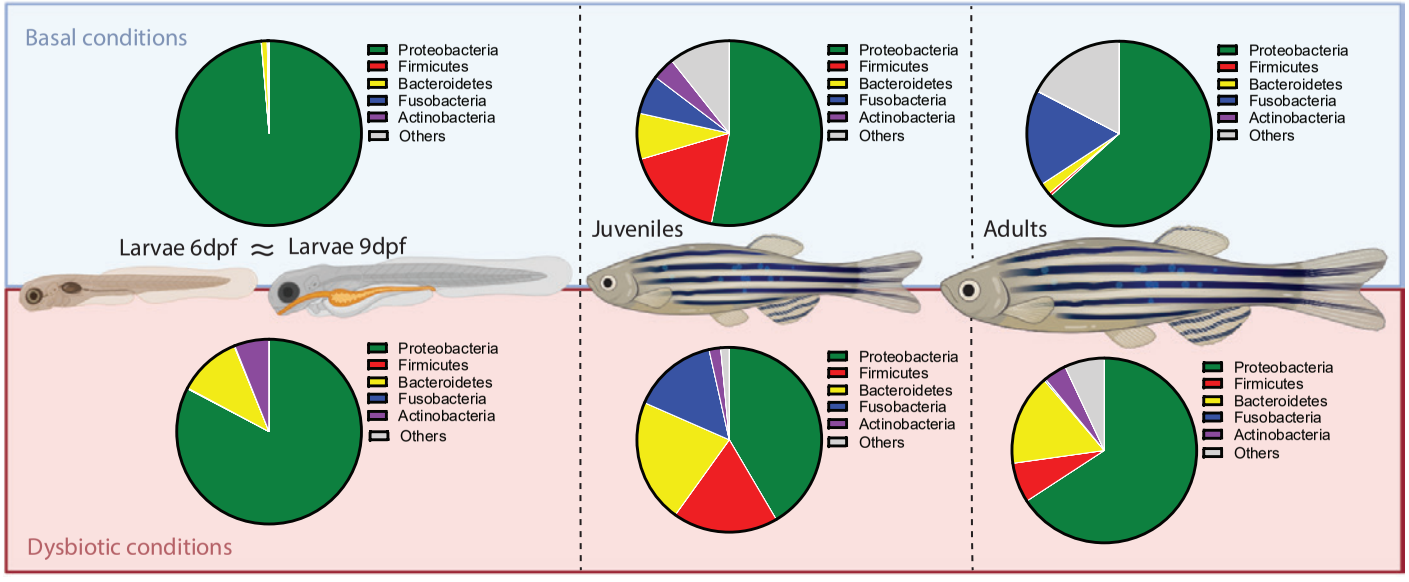


Figure 3: Graphical representation of the microbial composition (phylum level) of the zebrafish larvae (data averaged from **chapter 2, 3 6** and Roeselers et al., 2011, Stephens et al., 2016), juvenile zebrafish gut (data averaged from **chapter 7** and Roeselers et al., 2011; W Zac Stephens et al., 2016; Arias-Jayo et al., 2018 and adult zebrafish gut (data averaged from **chapter 6** and Roeselers et al., 2011, Stephens et al., 2016).

and showed that *cxcl8a* is crucial for the newly reported fever-like behaviour upon inflammation. Future research might address the role of HSF1 in the induction of *cxcl8a* expression in zebrafish as well as explore the other genes implicated in the interplay of immune cell recruitment, inflammation and febrile-like responses in fish. Our results in **chapter 4** suggest that thermal preference may be a novel valid non-invasive hallmark to assess (zebra)fish inflammation.

Microbiota composition as a hallmark for zebrafish gut health

The loss of structure in the gut epithelia leads to increased permeability that favours translocation of bacteria antigens promoting inflammation in the gut (Sitjà-Bobadilla *et al*., 2019) a process known as "leaky gut". Growing evidence shows that gut microbiota is important in supporting epithelial barrier function. Pathogenic bacteria facilitate a leaky gut condition and alter intestinal permeability making the host more susceptible to inflammation and autoimmune diseases (as reviewed in Mu et al., 2017). In an attempt to explore whether microbial composition changes upon inflammation, in **chapter 2** we showed that saponin and antibiotics disrupted the larval microbial composition and associated this with parameters associated with inflammation: increase in expression of pro-inflammatory cytokine and in the number of neutrophils and macrophages in the gut area. Furthermore, in **chapter 3** we showed that the innate immune response towards saponin-induced inflammation partly depended on the microbiota composition. A decrease in number of macrophages in the intestinal area as well as a decreased expression of *il22* were reported in larvae harbouring an altered microbial composition (larvae exposed to antibiotic-treated adult content). These results support the observation that the host microbiota composition plays a crucial role for mounting a proper (innate) immune response upon a pro-inflammatory challenge. Future research may investigate the microbial species-specific molecular mechanisms that ensure an uncompromised gut epithelial barrier that contributes to a large extent to fish gut health.

In this thesis, across our chapters, we examined the microbiota composition of fish tissues at different life stages: **chapter 2** (larvae), **chapter 3** (larvae and larvae exposed to adult gut content), **chapter 6** (larvae and adult gut) and **chapter 7** (juvenile gut). We summarized our findings and placed them in the context of previously published data in order to elaborate a user-friendly easy-to-interpret microbial composition roadmap (phylum level) in the context of zebrafish inflammation across development (**Figure 3**). Previous research established that across development, zebrafish undergo to two major microbial shifts (Stephens *et al*., 2016): **1)** from larval to juvenile stage and **2)** from late juveniles to adults (coinciding with sexual maturation). The first main shift coincides with the introduction of dry feed, the transfer of fish to a re-circulating water system and with the development of the adaptive immunity. The molecular mechanisms by which

immune cells and gut microbes interact are described in section "Microbiota and immune interactions in the zebrafish gut". The second microbial shift coincides with sexual maturation of the zebrafish as well as the switch to another dry feed regime (Stephens *et al.*, 2016).

In **chapter 2** and **chapter 3** I reported that microbiota composition at phylum level of zebrafish leave at 6 dpf (unfed) is almost identical than larvae at 9 dpf (fed with live feed) (integrated in pie chart "Basal conditions" for zebrafish larvae **Figure 3**). These observations indicate that zebrafish larvae are quite steady in terms of phyla composition prior to the abovementioned first microbial shift. Researchers showed that the first adaptations of gut-associated *Aeromonas veronii* when added to germ-free zebrafish larvae were to colonize the host by immigration from the environment and by interhost transmission, and only later microbes showed intra-host specific adaptations (Robinson *et al.*, 2018). These observation might indicate that the very high abundance of Proteobacteria (Enterobacteriaceae family) reported in "healthy" larvae (**chapter 2** and **3**) could be associated to a advantages of these species in terms of migration towards the gut and interhost transmission. At the larvae stage, zebrafish dysbiosis is characterized by a reduction of Proteobacteria and an increase in Actinobacteria and Bacteroidetes. Bacteroidetes have been previously associated to inflammatory condition in the gut and the liver of crucian carp (Kan *et al.*, 2015; Tang *et al.*, 2021). Within the Bacteroidetes phylum, the Flavobacterium genus has been associated with gut inflammation which resulted in death in grass carp (Tran *et al.*, 2018). We also observed an increase in Flavobacterium genus in butyrate-supplemented fed juvenile zebrafish that presented an inflamed gut (**chapter 7**).

At the juvenile stage, we observe that in basal "healthy" conditions Proteobacteria keeps on being the most abundant phylum (**Figure 3**). Interestingly, juvenile zebrafish showed aberrant gut microbial composition associated with inflammation (butyrate-supplemented diet) by reduced Proteobacteria and increased Bacteroidetes and Fusobacteria compared to the control fed fish (**chapter 7**). It is important to mention that our juvenile gut data of the microbial composition comes from different starting material (RNA extraction, converted to cDNA) than the rest of the data (DNA extraction) for the 16S rRNA profiling. When the starting material used is RNA we are investigating the microbial composition of the active bacteria and although the golden standard in the field still is DNA as a starting material, using DNA could potentially contribute to overestimations in the microbial communities in the zebrafish gut.

