UNIVERSITY *of* GLASGOW

SalmoSim:
the development of an *in vitro* simulator of the Atlantic Salmon (*Salmo salar*) GI tract and associated microbial communities.

A thesis submitted for the degree of Doctor of Philosophy

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Author's Declaration

I declare that, with the exception of the aid listed below, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

All HiSeq 2500 sequencing was carried out by Novogene. The gas chromatographic analysis of extracted Volatile Fatty Acids weas performed by MS-Omics (Denmark). Dr Jarred Lee Knapp and Matthew Watkins-Baker helped to collect real salmon samples in Averøy Norway. Julie Russell, Dr Chloe Heys, Dr Joseph Humble, Michele De Noia all helped to perform daily sampling from SalmoSim system. All of the resulting data, and all statistical analyses in the thesis were performed by me with a support from Dr Bachar Cheaib and Alex Kitts. Finally, all of the research performed in this thesis was overseen by Dr Martin Llewellyn and Dr Stephanie Connelly. The Chapter 1 is partly based on the review paper included in Appendix 3, and some of the data presented in this thesis is published in papers in included in Appendix 4 and 5, all three of which I was co-author.

Chapter 1 Overview

1.1 Atlantic salmon aquaculture worldwide and in Scotland

In 1950, the world population was estimated to be around 2.6 billion people, a figure that doubled by the 1990s and is estimated to increase further to 9.7 billion in 2050 and 11.2 billion by 2100 (United Nations 2019). Even though the Earth's population growth is predicted to plateau sometime in the next 100 years, producing enough food to feed 9 to 11 billion people poses an enormous global challenge. Aquaculture has the potential to contribute to the solution, as fish is an accessible and affordable source of nutrients, proteins, and energy. It was estimated that per capita fish consumption has almost doubled from 10 kilograms in the 1960s to over 19 kilograms in 2012 (FAO 2018). This increase in the demand for fish protein has put wild fish stocks under pressure (Froehlich et al. 2018). The aquaculture sector may have the capacity to alleviate this pressure and currently produces almost 50% of all fish for human consumption with expansion to 62% predicted by 2030 (Moffitt and Cajas-Cano 2014).

Commercial salmon farming started in the late 1960s in Norway, where the first sea cages were successfully used to raise salmon to market size (Liu et al. 2011). Large scale commercial operation launched in the 1980s (Bjorndal et al. 2000). Since then, salmon aquaculture has spread to Scotland, Ireland, the Faroe Islands, Canada, the USA (North Eastern seaboard), Chile and Australia (Tasmania coast only) and to a smaller extent in New Zealand, Spain and France (Moroney et al. 2015). Over a 25 year period (from 1982 to 2010) salmonid aquaculture production has expanded over ten-fold, with circa 2.5 million tonnes of Atlantic salmon production per year (Figure 1-1) (Food and Agriculture Organization 2016).

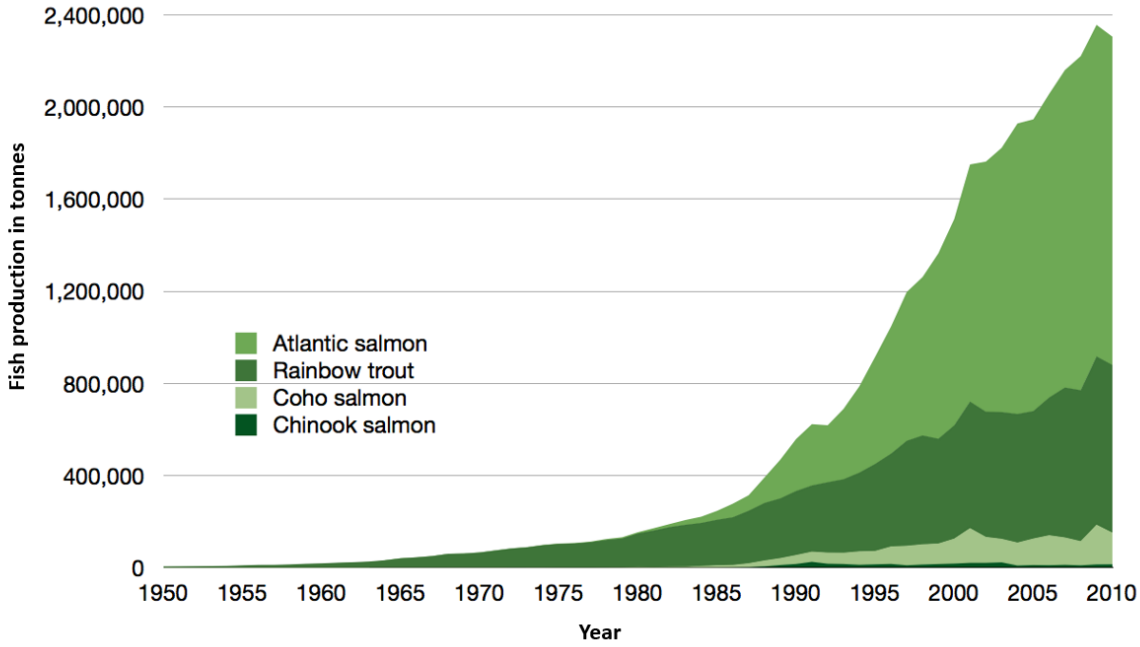


Figure 1-1 Aquaculture production of salmonids in tonnes 1950-2010 (adapted from Food and Agriculture Organization, 2016).