At the adult stage, zebrafish gut dysbiosis is depicted by increase of Bacteroides, Actinobacteria and Firmicutes and a decrease of Fusobacteria compared to the basal conditions. Other research also associated a decrease in Fusobacteria with an inflamed gut in adult zebrafish (Brugman *et al.*, 2009). Our data indicates that Fusobacteria may play

a different role in juvenile stage (associated with dysbiosis) than in the adult stage (associated with gut health). Interestingly, in the second shift reported from juvenile to adulthood Fusobacteria are increased (**Figure 3** and Stephens et al., 2016) suggesting a role upon zebrafish development towards adulthood. Altogether, our data suggest that each zebrafish developmental stage may have a certain "healthy" microbiota composition (as reviewed in Stephens et al., 2016). Interestingly, it seems that at all life stages an increase in relative abundances of the Bacteroidetes phylum and a reduction in the Proteobacteria are associated with dysbiosis and associated with an inflammatory condition in the in the zebrafish gut (**Figure 3**).

Our findings indicated that Proteobacteria are the main fish commensals. Contrary to this observation, Proteobacteria are considered a sign of dysbiosis in humans (as reviewed in Shin, Whon and Bae, 2015). Enterobacteriaceae family (Proteobacteria) are facultative anaerobes and bloom in aerobic environments promoting dysbiosis in the human (Zeng, Inohara and Nuñez, 2017). Interestingly, Proteobacteria is the dominant phylum in newborn mice. However, neo-colonization of germ-free mice revealed a Proteobacteria-specific IgA response triggered by immature microbiota that resulted in sustained intestinal inflammation (Mirpuri *et al.*, 2014). These difference may be explained by the fact that fish are much more tolerant to environments containing less oxygen and showed a delayed maturation of the adaptive immune system compared to mammals. In addition, an increase in Bacteroidetes is consistently associated with fish gut inflammation (by us **Figure 3** and others Kan et al., 2015; Tran et al., 2018, Tang et al., 2021) suggestive of a compromised fish gut health.

Besides the increased or decreased relative abundance of certain bacteria, in **chapter 7** I explored taxa connectivity as a hallmark of microbial stability and gut health status in fish. Taxa connectivity refers to the number of co- and anti-occurrences of pairs of bacterial taxons calculated from their relative abundances. A co-occurrence observed is that when Proteobacteria (*Vibrio*) increases correlates with a decrease in Bacteroides. Overall taxa connectivity was reduced after providing butyrate and saponin-supplemented feed to juvenile fish compared to controls, indicating an altered relationship of the bacteria present in their guts (**chapter 7**). Reduced taxa connectivity was associated with aberrant microbiota composition and inflammatory-like histological and transcriptomics features in saponin and butyrate-supplemented fed fish (**chapter 7**). Taken these observations together, taxa connectivity can potentially be used as a hallmark of gut dysbiosis and inflammation and future research may elaborate on the specific species connections important to promote and maintain fish gut health.

Factors affecting microbial composition analyses

In this thesis in **chapters 2**, **3**, **6** and **7** we studied the microbiota composition of zebrafish. We studied fluctuations in the abundance and dynamics of the consortia of bacteria rather than individual bacteria and their specific function. There is increasing evidence that microbial consortia and their interactions rather than single microbial species contribute to health and disease (reviewed in Gilbert et al., 2016). This is why in **Figure 3** of this **General Discussion** we placed our data and previously published studies in the context of gut basal conditions and inflammatory-associated gut dysbiosis. In **Figure 3** I averaged microbial compositions from several studies (Roeselers et al., 2011; W Zac Stephens et al., 2016; Arias-Jayo et al., 2018; López Nadal *et al.*, 2018; Ikeda-Ohtsubo *et al.*, 2020). Differences in microbiota composition across studies were found although the main phyla were always similarly represented. Several factors influence the microbial composition analyses complicating cross-studies comparisons and reproducibility of the results obtained. The main factors influencing consistency in microbial analyses are:

i) Lack of standard feeds: each research facility uses different compositions from the recommended guidelines and there is not a golden standard feed for zebrafish research (reviewed in Westerfield, 2007; and in Watts and D'Abramo, 2021).

ii) Housing factors: rearing density and water quality varies in each research facility and influences microbiota composition in zebrafish already at an early life (Wu *et al.*, 2022). Water temperature in which zebrafish are reared also have an impact in the zebrafish microbiota (data not shown and Wang et al., 2022).

iii) Zebrafish lines several genotypes of knockout zebrafish and transgenic zebrafish lines were used in a large quantity of studies (also in the studies of this thesis). Rag-deficient zebrafish have been shown to harbor a different gut microbiota than wild-type fish(Brugman *et al.*, 2014). However, a characterization of the microbiota of zebrafish transgenic lines or other knockout zebrafish is not reported so far.

iv) Taxonomic depth: the main factor that determines the depth at which researchers can taxonomically describe the microbiota composition is the length of the reads. In our studies in **chapter 2**, **3**, **6** and **7** we performed 16S rRNA profiling of the hypervariable region V3-V4 (100-300 base pairs) which allowed us to describe the microbiota composition at the genus level. To describe the bacteria composition at species level accurately longer reads are needed (Jeong *et al.*, 2021). In the future, we would like to use the 'third generation' sequencing technology from Oxford Nanopore Technologies named Nanopore sequencing. Nanopore sequencing generates long sequence read-lengths and it has been used for 16S rRNA analyses showing promising results (reviewed in Santos et al., 2020; and in Ciuffreda, Rodríguez-Pérez and Flores, 2021). Future research comparing costs, time, price and technical accuracy of 16S rRNA profiling of the zebrafish gut communities by Illumina and

Nanopore technology would help researchers to make an appropriate choice of sequencing method.

Microbiota and immune-interactions in the zebrafish gut

In the **General Introduction** (section: The development of the gut immunity in zebrafish) I summarized what is known about the maturation of the developing immune system of zebrafish. Although several adaptive immune cell types have been reported in the gut in the zebrafish already at the larval stage, functionality of such cells has not been proven so far. Therefore, the general consensus in the field is that zebrafish rely at least for the first two weeks of life solely on innate immunity. The host microbiota and the immune system are in an intimate and bidirectional interplay, here I revisit the molecular pathways in the host-microbe-immune interactions (also reviewed in **chapter 5**, López Nadal et al., 2020):

Innate immune system. Neutrophils are found in the gut in higher numbers in colonized zebrafish than in germ-free individuals (Bates *et al.*, 2007; Galindo-Villegas *et al.*, 2012; Kanther *et al.*, 2014; Koch *et al.*, 2018). In colonized larvae, intestinal neutrophils expressed higher number of transcripts encoding for pro-inflammatory cytokines, ROS production enzymes and anti-microbial peptides than in germ-free larvae (Rolig *et al.*, 2015; Murdoch *et al.*, 2019). Moreover, intestinal neutrophils in colonized larvae in colonized larvae moved with higher speed than germ-free individuals. Interestingly, neutrophil behavior is partly modulated by microbiota-induced secreted immunomodulatory protein Saa (Kanther *et al.*, 2014; Murdoch *et al.*, 2019). Microbiota-derived signals in the intestine signal via MyD88 (and Tnfr1) to potentiate neutrophil recruitment in the gut: in myd88-knockout larvae the abundance of myeloperoxidase-positive gut neutrophils decreased compared to controls at 6dpf (Bates *et al.*, 2007; Burns *et al.*, 2017). Interestingly, certain bacterial species modulated the number of neutrophils recruited in the gut in mono-associated germ-free zebrafish (Rolig *et al.*, 2015). For instance *Aeromonas* sp. or *Vibrio* sp. promoted higher numbers of neutrophils recruited in the gut than other bacteria like *Shewanella* sp. Moreover, the most abundant bacteria when using di-associations in germ-free zebrafish did not positively correlated with the intestinal number of neuropils indicating that certain bacteria contribute in a bigger extent to modulate host innate immune responses than other independently of their abundance. For instance, Shewanella sp. secreted a molecule that suppressed neutrophil recruitment to the gut. In another study, an immunomodulatory protein (AimA) secreted by Aeromonas was found to be required during gut colonization to prevent excessive inflammation that compromised the bacteria and the host (Rolig *et al.*, 2018).

Macrophages: adult irf8-deficient adult zebrafish showed a reduction in number of macrophages (measured by expression of mpeg1.1 promoter), in gene expression of complement system-associated genes (*c1q*) and severe dysbiosis (Fusobacteria, α and

γ-Proteobacteria diminished in favor of δ-Proteobacteria) compared to controls. Restauration of irf8 expression reverted *c1q* gene expression as well as the levels of commensal microbes (Earley, Graves and Shiau, 2018). In **chapter 3** I reported that zebrafish larvae exposed to oxytetracycline-treated adult gut content resulted in increased *Aeromonas*, *Pseudomonas*, *Curvibacter* and a decrease in *Escherichia-Shigella* abundance that correlated with a decrease in macrophage presence upon saponin-challenge compared to controls. The molecular mechanisms and the possible role of *il22* through which these species may influence macrophage presence require further investigation.

Adaptive immune system. T cells: adult zebrafish lacking adaptive immune system (rag1-deficient) showed more abundance of Proteobacteria (*Vibrio*) than controls. Adoptive transfer of T cells (but not B or NK-like cells) from wildtypes to rag1-knockout fish showed that T cells can diminish the abundance of Vibrio sp. (Brugman *et al.*, 2014) indicating a clear role of adaptive immune cells on regulating zebrafish gut pathobionts. The lack of adaptive immunity together with aberrant microbiota composition induced an inflamed state in the gut of aged zebrafish (Brugman *et al.*, 2014). Interestingly, when rag1-deficient zebrafish were housed separately microbial communities differed from wild-types but when they were cohoused the differences were not found (Stagaman *et al.*, 2017). This study seems to indicate that there are mechanisms of inter-host compensation when the adaptive immune system of some of the individuals are not functioning.

All these observations highlight the importance of the host immune maturation status then assessing the effect of host factors on the gut microbiota. To have a complete picture on how immunity interact with the host microbiota and vice-versa it is crucial to not only use the (not-fully immune developed) zebrafish larvae.

Societal relevance

In the context of an ever-growing world population the concept of protein transition for feeding the world in a sustainable manner have been gaining a lot of attention. Novel sources of plant-based proteins are needed to create balanced feeds for our cultivated animals as well as for humans. Aquafeed companies are actively researching novel raw sourced of plant-based proteins to feed fish. Here, we propose the zebrafish as an animal model to study the immuno-modulatory effects of these novel diets. Researchers stressed the use of zebrafish as a model for evaluating aquafeeds (reviewed in Ulloa et al., 2011; and in Ribas and Piferrer, 2014; and in Ulloa, Medrano and Feijo, 2014) as well as a model to characterize gut inflammation (reviewed in Brugman, 2016) and intestinal health (reviewed in López Nadal et al., 2020, **chapter 5**).

The assessment of the host-microbe-immune mechanisms uncovered by zebrafish research (discussed in **chapter 5** and **8**) also contribute to the understanding of the threats of bacterial diseases in fish. As a matter of fact, existing and (re)-emerging disease

The protein transition

The United Nations (UN) General Assembly adopted the 2030 Agenda for Sustainable Development (Desa, 2016). Food security and food sustainability together with transition towards circular economy are required to stay within planetary boundaries (reviewed in Aiking and de Boer, 2020). There is a pressing urgency to transition from animal to plant-based protein diets. Within the Wageningen University there are several research lines to establish more diverse and sustainable proteins for our future diets for animals as well as for humans. Here, we propose the usage of zebrafish as an *in vivo* model to visualize and investigate the host-microbe-immune interactions of novel proteins coming from plant-based diets as well as food waste..

are hampering fish farming. Bacterial fish disease are mostly treated by antibiotics (reviewed in Romero, Feijoó and Navarrete, 2012). The risk of antibiotic resistance and the transfer of antibiotic resistance genes to other fish together with the concern for the environment and the consumer safety potentiates the development for novel and sustainable control measures (reviewed in Brandt et al., 2015). In **chapter 2** and **chapter 3** we employed antibiotics to show the detrimental effects of antibiotics when combined with immune-challenging feed-derived compounds (saponin). The supplementation of feed additives in forms of probiotics, prebiotics and synbionts in aquaculture practices (reviewed in Amenyogbe et al., 2020) may contribute to the generation of healthier aquafeeds boosting the immune system of fish and eliminating the need of using antibiotics. However, future research is needed to properly integrate all this knowledge and to depict the main molecular pathways implicated in these interactions. Taking into account the scientific notions gained from the zebrafish model, it seems clear that there are still a large number of lessons to be learned from these small cyprinids.

References

Aiking, H. and de Boer, J. (2020) 'The next protein transition', *Trends in Food Science & Technology*, 105, pp. 515–522.

Alvers, A.L. *et al.* (2014) 'Single continuous lumen formation in the zebrafish gut is mediated by smoothened-dependent tissue remodeling', *Development*, 141(5), pp. 1110–1119.

Amenyogbe, E. *et al.* (2020) 'The exploitation of probiotics, prebiotics and synbiotics in aquaculture: present study, limitations and future directions.: a review', *Aquaculture International*, 28(3), pp. 1017–1041.

Baeverfjord, G. and Krogdahl, Å. (1996) 'Development and regression of soybean meal induced enteritis in Atlantic salmon, Salmo salar L., distal intestine: a comparison with the intestines of fasted fish', *Journal of Fish Diseases*, 19(5), pp. 375–387.

Bates, J.M. *et al.* (2007) 'Intestinal Alkaline Phosphatase Detoxifies Lipopolysaccharide and Prevents Inflammation in Zebrafish in Response to the Gut Microbiota', *Cell Host and Microbe*, 2(6), pp. 371–382. Available at: https://doi.org/10.1016/j.chom.2007.10.010.

Brandt, K.K. *et al.* (2015) 'Ecotoxicological assessment of antibiotics: a call for improved consideration of microorganisms', *Environment International*, 85, pp. 189–205.

Brugman, S. *et al.* (2009) 'Oxazolone-Induced Enterocolitis in Zebrafish Depends on the Composition of the Intestinal Microbiota', *Gastroenterology*, 137(5), pp. 1757-1767.e1. Available at: https://doi.org/10.1053/j.gastro.2009.07.069.

Brugman, S. *et al.* (2014) 'T lymphocytes control microbial composition by regulating the abundance of Vibrio in the zebrafish gut', *Gut Microbes*, 5(6), pp. 737–747. Available at: https://doi.org/10.4161/19490976.2014.972228.

Brugman, S. (2016) 'The zebrafish as a model to study intestinal inflammation', *Developmental and Comparative Immunology*, 64, pp. 82–92. Available at: https://doi.org/10.1016/j.dci.2016.02.020.

Burns, A.R. *et al.* (2017) 'Interhost dispersal alters microbiome assembly and can overwhelm host innate immunity in an experimental zebrafish model', *Proceedings of the National Academy of Sciences*, 114(42), pp. 11181–11186. Available at: https://doi.org/10.1073/pnas.1702511114.

Ciuffreda, L., Rodríguez-Pérez, H. and Flores, C. (2021) 'Nanopore sequencing and its application to the study of microbial communities', *Computational and Structural Biotechnology Journal*, 19, pp. 1497–1511.

Cocchiaro, J.L. and Rawls, J.F. (2013) 'Microgavage of Zebrafish Larvae', *Journal of Visualized Experiments*, (72), pp. 1–12. Available at: https://doi.org/10.3791/4434.