Salmon is currently the largest food export in value in Scotland (The Scottish Government 2019). 203,881 tonnes of salmon were produced in 2019 in Scotland, with the aim to increase this number to 350,000 tonnes per year by 2030 (Munro 2019). In 2018 there were 121 active Atlantic salmon farms on the Northern and Western Scottish coast, and over the last 10 years employment within Scottish salmon aquaculture increased by 52% (Figure 1-2). To support the salmon aquaculture sector, the Scottish government has developed a strategy to double the economic contribution of the Scottish aquaculture sector from £1.8 billion in 2016 to £3.6 billion by 2030 (Scotland food and drink 2016).

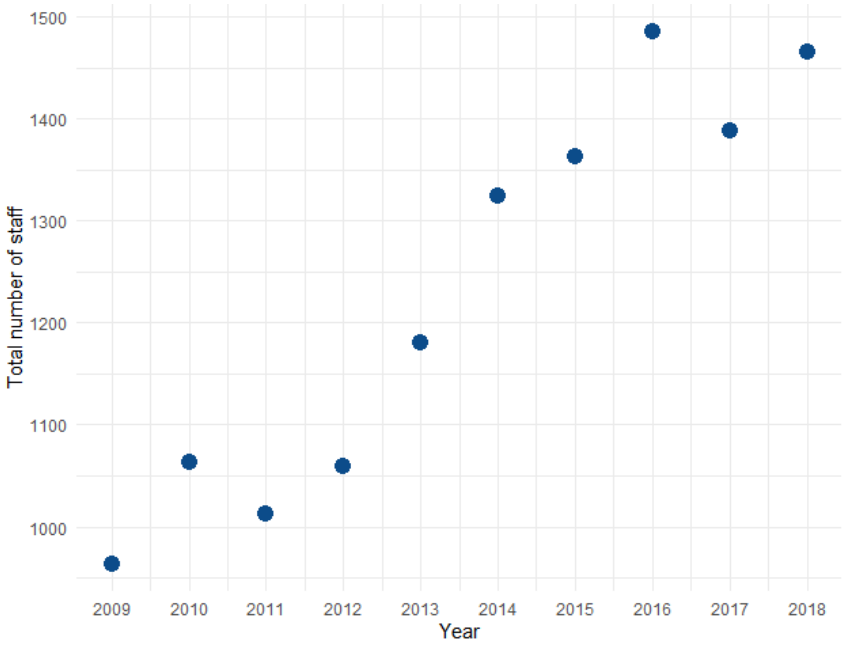


Figure 1-2 Number of staff employed in the production of salmon during 2009-2018 in Scotland (adapted from Munro, 2019).

1.2 Atlantic Salmon

1.2.1 Atlantic salmon life cycle

The life cycle of *Salmo salar* involves seven major stages: egg, alevin, fry, parr, smolt, post-smolt, and adult salmon (Figure 1-3). The majority of wild Atlantic salmon are iteroparous, anadromous fish that migrate between freshwater and marine habitats at different stages in their life cycle: egg, alevin, fry and parr life stages occur in freshwater, and the main feeding and growth phases (smolt and adult stages) occur in a marine water environment. These maturing smolts usually spend one or more winters in the ocean to grow to the adult phase before returning to the freshwater for spawning (Thorstad et al. 2011). Some Atlantic salmon repeat this migration and spawning pattern several times, however the majority spawn only once or twice per lifecycle (Bordeleau et al. 2020). Furthermore, some Atlantic salmon, especially in Canada and Russia, complete their entire lifecycle in freshwater only (Carr et al. 2005, Ozerov et al. 2010, Hutchings et al. 2019).

While initial salmon life stages (from egg to parr) take from two to five years in the wild, Atlantic salmon raised for aquaculture sector require only 8 to 16 months to reach smolt phase and then are transferred to sea cages for around two years

to reach harvestable size (FAO 2012). The weight of Norwegian farmed smolt has more than doubled from 20-50 g in 1985 to 70-120 g in 2000, and more recently, the land-based phase has been lengthened even further to produce post-smolts of up to 1,000 g before release in sea-water to reduce the exposure to sea lice (Bergheim et al. 2009, Bjørndal and Tusvik 2020).