Coronado, M. *et al.* (2019) 'Soybean Meal-Induced Intestinal Inflammation in Zebrafish Is T Cell-Dependent and Has a Th17 Cytokine Profile', *Frontiers in Immunology*, 10, p. 610. Available at: https://doi.org/10.3389/fimmu.2019.00610.

Desa, U.N. (2016) 'Transforming our world: The 2030 agenda for sustainable development'.

Diekmann, H. and Hill, A. (2013) 'ADMETox in zebrafish', *Drug discovery today: disease models*, 10(1), pp. e31–e35.

Earley, A.M., Graves, C.L. and Shiau, C.E. (2018) 'Critical Role for a Subset of Intestinal Macrophages in Shaping Gut Microbiota in Adult Zebrafish', *Cell Reports*, 25(2), pp. 424–436. Available at: https://doi.org/10.1016/j.celrep.2018.09.025.

Enami, H.R. (2011) 'A review of using canola/rapeseed meal in aquaculture feeding', *Journal of Fisheries and Aquatic Science* [Preprint], (1) 6).

Estepa, A. *et al.* (2013) 'Behavioural fever is a synergic signal amplifying the innate immune response', *Proceedings of the Royal Society B: Biological Sciences*, 280(1766), p. 20131381. Available at: https://doi.org/10.1098/rspb.2013.1381.

Evans, S.S., Repasky, E.A. and Fisher, D.T. (2015) 'Fever and the thermal regulation of immunity: The immune system feels the heat', *Nature Reviews Immunology* [Preprint]. Available at: https://doi.org/10.1038/nri3843.

FAO (2020) *The State of World Fisheries and Aquaculture 2020*. Sustainabi. Rome.

Ferdous, F. and Scott, T.R. (2015) 'A comparative examination of thrombocyte/platelet immunity', *Immunology letters*, 163(1), pp. 32–39.

Froehlich, H.E. *et al.* (2018) 'Comparative terrestrial feed and land use of an aquaculture-dominant world', *Proceedings of the National Academy of Sciences*, 115(20), pp. 5295–5300.

Galindo-Villegas, J. *et al.* (2012) 'Regulation of immunity and disease resistance by commensal microbes and chromatin modifications during zebrafish development', *Proceedings of the National Academy of Sciences*, 109(39), pp. E2605–E2614. Available at: https://doi.org/10.1073/pnas.1209920109.

Gilbert, J.A. *et al.* (2016) 'Microbiome-wide association studies link dynamic microbial consortia to disease', *Nature*, 535(7610), pp. 94–103.

Ikeda-Ohtsubo, W. *et al.* (2020) 'Intestinal microbiota and immune modulation in zebrafish by fucoidan from Okinawa mozuku (Cladosiphon okamuranus)', *Frontiers in nutrition*, 7, p. 67.

Jeong, J. *et al.* (2021) 'The effect of taxonomic classification by full-length 16S rRNA sequencing with a synthetic long-read technology', *Scientific Reports*, 11(1), p. 1727. Available at: https://doi.org/10.1038/s41598-020-80826-9.

Kan, H. *et al.* (2015) 'Correlations of gut microbial community shift with hepatic damage and growth inhibition of Carassius auratus induced by pentachlorophenol exposure', *Environmental Science & Technology*, 49(19), pp. 11894–11902.

Kanther, M. *et al.* (2014) 'Commensal microbiota stimulate systemic neutrophil migration through induction of Serum amyloid A', *Cellular Microbiology*, 16(7), pp. 1053–1067. Available at: https://doi.org/10.1111/cmi.12257.

Koch, B.E.V. *et al.* (2018) 'Intestinal microbiome adjusts the innate immune setpoint during colonization through negative regulation of MyD88', *Nature Communications*, 9(1). Available at: https://doi.org/10.1038/s41467-018-06658-4.

Lickwar, C.R. *et al.* (2017) *Genomic dissection of conserved transcriptional regulation in intestinal epithelial cells*, *PLOS Biology*. Available at: https://doi.org/10.1371/journal.pbio.2002054.

López Nadal, A. *et al.* (2018) 'Exposure to Antibiotics Affects Saponin Immersion-Induced Immune Stimulation and Shift in Microbial Composition in Zebrafish Larvae', *Frontiers in Microbiology*, 9(October), pp. 1–16. Available at: https://doi.org/10.3389/fmicb.2018.02588.

López Nadal, A. *et al.* (2020) 'Feed, Microbiota, and Gut Immunity: Using the Zebrafish Model to Understand Fish Health ', *Frontiers in Immunology* . Available at: https://www.frontiersin.org/article/10.3389/fimmu.2020.00114.

Martinez, F.O. *et al.* (2006) 'Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression', *The Journal of immunology*, 177(10), pp. 7303–7311.

Mirpuri, J. *et al.* (2014) 'Proteobacteria-specific IgA regulates maturation of the intestinal microbiota', *Gut microbes*, 5(1), pp. 28–39.

Mortha, A. *et al.* (2014) 'Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis', *Science*, 343(6178), p. 1249288. Available at: https://doi.org/10.1126/science.1249288.

Mu, Q. *et al.* (2017) 'Leaky gut as a danger signal for autoimmune diseases', *Frontiers in immunology*, p. 598.

Mucida, D. *et al.* (2005) 'Oral tolerance in the absence of naturally occurring Tregs', *The Journal of clinical investigation*, 115(7), pp. 1923–1933.

Murdoch, C.C. *et al.* (2019) 'Intestinal serum amyloid a suppresses systemic neutrophil activation and bactericidal activity in response to microbiota colonization', *PLoS Pathogens*, 15(3), pp. 1–30. Available at: https://doi.org/10.1371/journal.ppat.1007381.

Nguyen-Chi, M. *et al.* (2014) 'Transient infection of the zebrafish notochord with E. coli induces chronic inflammation', *Disease Models & Mechanisms*, 7(7), pp. 871–882.

Nguyen-Chi, M. *et al.* (2015) 'Identification of polarized macrophage subsets in zebrafish', *Elife*, 4, p. e07288.

Nogales-Mérida, S. *et al.* (2019) 'Insect meals in fish nutrition', *Reviews in Aquaculture*, 11(4), pp. 1080–1103.

Norambuena, F. *et al.* (2015) 'Algae in fish feed: performances and fatty acid metabolism in juvenile Atlantic salmon', *PloS one*, 10(4), p. e0124042.

de Oliveira, S. *et al.* (2013) 'Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response.', *Journal of immunology (Baltimore, Md. : 1950)*, 190(8), pp. 4349–4359. Available at: https://doi.org/10.4049/jimmunol.1203266.

Øverland, M. *et al.* (2009) 'Pea protein concentrate substituting fish meal or soybean meal in diets for Atlantic salmon (Salmo salar)—effect on growth performance, nutrient digestibility, carcass composition, gut health, and physical feed quality', *Aquaculture*, 288(3–4), pp. 305–311.

Rakus, K. *et al.* (2017) 'Conserved fever pathways across vertebrates: a herpesvirus expressed decoy TNF-α receptor delays behavioral fever in fish', *Cell host & microbe*, 21(2), pp. 244–253.

Rey, S. *et al.* (2017) 'Behavioural fever in zebrafish larvae', *Developmental and Comparative Immunology* [Preprint]. Available at: https://doi.org/10.1016/j.dci.2016.09.008.

Reynolds, W.W., Casterlin, M.E. and Covert, J.B. (1976) 'Behavioural fever in teleost fishes', *Nature* [Preprint]. Available at: https://doi.org/10.1038/259041a0.

Ribas, L. and Piferrer, F. (2014) 'The zebrafish (Danio rerio) as a model organism, with emphasis on applications for finfish aquaculture research', *Reviews in Aquaculture*, 6(4), pp. 209–240. Available at: https://doi.org/10.1111/raq.12041.

Rice, P. *et al.* (2005) 'Febrile-range hyperthermia augments neutrophil accumulation and enhances lung injury in experimental gram-negative bacterial pneumonia', *The Journal of Immunology*, 174(6), pp. 3676–3685.

Robinson, C.D. *et al.* (2018) 'Experimental bacterial adaptation to the zebrafish gut reveals a primary role for immigration', *PLoS Biology*, 16(12), pp. 1–26. Available at: https://doi.org/10.1371/journal.pbio.2006893.

Roeselers, G. *et al.* (2011) 'Evidence for a core gut microbiota in the zebrafish', *ISME Journal*, 5(10), pp. 1595–1608. Available at: https://doi.org/10.1038/ismej.2011.38.

Rolig, A.S. *et al.* (2015) 'Individual members of the microbiota disproportionately modulate host innate immune responses', *Cell Host and Microbe*, 18(5), pp. 613–620. Available at: https://doi.org/10.1016/j.chom.2015.10.009.

Rolig, A.S. *et al.* (2018) 'A bacterial immunomodulatory protein with lipocalin-like domains facilitates host–bacteria mutualism in larval zebrafish', *eLife*, 7, pp. 1–26. Available at: https://doi.org/10.7554/elife.37172.

Romero, J., Feijoó, C.G. and Navarrete, P. (2012) 'Antibiotics in aquaculture–use, abuse and alternatives', *Health and environment in aquaculture*, 159, pp. 159–198.

Santos, A. *et al.* (2020) 'Computational methods for 16S metabarcoding studies using Nanopore sequencing data', *Computational and Structural Biotechnology Journal*, 18, pp. 296–305.

Shin, N.-R., Whon, T.W. and Bae, J.-W. (2015) 'Proteobacteria: microbial signature of dysbiosis in gut microbiota', *Trends in biotechnology*, 33(9), pp. 496–503.

Silva, N.J. *et al.* (2020) 'Inflammation and matrix metalloproteinase 9 (Mmp-9) regulate photoreceptor regeneration in adult zebrafish', *Glia*, 68(7), pp. 1445–1465.

Singh, I.S. *et al.* (2008) 'Heat shock co-activates interleukin-8 transcription', *American journal of respiratory cell and molecular biology*, 39(2), pp. 235–242.

Sitjà-Bobadilla, A. *et al.* (2019) 'Disruption of gut integrity and permeability contributes to enteritis in a fish-parasite model: a story told from serum metabolomics', *Parasites & Vectors*, 12(1), p. 486. Available at: https://doi.org/10.1186/s13071-019-3746-7.

Sommer, F. *et al.* (2021) 'Inhibition of macrophage migration in zebrafish larvae demonstrates in vivo efficacy of human CCR2 inhibitors', *Developmental & Comparative Immunology*, 116, p. 103932. Available at: https://doi.org/https://doi.org/10.1016/j.dci.2020.103932.

Stagaman, K. *et al.* (2017) 'The role of adaptive immunity as an ecological filter on the gut microbiota in zebrafish', *ISME Journal*, 11(7), pp. 1630–1639. Available at: https://doi.org/10.1038/ismej.2017.28.

Stephens, W.Z. *et al.* (2016) 'The composition of the zebrafish intestinal microbial community varies across development', *The ISME Journal*, 10(3), pp. 644–654. Available at: https://doi.org/10.1038/ismej.2015.140.

Tang, J. *et al.* (2021) 'Diazinon exposure produces histological damage, oxidative stress, immune disorders and gut microbiota dysbiosis in crucian carp (Carassius auratus gibelio)', *Environmental Pollution*, 269, p. 116129. Available at: https://doi.org/https://doi.org/10.1016/j.envpol.2020.116129.

Tordesillas, L. *et al.* (2017) 'Epicutaneous immunotherapy induces gastrointestinal LAP+ regulatory T cells and prevents food-induced anaphylaxis', *Journal of Allergy and Clinical Immunology*, 139(1), pp. 189–201.

Tordesillas, L. and Berin, M.C. (2018) 'Mechanisms of oral tolerance', *Clinical reviews in allergy & immunology*, 55(2), pp. 107–117.

Tran, N.T. *et al.* (2018) 'Altered gut microbiota associated with intestinal disease in grass carp (Ctenopharyngodon idellus)', *World Journal of Microbiology and Biotechnology*, 34(6), pp. 1–9.

Tulapurkar, M.E. *et al.* (2012) 'Febrile-range hyperthermia modifies endothelial and neutrophilic functions to promote extravasation', *American journal of respiratory cell and molecular biology*, 46(6), pp. 807–814.

Ulloa, P.E. *et al.* (2011) 'Zebrafish as a model organism for nutrition and growth: Towards comparative studies of nutritional genomics applied to aquacultured fishes', *Reviews in Fish Biology and Fisheries*, 21(4), pp. 649–666. Available at: https://doi.org/10.1007/s11160-011-9203-0.

Ulloa, P.E., Medrano, J.F. and Feijo, C.G. (2014) 'Zebrafish as animal model for aquaculture nutrition research', *Frontiers in Genetics*, 5(SEP), pp. 1–6. Available at: https://doi.org/10.3389/fgene.2014.00313.

Wallace, K.N. and Pack, M. (2003) 'Unique and conserved aspects of gut development in zebrafish', *Developmental Biology*, 255(1), pp. 12–29. Available at: https://doi.org/10.1016/S0012-1606(02)00034-9.

Wang, B. *et al.* (2022) 'Ambient temperature structures the gut microbiota of zebrafish to impact the response to radioactive pollution', *Environmental Pollution*, 293, p. 118539.

Wang, Z. *et al.* (2010) 'Morphological and molecular evidence for functional organization along the rostrocaudal axis of the adult zebrafish intestine', *BMC Genomics*, 11(1). Available at: https://doi.org/10.1186/1471-2164-11-392.

Watts, S.A. and D'Abramo, L.R. (2021) 'Standardized reference diets for Zebrafish: Addressing nutritional control in experimental methodology', *Annual review of nutrition*, 41, pp. 511–527.

Westerfield, M. (2007) *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio).* 5th edn. Eugene: University of Oregon Press, Eugene.

Wu, C. *et al.* (2022) 'Point-of-use carbon-block drinking water filters change gut microbiome of larval zebrafish', *Environmental Microbiology Reports* [Preprint].

Xu, S. *et al.* (2018) 'Matrix metalloproteinases (MMPs) mediate leukocyte recruitment during the inflammatory phase of zebrafish heart regeneration', *Scientific reports*, 8(1), pp. 1–14.

Zeng, M.Y., Inohara, N. and Nuñez, G. (2017) 'Mechanisms of inflammation-driven bacterial dysbiosis in the gut', *Mucosal immunology*, 10(1), pp. 18–26.

Summary

Fish consumption derived from aquaculture practices already surpassed wild-catch fish. The raw material to produce fish diets are of great interest to promote healthier fish for human consumption. Aquafeeds are formulated aiming to boost fish health and reduce the usage of medicines (such as antibiotics). A robust and reliable fish model is needed to pre-screen more sustainable ingredients to design aquafeeds. Here, we propose zebrafish (*Danio rerio*) as a model to pre-screen novel feed ingredients and assess gut health in fish. Moreover, due to its unique advantages (transgenesis, easy to manipulate, knock-out generation by CRISPR-Cas9 technique, etc.) zebrafish can greatly contribute to the study of host-microbe-immune interactions to unravel the molecular mechanisms by which some feeds may be (un)healthy.

The main aims of these thesis were to set-up an *in vivo* fish model to study host-microbe-immune interactions and to develop a tool-box of several readouts to evaluate feed-derived inflammation.