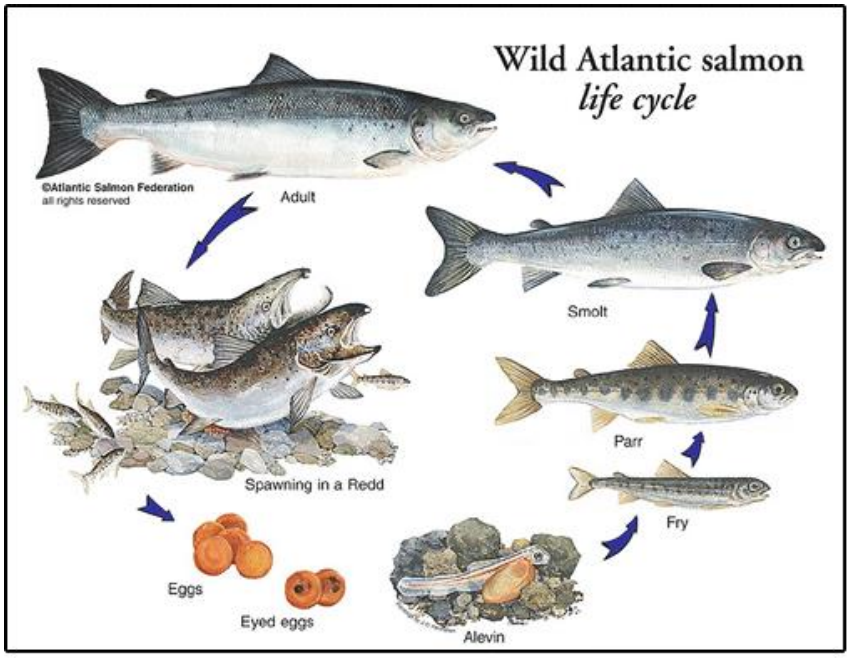


Figure 1-3 The life cycle of the Atlantic salmon (Salmo Salar) (Atlantic Salmon Federation ©). *Figure illustrates the major life stages of the Atlantic salmon, including eggs, eyed eggs, alevin, fry, parr, smolt, adult and spawning.*

1.2.2 Gastrointestinal tract of salmon

Due to a wide variety of habitats and foods that fish consume (ranging from bottom-living seaweeds and plankton organisms to actively swimming animals) fish have a wide array of gut morphology (Horn and Gawlicka 2001). Fish stomachs can be classified into four general configurations: a straight stomach with enlarged lumen (e.g. *Esox lucius*), Y-shaped stomach (e.g. *Anguilla anguilla*), the absence of stomach (e.g. *Cyprinus carpio*) and U-shaped sack-like stomach with enlarged lumen, such as is found in Atlantic salmon, receiving food via the oesophagus (Smith 1980). Together with the oesophagus, the stomach makes 42% of the length of the entire gastrointestinal tract in *Salmo salar* (Lkka et al. 2013). As illustrated in Figure 1-4, the Atlantic salmon gastrointestinal tract can be divided into several functionally and morphologically distinct segments. The acidic stomach is responsible for initial unspecific digestion of incoming food by secretion of hydrochloric acid and endopeptidase pepsin from gastric mucosa glands in the

stomach lining (Krogdahl, Sundby, et al. 2015). In *Salmo salar*, the stomach is connected to the pyloric caecum (absent in fish which lack stomach), which consists of multiple longitudinally arranged blind-ended finger-like projections in a net-like pattern that increase the mucosal surface area for digestive and absorptive capacity (Krogdahl, Sundby, et al. 2015). The surface area-increasing structures have proven to be important in enzymatic breakdown by secreting digestive enzymes (such as proteases (e.g. trypsinogen), glucosidases (e.g. α-amylase) and lipases (e.g. bile salt-dependent lipase)) (Sahlmann et al. 2015) and to utilize the counter-current multiplication in generating osmoregulatory mechanisms for absorption of glucose, amino acids, dipeptides and medium chain fatty acids (Buddington and Diamond 1986, 1987, Bakke-McKellep et al. 2000, Denstadli et al. 2004). The pyloric caecum accounts for around 70% of the total nutrient absorption in *Salmo salar* (Veillette et al. 2005). The gastrointestinal tract ends with mid and distal intestines that further digest and absorb nutrients with the help of brush boarder membrane bound and cytosolic digestive enzymes, such as alkaline phosphatase (ALP) and leucine aminopeptidase (LAP) (Refstie et al. 2006, Krogdahl, Sundby, et al. 2015).