In **chapter 1** I first explain that fish present a high physiological heterogeneity in their gastrointestinal tracts (GITs) depending on their naturally-occurring diet. I summarize the cell types and the bacteria found in the guts of the fish. Then, I focus on the GIT of zebrafish and the immune cell types that were visualized and characterized due to the generation of transgenic zebrafish and their larval transparency. After expanding on the immune maturation in zebrafish, I summarize the immune responses of zebrafish upon chemically-induced models. Then, I explore the usage of zebrafish as a model for feed-induced inflammation. Finally, I introduce the current concept of fish gut health and its limitations, providing the background that leads to the formulations of the aims of this thesis.

In **chapter 2** we setup a feed-derived inflammatory model by using soy saponin in zebrafish larvae. We characterized the fish immune response to saponin by gene expression, *in vivo* imaging and quantification of innate immune cells and the microbiota composition fluctuations. This was the first time that microbiota disruptions were reported upon saponin exposure in zebrafish. We also pre-exposed the fish to antibiotics before the saponin challenge and we assessed a larger disruption of the microbiota composition than controls getting some preliminary insights on host-microbe-immune interactions.

In **chapter 3** we explored how the degree of the response towards the inflammatory condition was affected by the microbial composition of the host larvae. We examined whether exposure in media of disrupted parental microbiota (by antibiotics) would modify the microbial composition of zebrafish larvae. After assessing that transferred adult microbiota changed the relative abundance of the taxa in the larvae, we examined the responses of these differently colonized larvae upon our saponin-induced inflammation. We showed that larvae colonized with antibiotic-treated adult gut content

recruited less macrophages and expressed less *il22* than controls upon saponin-induced inflammation.

In **chapter 4** we explored less invasive readouts to monitor inflammation in the zebrafish model. Using a unique prototype of live-tracking infrared camera connected to temperature control units, we studied the locomotion parameters and thermal preference of saponin-inflamed fish. We reported a novel (zebra)fish behaviour where fish spent more time in high temperature waters upon saponin-induced inflammation. Such behaviour was reversed back to control levels upon co-exposure with an anti-inflammatory glucocorticoid (dexamethasone). Similarly, increased expression of *cxcl8a* and increased number of neutrophils after saponin-induced inflammation were reserved back to control levels after dexamethasone co-exposure. We engineered a *cxcl8a* knockout by CRISPR-Cas9 technology and showed the crucial contribution of this cytokine to the inflammatory-associated behaviour described.

In **chapter 5** after the extensive characterization of our saponin-induced model of inflammation we revised the already published zebrafish literature concerning feeding interventions and their host-microbe-immune interactions. We critically reviewed the experimental designs and we described the necessity to include different development stages of the fish (not only larvae) and combine several laboratory techniques with large omics datasets to properly evaluate the effect of novel feeds in the context of (zebra)fish gut health.

In **chapter 6** to better understand the effect of feed ingredients at different developmental stages we employed larvae as well as adult zebrafish to investigate the effects of a fucoidan (brown algae extract) on their immune response and microbiota composition. Fucoidan exposure in media increased the expression of pro-inflammatory cytokines in zebrafish larva while fucoidan supplementation decreased the expression of *il1b* in the gut of adult zebrafish. Fucoidan exposure in media mildly disturbed the microbiota composition of larvae while fucoidan supplementation disrupted the gut microbiota composition to a greater extent in adult zebrafish

Then, in **chapter 7** we employed novel butyrate- and saponin-supplemented diets (oil-coated pellets) to comprehensively evaluate the intestinal health of juvenile zebrafish. Among the readouts we included: microbiota profiling and taxa connectivity with matched host gut transcriptomics and high-throughput quantitative histology and we combined these datasets with *in vivo* imaging of innate immune cells in larvae. Butyrate-supplemented feed elicited a stronger pro-inflammatory-like reaction in the zebrafish gut than saponin-supplemented feed and the control feed.

Finally, in **chapter 8** of this thesis we discuss the potential of zebrafish larvae to study processes such as intestinal barrier integrity or bowel movement as well as quantification of the feeding intake. Then I revised the work performed across the chapters of this thesis

and I embedded it in the currently existing literature to suggest hallmarks of inflammation for further studies. Subsequently, I integrated our microbiota composition data by developmental stage from different experiments of healthy and inflamed zebrafish to explore the idea of hallmarks of taxa related to inflammation. I explored the possible uses for the aquaculture industry with the relevance of testing novel and more sustainable diets contributing to the protein transition. Lastly, I stressed the usage of zebrafish to study host-microbe-immune interactions and to study gut health.

About the author

Adrià López Nadal was born on the 7th of May 1991, in Valls (Tarragona). During high school, Adrià was awarded an AGAUR mobility grant "from the Generalitat of Catalunya" to go to Cheltenham (UK) to improve his knowledge of the English language. Afterwards, he studied Biology at the Universitat Autònoma de Barcelona where he learnt many disciplines, from Immunology to Zoology, from Statistics to Botany. Moreover, Adrià enjoyed an exciting live in the Catalan capital combining many side projects, formal education, work, and socializing activities. During his Bachelor, Adrià was awarded an Erasmus scholarship to study at the Universitá degli Studi Roma Tre (Italy), where he learnt Italian, and studied Immunology, Microbiology and Bioinformatics among other subjects. Soon after returning from Italy, Adrià was awarded an Erasmus Internship grant, and he moved to the Vrije Universiteit in Amsterdam (The Netherlands) to study the effects of starvation on breast cancer cells as a final project for his Biology BSc.

After some time thinking about his next step, he moved to Wageningen and got enrolled in the MSc of Molecular Life Science, with specialization in Biomedical Research. Adrià wanted to pursue a PhD degree and he coursed a major MSc thesis in the Human Nutrition department about the effects of caloric restriction in the gut of mice, and a minor thesis in the Cell Biology and Immunology group about the effects of msp-/- zebrafish on intestinal inflammation. After completing his MSc degree, he got offered a job as Research Assistant at the Cell Biology and Immunology group. Later, he took time off and travelled around Colombia. After returning from his travels, he was offered a PhD position with Sylvia Brugman at the Cell Biology and Immunology group/Aquaculture and Fisheries Group to study hostmicrobe-immune interactions in the context of nutrition using zebrafish (this thesis). Upon the completion of the PhD thesis, Adrià started a Postdoc fellowship at the Host-Microbe Interactomics in Wageningen University to study the function of cxcl8a in the host-microbe-immune interactions using zebrafish among other subjects.

List of publications

López Nadal, A., Peggs, D., Wiegertjes, G. F., & Brugman, S. (2018). Exposure to antibiotics affects saponin immersion-induced immune stimulation and shift in microbial composition in zebrafish larvae. *Frontiers in microbiology*, *9*, 2588.

Ikeda-Ohtsubo, W., **López Nadal, A.**, Zaccaria, E., Iha, M., Kitazawa, H., Kleerebezem, M., & Brugman, S. (2020). Intestinal microbiota and immune modulation in zebrafish by fucoidan from Okinawa mozuku (Cladosiphon okamuranus). *Frontiers in nutrition*, *7*, 67.

López Nadal, A., Ikeda-Ohtsubo, W., Sipkema, D., Peggs, D., McGurk, C., Forlenza, M., Wiegertjes, G. F & Brugman, S. (2020). Feed, microbiota, and gut immunity: using the zebrafish model to understand fish health. *Frontiers in immunology*, 114.

López Nadal, A, Kidess E, Boekhorst J, Brugman S. Environmental microbes determine macrophage response towards saponin-induced inflammation in zebrafish larvae. *Submitted*.

López Nadal A, Boekhorst J, Lute C, van den Berg F.,Schorn M. A., Bergen Eriksen T, Peggs D., McGurk C, Sipkema D, Kleerebezem M, Wiegertjes G. F., Brugman S. Omics and imaging combinatorial approach reveals butyrate-induced inflammatory effects in the zebrafish gut. *In press*

López Nadal A., Beekman F, Sipkema D, Wiegertjes G. F., Brugman S; Zebrafish display cxcl8a-dependent selective thermal preference during inflammation. *In preparation*.