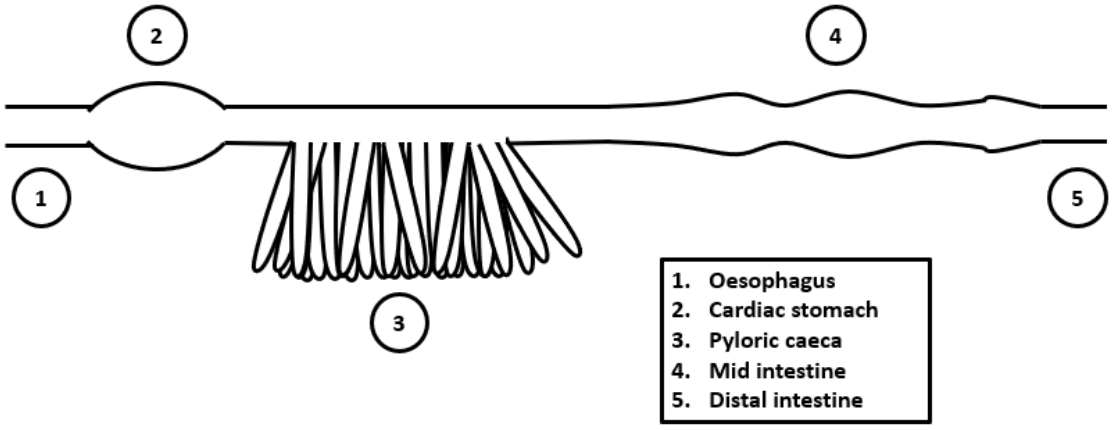


Figure 1-4 Gastrointestinal track of Atlantic Salmon. *Figure illustrates a schematic drawing of the salmon gastrointestinal tract with the oesophagus (1), cardiac stomach (2), pyloric caeca (3), mid intestine (4), and distal intestine (5).*

1.2.3 Gastrointestinal microbiota of Atlantic salmon

Animal guts contain dense, extremely complex, and dynamic microbial communities that are not just a collection of the passenger microorganisms, but

can include organisms with active roles in driving immunity and physiology of vertebrates (McFall-Ngai et al. 2013, Murdoch and Rawls 2019). The gastrointestinal microbiota is defined as the community of microorganisms that reside within the gut of the host, which include bacterial communities, archeae, viruses and eukaryotes, such as yeast (Walter et al. 2011). However, in this thesis we focus on only microbial communities residing in the gut. These microorganisms exhibit a lower density (10^4-10^9 colony forming units), compared to homoeothermic animals (10^{13}-10^{14} colony forming units) (Egerton et al. 2018).

Significant research effort has been mobilised in recent years to explore the role of the intestinal microbiome in host biology. Innumerable studies in vertebrates suggest a role for gut microbes in host development, physiology and health (Yu et al. 2012, Suzuki 2017, Sharpton 2018). However, most of these findings are derived from studies performed on mammals - in particular, rodents - with few derived from studies performed on teleosts, leading to limited evidence of functional capacity of fish gut microbiomes (Llewellyn et al. 2014b, Ringø et al. 2016a). Recent studies indicate the microbiome of Atlantic salmon plays a possible role in nutrition and immunity (Llewellyn et al. 2016).

1.2.3.1 Microbiome in nutrition

Several reviews have pointed out that the microbiome of teleosts plays a crucial role in fish nutritional harvest. It was shown that microbial communities inside the gastrointestinal tract of fish produce a wide range of different enzymes (such as carbohydrases, phosphatases, esterases, lipases and peptidases, cellulase, lipase and proteases) which could contribute to feed digestion (Ray et al. 2012). These enzyme-producing bacteria were found in the guts of four brackish water teleost species (*Scatophagus argus, Terapon jarbua, Mystus gulio, and Etroplus suratensis*) as well as in the Atlantic salmon gut (Askarian et al. 2012, Das et al. 2014).

It was also suggested that anaerobic bacteria living inside the fish gut could have a role in feed digestion and absorption of nutrients, by supplying the host fish with volatile fatty acids that are an end-product of anaerobic fermentation involved in nourishing enterocytes (Ramirez and Dixon 2003, Clements 2011, Tran et al. 2020). These volatile fatty acids were found in the intestines of common carp (*Cyprinus*

carpio), shad (*Dorosoma cepedianum*) and largemouth bass (*Micropterus salmoides*), as well as Atlantic salmon (salmon farmed in Irish waters) (Smith et al. 1996, Fogarty et al. 2019).

Finally, the microbiota was shown to be able to synthesise vitamins and amino acids in the gut of aquatic vertebrates which otherwise would not be available to the host (Balcázar, Blas, et al. 2006, Nayak 2010). For instance, it was found that microorganisms within different freshwater fish can produce vitamin B_{12} (Sugita et al. 2017). However, a knowledge gap exists between determining the composition of the microbiome and understanding its function, partially due to the complex and variable ecology of teleost gastrointestinal tracts and unknown bacterial taxa (Nayak 2010).

1.2.3.2 Microbiome in immunity

It is known that the vertebrate gut microbiota plays an important role in the immunity of the host (Raulo et al. 2018). This role is especially vital in fish, as they are in constant contact with a wide variety of microorganisms, including pathogenic, and opportunistic bacteria which may colonize the gut, and thus, a strong immune system is critical (Ellis 2001). Thus, a robust gut microbiome in fish may be important to prevent invader colonization and maintain gut health (Balcázar, Decamp, et al. 2006, Zheng et al. 2020).

The role of the microbiome in immunity is particularly important during initial microbiome colonization at the early stages of teleost development. It was found that the colonizing microorganisms in the gut can modulate gene expression to create a favourable environment for themselves and avoid invasion by other pathogenic and opportunistic bacteria coming into the ecosystem later in the hosts' life (Balcázar, Blas, et al. 2006). This idea was supported by a study in which the effect of colonization by components of the microbiota in gnotobiotic zebrafish (*Danio rerio*) was studied (Rawls et al. 2004). This study suggested that the expression of some of the zebrafish genes are bacteria-specific (the genes expressions are induced by unknown factors produced by the specific gut bacteria). Finally, it was found that *Lactobacillus* inside the gut of various fish (and naturally found in *Salmo salar* gut) are able to stimulate the immune response and in this way protect the host against diseases (Llewellyn et al. 2016, He et al.