Training and Supervision Plan (TSP)

The Basic Package	**year**	**credits ***
WIAS Introduction Day	2018	0,3
Scientific Integrity & Ethics in Animal Sciences	2018	1,5
Subtotal Basic Package		2
Disciplinary Competences *(minimum 2 credits)*	**year**	**credits**
PhD project proposal	2018	6,0
Laboratory Animal Science: Design and Ethics in Animal Experimentation. WUR (The Netherlands)	2018	3,0
Laboratory Animal Science: Species Specific Course on Fish. Utrecht (The Netherlands)	2018	0,6
Fish Immunology and Vaccination Workshop. Wageningen (The Netherlands)	2018	1,5
Course Advanced Immunology. University of Utrecht (The Netherlands)	2019	1,5
Internship at Skretting, learning histology. Stavenger (Norway)	2020	2,0
Subtotal Disciplinary Competences		15
Professional Competences *(minimum 2 credits)*	**year**	**credits**
Committee member of the organization team for the WIAS Science Day	2019	2,0
Brain friendly working and writing. WUR (The Netherlands)	2020	0,3
Project and time management. WUR (The Netherlands)	2019	1,5
The Choice: Un-box your PhD process & take charege of your performance. WUR (The Netherlands)	2020	0,5
The Final Touch: Writing the General Introduction and Discussion. WUR (The Netherlands)	2021	0,6
Subtotal Professional Competences		5
Societal Relevance *(recommended)*	**year**	**credits**
Societal impact of your research WUR (The Netherlands)	2019	1,5
Subtotal Professional Competences		2

Presentation Skills *(maximum 4 credits)*	**year**	**credits**
Saponin induces an inflammatory phenotype and a microbial switch in zebrafish larvae, 11th Zebrafish Disease Model , Leiden (The Netherlands), July 2018, Poster presentation	2018	[0.5]
Saponin and antibiotics exposure to zebrafish larvae: immunity and microbiota, 3rd International Conference on Fish and Shellfish Immunology, Gran Canaria (Spain), June 2019, Oral presentation	2019	[1]
Microbial and immune modulation by saponin and antibiotics in zebrafish larvae, 2nd Fish Microbiota Workshop, Eugene (OR, USA), September 2019, Oral presentation	2019	1,0
Zebrafish responses towards feed components: a screening model, WIAS Annual Conference, Lunteren (The Netherlands), February 2020, Oral presentation	2020	1,0
Understanding zebrafish gut health: feed, microbiota and immune system. 16th annual Zebrafish Husbandry Workshop. San Diego (CA, USA). Oral online presentation (Invited Speaker)	2021	1,0
Butyrate and Saponin in zebrafish feeds: understanding gut health-related effects of dietary components. Bejing (China). Oral online presentation	2021	1,0
Subtotal presentations		4
F. Teaching competences *(max 6 credits)*	**year**	**credits**
Cell Biology and Health (Dutch). PO Group.	2018	0,5
Cell Biology and Health (English). PO Group.	2018	0,5
Human and Veterinary Immunology. Wet Practicals.	2018-19-20	1,5
Thesis Master Supervision. Charlotte Berendsen	2018-19	1,5
Thesis Master Supervision. Fanny Beekman	2020	1,5
Thesis Master Supervision. Roos van t'Spijker	2020-21	[1.5]
Thesis Master Supervision. Gerben Scholman	2021-22	[1.5]
Subtotal Teaching competences		6
Education and Training Total *(minimum 30 credits)**		32

Acknowledgments

In this section I would like to thank all the people who gave me support during these five years of my PhD. In this period, I have developed myself as a scientist, and as a professional with a wide range of skills, from managing projects to collaborating with several teams from academia as well as the private sector. Although the scientific contribution of certain people has been crucial for the completion of my PhD, I must admit that without the love of my family, my friends, and my partner, I would have struggled much more to complete this PhD satisfactorily.

The first person I would like to thank is my daily supervisor, **Sylvia Brugman**. Once I read that a great scientist is someone who besides being curious and methodical is able to provide an empathic supervision. In 2016, I started working with Sylvia for my MSc minor thesis and I immediately understood the meaning of that quote. Sylvia has always been there for me, while giving me the freedom to develop my scientific curiosity at the same time. I have enjoyed alike the scientific discussions, the excitement of promising results, and the conferences and their corresponding parties with plenty of beers and "networking". Above all, I have relished sharing our personal points of view on different topics in and outside of academia. It has been a great pleasure to work with Sylvia, and the best part of all is that we are going to work for some more time together. Another indispensable person for the completion of my PhD thesis is my promotor **Geert Wiegertjes**. Geert is an excellent scientist who always has an interesting take on the scientific stories, a quality that often inspires the people around him to look at things form a new angle. Geert is very sharp man, and he possesses a very sarcastic humour that took me a bit of time to grasp at the beginning. Although he has been very busy since he became the chairholder of AFI, he always made some time to discuss my latest results, and to figure out how hora finita works. Thank you very much for your time and support, Geert! I would also like to thank the contribution of **Detmer Sipkema**, part of my promoting team. I remember that the first time I met Detmer I thought: "Here he comes, the cool guy from the Microbiology group." And I was not entirely wrong. Detmer always provided extremely helpful feedback. From our personal interactions, it quickly became clear that Detmer is a very understanding and caring person with a weird (and very cool) obsession with sponges.

I would also like to thank several other incredible scientists I have had the opportunity to work with during my PhD thesis. **Jos Boekhorst**, for our never-ending hours spent in the bioinformatic analyses. I will always remember your question just before clicking on the last step in the script: "What can possibly go wrong?" Spoiler: it does, and often! Thank you so much for being patient with me and teaching me so many cool software analyses. **Michiel Kleerebezem**, thank you for your critical view on my work, it has helped me

tremendously to re-think and question many choices I have made with my data. I also appreciate your sense of humour very much – for the ones you do not know him, Michiel is the kind of guy who seems very serious at first impression, but quickly reveals his true, inner comedian. **Michelle Schorn**, thank you very much for all the help while navigating R when analysing the 16S results. It was simultaneously a frustrating and rewarding experience. Thank you for comforting me there during covid times when research was halted. **Maria Forlenza**, thank you for your enthusiasm about fish immunology. When I am writing I always keep in mind that "A fish is not a mammal" and I start visualizing the whole story from a zebrafish-point of view. **Carolien Lute**, thank you very much for brainstorming many experiments together, and for helping me with different experimental preparations. I have learnt a lot from you (already from my MSc thesis!) on how to properly work in a laboratory, I really appreciate you. Thanks to **Wakako Ikeda-Ohtsubo**, for transmitting her passion about microbiology and for working so well with me. Looking forward to visiting you in Japan.

Part of this thesis was possible because of the input of **Charles McGurk**, **David Peggs** and **Tommy Bergen Eriksen**. Thank you, Charles and David, for being so supportive in every users' meeting, for providing me with the experimental diets on time, and for hosting me in the incredible automated laboratory facilities in Skretting (Norway). Thank you, Tommy, for being so passionate and such good teacher in the histological analyses. It was a pleasure to work with you, and to figure out how we could automatically quantify many cell types in the small zebrafish guts. Also, many thanks to **Albert Willemsen** and **Patrick Zimmerman,** from Noldus, for helping me to set the DanioVision up. After many years of experiments with this very cool device, I think a very interesting story is coming!

I was lucky enough to be affiliated in two research groups, CBI and AFI. I would like to thank all my colleagues from CBI, especially **Julia Teresa** for being my very good friend, and becoming my paranymph. Muchas gracias querida, eres una mujer muy fuerte y siempre me transmites una alegría enorme. **Mojtaba**, thank you for all the advice, and all the cookies whenever I was low (in mood and in blood sugar), you always made my day. **Paulina**, thank you for being so caring and always bringing with you so much positivity. **Cresci**, thank you for being such a good officemate and my best partygoer, it is always a lot of fun with you. **Tess**, thanks for all the beers and support when we complain about our PhDs. Also, thanks to **Alvja** and **Navya** for bringing new energy to CBI. Many thanks to the oldies from CBI (**Jules**, **Annelieke**, **Carmen**, **Eva**, **Sem**, **Marloes** and **Danilo**), it was great to enter the group when you were there.