2017). However, existing knowledge about the microbiome's role in immunity has many gaps, due to the complex interactions between the immune system, environment, and the gut microbiome; thus, a more thorough understanding of microbial-host-environmental interactions are required (Perry et al. 2020).

1.3 Manipulating teleost gut microbiome

As described in section 1.2.3, while present knowledge of gut microbiota in fish is still limited, it is known that microbiota may affect enzyme production (Ray et al. 2012), nutrient digestion and utilization (Semova et al. 2012, Falcinelli et al. 2015), and contribute to immune status (Ringø et al. 2016b). In vertebrates microbial communities and processes can be modulated by consumption of different foods, dietary ingredients (prebiotics and probiotics) and drugs, including, of course, antimicrobials (Jernberg et al. 2010, Grootaert et al. 2011, Versalovic 2013).

1.3.1 Diet

Due to the carnivorous nature of the Atlantic salmon, wild pelagic fish stocks are used as a marine protein source to feed farmed salmon. However, over the years, wild fish stocks have been depleted in marine ecosystems, making feeding farmed salmon on these ingredients unsustainable and cost-ineffective (Worm et al. 2006). The high demand of farmed salmon for marine ingredients underpins the negative environmental consequences of the industry worldwide. To address this issue farmed salmon feed composition has changed considerably during a relatively short history of intensive salmon farming in Norway, reducing the ratio of the marine origin components within salmon feed from around 90% in 1990 to 30% in 2013 (Ytrestøyl et al. 2015a). It was found that dietary changes do modify fish gut microbial community composition and activity, and, there have been several studies investigating the influence of alternative protein sources on the salmon microbiome (Ingerslev, Strube, et al. 2014).

Previously solvent-extracted soybean meal (SBM) was an attractive protein source used in Atlantic salmon aquaculture sector due to its' high protein content, desirable amino acids profile, and low cost (Booman et al. 2018). However, it was found that SBM diet does not only induce changes in the microbial population

within the gut of Atlantic salmon, but also alter intestinal health by affecting feed digestibility and intestinal immunity (Nayak 2010, Green et al. 2013, Booman et al. 2018). This SBM effect could be explained by the fact that the SBM diet contains antinutritional factors (factors that reduce the availability of nutrients) that alter the microbiome, lipid digestion, absorption and induce enteritis in Atlantic salmon (Refstie et al. 2005, Gu et al. 2014, Krogdahl, Gajardo, et al. 2015, Booman et al. 2018). Current alternative plant-based materials to SBM include soybean protein concentrate, wheat and wheat gluten, along with corn, faba beans, sunflower meal, pea protein concentrate, and other vegetable proteins, as well as feeds containing novel protein sources; e.g. from insects (Aas et al. 2019).

Similarly to marine sources being used as a protein source, for many years fish oils were the predominating lipid source for farmed salmon. However, even though a reduction has been seen in wild fish stocks use as a protein source, their use as a lipid source has drastically increased from 16% in 1988 to 81% in 2002 (Tacon et al. 2006). Thus, there is an increasing demand for sustainable alternative lipid sources to reduce the use of fish oil. One of the candidates is vegetable oils that are produced at a higher and cheaper rate than fish oils (Tacon et al. 2006). However, in Atlantic salmon, vegetable oils are known to alter not only gut microbiome composition but gastrointestinal morphology and lipid absorption as well (Ringø et al. 2002, Moldal et al. 2014, Hansen, Kortner, Denstadli, et al. 2020). Furthermore, n-3 fatty acids are essential for salmon and are not present in plant oil; thus, supplementation of these fatty acids is required for optimal growth in plant-based feeds (Rosenlund et al. 2016). It is worth noting that recently microalgae, which are rich in n-3 fatty acids, have been used as an effective replacement of fish oil in salmon feeds (Norambuena et al. 2015, Gong et al. 2020).

1.3.2 Probiotics

Probiotics are the live microbial strains that are incorporated into feed as dietary supplements and are thought to suppress the growth of pathogenic bacteria and improve gut health by producing antimicrobial compounds, although there is a considerable lack of scientific understanding of their mode of action in many cases (Dunne et al. 2001, Spinler et al. 2008, Scott et al. 2015). Whether guided by a clear evidence base or not, the use of probiotics has nonetheless become a popular

tool to support disease resilience to exert beneficial effects on fish health. Many of the probiotics used in Atlantic salmon farming are lactic acid bacteria that are thought to maintain gut health by producing lactic acid used as a food source for short-chain fatty acid (SCFAs)-producing bacteria (Gatesoupe 2007, Ringø et al. 2018). As discussed above these SCFAs contribute to hosts' health maintenance, such as butyrate that has anti-inflammatory effects and is used as an energy source by the gut epithelial cells (Louis et al. 2014). It was shown that feeding Atlantic salmon with probiotic *Lactobacillus,* modulated the composition and interaction of the microbial communities within the gut and increased the bacterial diversity in the intestinal mucus of the fish (Gupta et al. 2019). Similar results were demonstrated in Atlantic salmon transitioning from freshwater to seawater by supplementing their diet with probiotic *Pediococcus acidilactici MA18/5M* which affected not only the composition of the gut microbiome but also modulated antiviral response (Jaramillo-Torres et al. 2019). However, the full potential of probiotics, appropriate modes of treatment (oral, or in the water), doses, and the characterization of mechanisms of action of individual probiotic organisms, need to be explored in more detail (Quigley and Shanahan 2014, Jahangiri and Esteban 2018, Talwar et al. 2018, Butt and Volkoff 2019).

1.3.3 Prebiotics

Prebiotics are defined as food ingredients that are metabolised by host microbiota and used to induce the proliferation and activity of gut bacteria to improve the hosts' health (Hill et al. 2014). As discussed above, substituting a fish meal with feeds containing alternative protein sources do affect gut microbiome and health; thus prebiotics are usually used in combination with the alternative feeds to mitigate unwanted symptoms by acting in a protective manner on the gut epithelium (Ringø, Olsen, et al. 2010). Modulation of the intestinal environment can, in turn, shape the gut microbiota composition (Grisdale-Helland et al. 2008, Dimitroglou et al. 2011). For instance, it was found that inclusion of the prebiotic lecithin in Atlantic salmon diet supports lactic acid-producing bacteria (Pratoomyot et al. 2010). However, the same as with probiotics, while there are various prebiotics available commercially, the scientific understanding of mode of action is lagging behind (Martinez et al. 2015).

1.3.4 Antimicrobial compounds

Antimicrobial compounds are the pharmaceutical substances used to kill or inhibit the growth of microorganisms and include antibiotics, antivirals, antifungals, and antiprotozoals (Henriksson et al. 2018). In the past decade, antimicrobial compounds were increasingly used in agriculture and aquaculture to prevent and treat diseases as well as promoting animal growth (Van Boeckel et al. 2015). However, growing evidence indicates that these substances are linked to the increase in antimicrobial resistance in many farming sectors that does not only weaken the treatments against unwanted pathogens within the sector but also increase the risk of transferring antimicrobial resistance to humans (Henriksson et al. 2018). Due to this reason, over the years the usage of the antimicrobial compounds in Atlantic salmon farming in the Northern hemisphere has decreased. However, in Chile, the usage of these substances keeps increasing (Figure 1-5) (Henriksson et al. 2018). This extended and high use of the antibiotics florfenicol and oxytetracycline in Chilean salmon farms selects multiresistant bacteria in the *Salmo salar* guts (Higuera-Llantén et al. 2018).

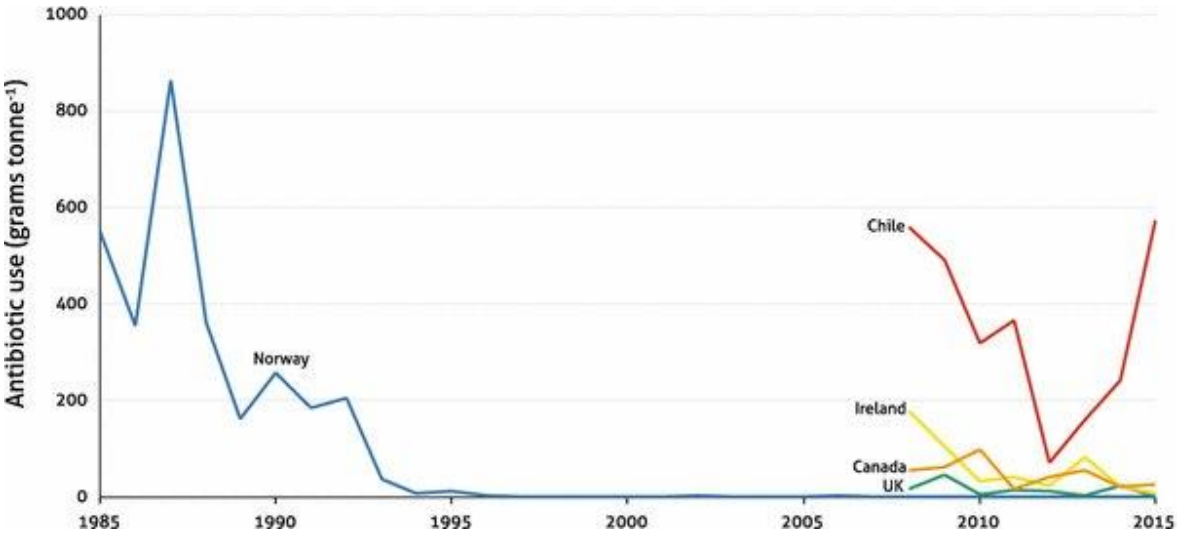


Figure 1-5 Antibiotic use in Atlantic salmon farming in the five top producing countries (Henriksson et al. 2018)**.** *Antibiotics in grams per ton in five top Atlantic salmon producing countries are shown from 1985 to 2015. Norway values represented in blue, Chile in red, Ireland in yellow, Canada in orange and UK in green.*