I would like to thank **Vivi Koletsi** from AFI for being a strong and brave woman, and for becoming my paranymph. I really appreciate you, and I would love to spend more time together. **Corrie**, thank you for many evenings philosophizing about live and sharing

points of view; I am looking forward to many more. **Elisa**, grazie tante per ascoltarmi sempre, per relativizzare tutto, per appogiarmi anche coi pensieri piu pazzesci. **Mark**, thank you for being there since the beginning. We went together through so many changes, laboratory practicals, conferences and many more! **Kaylee**, you are my zebrafish mate. We had so much fun in Sheffield. **Zhang**, thank you for the trip we did and the canoeing, it was great to get to know you a bit more. **Gauthier**, thank you for being my best squash mate at the beginning, and for being so brave at the end of your PhD. I hope we keep on sharing more moments soon.

Since my daily supervisor moved to HMI, I started to get to know the people in this group and I got integrated pretty easily. Thank you very much to **Maria Juanpere,** per estar sempre quan et necessito, per estar tant boja i a la vegada ser tant responsable i per ensenyar-me a no jutjar als altres per les decissions que prenen en les seves vides. Gracias a **Blanca** por estar tan cuerda (o no), o por al menos parecerlo cuando los de tu alrededor están entrando en pánico. Me alegra mucho que seas mi nueva officemate. **Evelien**, I am so glad that you also started working with zebrafish! I had a blast when we shared conferences (and parties), meetings and random conversations, looking forward to many more. Also, many thanks to **Isa**, **Oshin**, **Alex**, **Arabela**, **Bart** and all the other colleagues from HMI for making me feel so welcome in this group.

I consider myself a very lucky person because I have a very extensive network of friends that supported me in the process of getting my PhD. We have also had a lot of fun together. Before moving to Wageningen, I did my BSc thesis in Amsterdam, and I would like to thank **Mohammed** and **Corinne** for introducing me to the Dutch culture from a foreigner's point-of-view. **Maria Redondo** y **Pablo,** que os llevo siempre conmigo vaya donde vaya, desde New Zealand a Valladolid. I sobretot a la **Marta Clotet** i la **Laura Trapero** per ser les meves indispensables tots aquests anys en terres holandeses. Us estimo moltíssim nenes! When I started living in Wageningen, I lived in Droevendaal and there I met some of my favourite people: **Anouk**, **Lian**, **Vero**, **Orestis**, **Silvia Quarta**. I am so happy to still have you all in my life! I would also like to thank many more friends I've met during these years because it has been a wonderful period. In particular, my catalan/ spanish crew: **Vicky**, **Martí**, **Debs**, **Tom**, **Celia** i **Marta** (tela lo de la kombucha lol). M'encanta tenir-vos a prop i a veure si ara ens trobem més. Afterwards, I moved to The Farm "Boerderie" where I immediately felt part of this big and incredible **Farmily**. As a foreigner living in The Netherlands, this group of people has been the biggest blessing in my live. Several generations of farmies stand before me, and I am so grateful they have taught me so many valuable things, such as being proud of who I am, never stop being curious, spending time on the things I like (that although it may sound obvious, we keep on forgetting in our busy lives) and, above all, respecting our differences and loving each other. Thank you to: **Alba**, **Laurens**, **Maria**, **Foskea**, **Louis**, **Linde**, **Kalijn**, **Isa**, **Loes**, **Ivo**,

Marco, **Audrey**, **Paula**, **Raquel**, **Charly**, **Annemiek**, **Jean-Yves**, **Heitor**, **Ilsa**, **Sara**, **Elske**, **Erwin**, **Bernice**, **Wouter**, **Carlos**, **Moritz**, **Hestia**, **Elsbeth**, **Bart**, and I am sure I am forgetting many more of you, but I keep you all in my heart.

Part del meu cor segueix estan a Barcelona i vull agraïr que hi ha varies persones que em fan sentir que encare pertanyo a aquestes terres. Gràcies al **Jordi** per ser tant pesat i per sempre desitjar-me el millor. Gràcies a la **Maria Borràs** per ser una indispensable de fa tants i tants anys. T'estimo moltíssim i sempre ets per a mi un exemple de valentia i de coratge i un exemple a seguir. A la **Maria Cutilles**, **Laura Julibert**, **Letti** gràcies per estar sempre ni que ens veiem tant poc. Us estimo molt! Gràcies a la **Maria Jou** per ser tant artista i tant empàtica amb els altres i brindar-me tant riures i aventures. Gràcies a l'**Artura** per ser tant tremeneda i deconstruida (o no!). Moltes gràcies a la **Rosa Ulldemolins** per ser una amiga excepcional, com de les que se'n troben poques i també a la **Marta** per obrir-me sempre les protes de casa i per tantes converses sobre ciència i art. Moltes gràcies als meus amics indispensables de Valls, els que any rere any estan allà i que són el sostén de la meva identitat des de que tinc us de raó. Gràcies **Ari**, **Cris**, **Anna**, **Xènia**, **Dúnia**, **Carol** i **Irene** no sé que faria sense vosaltres.

Vull donar les gràcies a la meva familia per tot el suport rebut. A la **mare** i al **pare** per tant d'amor incondicional que em fa ser com sóc. Per confiar amb mi i per intentar entendrem sempre fins i tot quan no faig el que s'espera de mi. Per creure que sóc capaç de fer el que em proposi. Per donar-me la mà cada cop que he tornat a casa i estava perdut i per dir-me que no passa res i que poc a poc trobaré el que estic buscant. Gràcies per donar-me tant de suport en els moments més difícils i sobretot per respectar-me en les meves decisions. Us estimo moltíssim. Moltes gràcies al meu germà **Albert** i a l'**Helena** i a les meves dos precioses nebodes **Carlota** i **Irene**. Per ser casa, un pilar sòlid i per estar sempre per celebrar els bons moments i plorar junts en els dolents. Tinc moltes ganes de que estiguem més a prop i ens tinguem més sovint. Gràcies a l'**avi** (allà on descansi) i a l'**àvia** per cuidar-nos sempre i per prioritzar la familia en tot moment. Gràcies als meus cosins **Manel**, **Berta**, **Xavi** i **Gine** i a les meves tietes **Anna** i **Sussi** que tot i que ens veiem poc us estimo molt.

Finalmente, me gustaría agradecer a **Ángel** tantas cosas que no sé ni por dónde empezar. Gracias por tanta luz que desprendes y que me hace brillar al estar cerca tuyo. Gracias por ser tan bonito y paciente, por quererme y aceptarme como soy. Gracias por aguantarme con el doctorado sin ni siquiera entender del todo qué hago. Ha sido sin duda una etapa complicada, pero estoy tan orgulloso de todo lo que hemos conseguido que sé que ha valido la pena. Eres el mejor compañero que nunca hubiese podido imaginar. Cada rato a tu lado es un aprendizaje, y estoy tan feliz de que sigamos mano a mano intentando surfear tantas incertidumbres y dudas vitales que si lo hacemos juntos me siento capaz de todo. ¡Te quiero muchísimo y tengo muchas ganas de seguir sumando y viviendo aventuras a tu lado!

Colophon

The research described in this thesis was financially supported by the Netherlands Organisation for Scientific Research (NWO) and Skretting (ARC), Noldus and Future Genomics as part of the NWO-TTW Applied and Engineering Sciences (project number 15566).
Financial support from Wageningen University and for printing this thesis is gratefully acknowledged.

Cover design by Maria Jou Sol | www.mariajousol.com
Lay-out by Adrià López Nadal and Promotie In Zicht | www.promotie-inzicht.nl
Printed by Promotie In Zicht on FSC-certified paper