1.4 Modelling the GI tract using synthetic gut models

To study the impact of the gut microbial diversity and composition on the digestion of food or drugs, *in vivo* animal and human trials are standard approaches due to

their practicality and physiological relevance (Franklin and Ericsson 2017, King et al. 2019). However, such *in vivo* work has several disadvantages: ethical constraints (Ma et al. 2018); lack of easy access to the gut contents (in non-terminal and ethical way) (Sousa et al. 2008); and the restriction to only faecal samples that cannot provide information on the dynamic microbial communities and processes in different regions of the gut. Thus, *in vitro* gut model systems can be chosen as an alternative or complementary approach to *in vivo* studies. Despite not fully replicating the physiological host environment, they are still considered an excellent tool to mimic microbial activity and composition in different compartments in the gut (Pearce et al. 2018). This section will review the progress of current *in vitro* gut models, their advantages and disadvantages, and different methods that can be used to study the processes happening in the synthetic gut models.

1.4.1 Progress of synthetic gut models

1.4.1.1 Batch-type simulators

In vitro digestion models are commonly employed to examine the structural changes, digestibility, and release of dietary elements under simulated gastrointestinal conditions (Hur et al. 2011). One of the first examples of *in vitro* enzymatic digestion models was developed in 1995 by five European laboratories and aimed to analyse human colonic fermentation of different dietary sources, including cellulose, sugar beet and soybean fibres, maize bran and pectin (Barry et al. 1995). The *in vitro* model developed by Barry et al. (1995) are the most common and the most straightforward simulators to study the fermentative activity in the vertebrate colon. This single compartment fermentation model, also known as a batch-type simulator, is a closed anaerobic system replicating only a single gastrointestinal tract compartment (Figure 1- 6 A). These types of simulators are typically used to study the response of different substrates or their doses on the physiology and biodiversity of the defined single or mixed intestinal microorganisms (including faeces) and *vice versa*. For instance, the batch-type simulator was successfully applied to simulate human colonic microbiota by seeding the reactor with faecal samples to evaluate probiotics (Takagi et al. 2016). DNA-based molecular quantitative and qualitative techniques are used to evaluate the impact of different substrate compositions on the composition of

microbial communities. The effects of different substrates on the metabolic activity of gut microbes are often analysed by measuring the formation of various microbial metabolites, such as methane or short-chain fatty acids.

The major advantages of the batch-type simulators are that they are inexpensive to operate, easy to set-up, and allow a rapid turnaround and throughput of samples, making them a useful tool for the analysis of a wide variety of different substrates or faecal samples (Macfarlane and Macfarlane 2007). However, batch-type simulators are limited by characteristic substrate depletion as well as the accumulation of fermentation by-products over time that may alter or inhibit the metabolic processes of the organisms that produce them. Such limitations influence the internal reactor conditions, leading to the inability to maintain steady-state conditions, limiting their use to short-term testing.

1.4.1.2 Chemostat-type simulators

To eliminate problems associated with batch-type simulators, open systems, commonly referred to as single-stage chemostats, can be used. Although similar to batch-type simulators, single-stage chemostats are characterised by the possibility to further control environmental parameters by supplying fresh growth medium as well as removing waste (Figure 1- 6 B). These properties make chemostats more suitable for the prolonged maintenance of the steady-state conditions while replicating the environmental conditions characteristic of the selected gut compartment (Freter et al. 1983).

Dynamic multistage-fermentation models can be used to simulate the fermentative/metabolic activity of multiple gut compartments. The most common type of multistage-fermentation models is typically referred to as the Reading model. It is based on a design developed by Gibson *et al*. in 1988 to study human gut bacteria (Gibson et al. 1988). The original model consisted of three interconnected vessels having different operating volumes and pH, thereby allowing a more accurate simulation of the physiological conditions of different human gut compartments. An advanced derivative of the three-stage fermentation model is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) developed by Van de Wiele and colleagues at the University of Ghent (van de Wiele et al. 2015) (Figure 1- 6 C). The SHIME *in vitro* model aims to simulate

the human digestion system and provides information about the digestion of food and related changes in the human gut microbiota for a period ranging from 2 to 3 weeks. The SHIME model consists of five reactors (the first two simulating stomach and the small intestine and the last three mimicking three colon compartments). The growth media present in the vessels replicating the stomach and small intestine is enriched with pancreatic enzymes and bile allowing for a further degree of accuracy in simulating the physicochemical conditions of these compartments as well as allowing the system to operate for more extended periods of time when compared to a batch model. However, this type of system lacks characteristic host functions, such as immunomodulatory and neuroendocrine responses.

1.4.1.3 Gut models incorporating the mucosal compartment

Several recent studies have shown that a portion of the gut microorganisms adhere and colonize the mucosal layer lining different gut compartments. This distinct community has been termed mucosa-associated microbial community (MAMC) (Zoetendal et al. 2002, Macfarlane 2008). The MAMC are protected from disturbances in the lumen, such as the washout resulting from the peristaltic activity, leading to prolonged and stable colonization of certain tracts. Furthermore, the MAMC layer can act as 'shield' against pathogens, activating mucosal immune responses and physically blocking colonization of potentially harmful bacteria (Canny and McCormick 2008). The incorporation of the mucosal compartment in synthetic gut models represents a fundamental element needed for a more accurate simulation of gastrointestinal systems. In 2012, Van den Abbeele *et al*. incorporated the mucosal environment into the SHIME system, establishing the mucosal-SHIME (M-SHIME) allowing a portion of the microbiota present in the vessels to adhere to a gut mucus layer (Van den Abbeele et al. 2012). Incorporation of the mucosal environment to the model allowed the colonization and expansion of the microorganisms that are able to adhere to the mucous, such as *Lactobacillus mucosae* that contains a mucus binding domain (Nishiyama et al. 2016). M-SHIME also demonstrated that different bacteria colonize the luminal and mucosal environments, resulting in less than 60% similarity between those two environments, but similar within the luminal environment (≈90% similarity). The results provided by M-SHIME indicate the importance of the addition of mucus to the *in vitro* system to enhance the

simulated three-dimensional complexity and achieve broader bacterial diversity seen in *in vivo* environment.

1.4.1.4 Microfluidics-based *in vitro* models

One of the most recent advances in *in vitro* gut model systems was developed in 2012 and involved the development of a microfluidic approach termed gut-on-a-chip (Kim et al. 2012). This system comprises of a transparent, hollow-channelled microfluid device that is lined with intestinal cells and subjected to peristaltic motion. Even though this system is more complex, less established and less recognized, it offers the capacity to analyse the response of the core microorganisms of the intestinal tract to pathogenic and immune cells by adding live pathogenic microbes and immune cells (Kim et al. 2016). The system also allows the incorporation of other types of host cells, such as vascular and lymphatic endothelium or immune components, to replicate organ-level interaction in the lumen-capillary interface. The gut-on-a-chip system can therefore be leveraged to analyse the interconnection between the microbiome, human intestinal cells, and human immune components *in vitro*.

A similar microfluid-based *in vitro* system was developed in 2016 by Shah *et al.* and called HuMiX (Figure 1- 6 D). This system allows co-culturing of the human gut cells and microbial cells under conditions and processes mimicking gastrointestinal human-microbiome interface (Shah et al. 2016). The HuMiX system consists of three layers: a top layer containing a supply chamber, where nutrients flow continuously, a middle layer containing human gut cells and a bottom layer containing growing bacteria. This system allows the analysis of the interaction between host gut cells and the gut microbiome, including the impact of how different bacterial strains affect the metabolism of intestinal epithelial cells.

Recently a teleost gut-on-chip model was developed that reconstructs the intestinal barrier of the rainbow trout (*Oncorhynchus mykiss*) in an artificial microenvironment (Drieschner et al. 2019). This system includes a micro-well plate-based microfluid bioreactor that contains a culture of two intestinal cell lines from rainbow trout and electrodes used to sense the response of those cells to experimental conditions (for example, *in vivo*-like fluid share stress conditions - representing the stress caused by nutrients progressing throughout the gut

lumen). However, this system currently does not involve microbial communities isolated from the fish's gut.

1.4.1.5 Models allowing absorption

Arguably the most sophisticated synthetic gut model was developed by The Netherlands Organisation for Applied Scientific Research, known as TNO, more than 18 years ago referred to as the TNO computer-controlled, dynamic *in vitro* gastro-Intestinal Model of the colon, or TIM-2 (Minekus et al. 1999) (Figure 1- 6 E). TIM-2 offers the ability to reproduce the digestion of monogastric animals or humans in specific age ranges (from new-born to elderly) under physiological or diseased conditions (Roussel et al. 2016). This model has been successfully applied in many different fields, such as nutritional studies (aim to evaluate the digestibility of food) and pharmaceutical applications (analysing food-drug interaction and drug release, solubility and bioavailability and availability) (Blanquet-Diot et al. 2009, 2012, Déat et al. 2009, Shani-Levi et al. 2017).

The TIM-2 system has several advantages over other synthetic gut models discussed in previous sections. First, it consists of four connected glass jackets with silicon membranes inside that can be moved by applying pressure, leading to the peristaltic movement simulation. This type of movement causes the mixing of the luminal content through the entire system, unlike other systems that mix only by stirring. Secondly, TIM-2 is the only synthetic gut system that contains a dialysis system, which helps to maintain a physiological concentration of metabolites and prevents inhibition of the microbial growth by microbial metabolite accumulates (Lefebvre et al. 2015). Finally, TIM-2 is computer-controlled, making it highly reproducible and easier to regulate temperature and pH to mimic the environmental conditions in the proximal colon. However, the major drawbacks of this system include the ability to mimic only the proximal colon (stomach and three parts of the small intestine) and lack of mucus, epithelial or immune cells (Lefebvre et al. 2015).

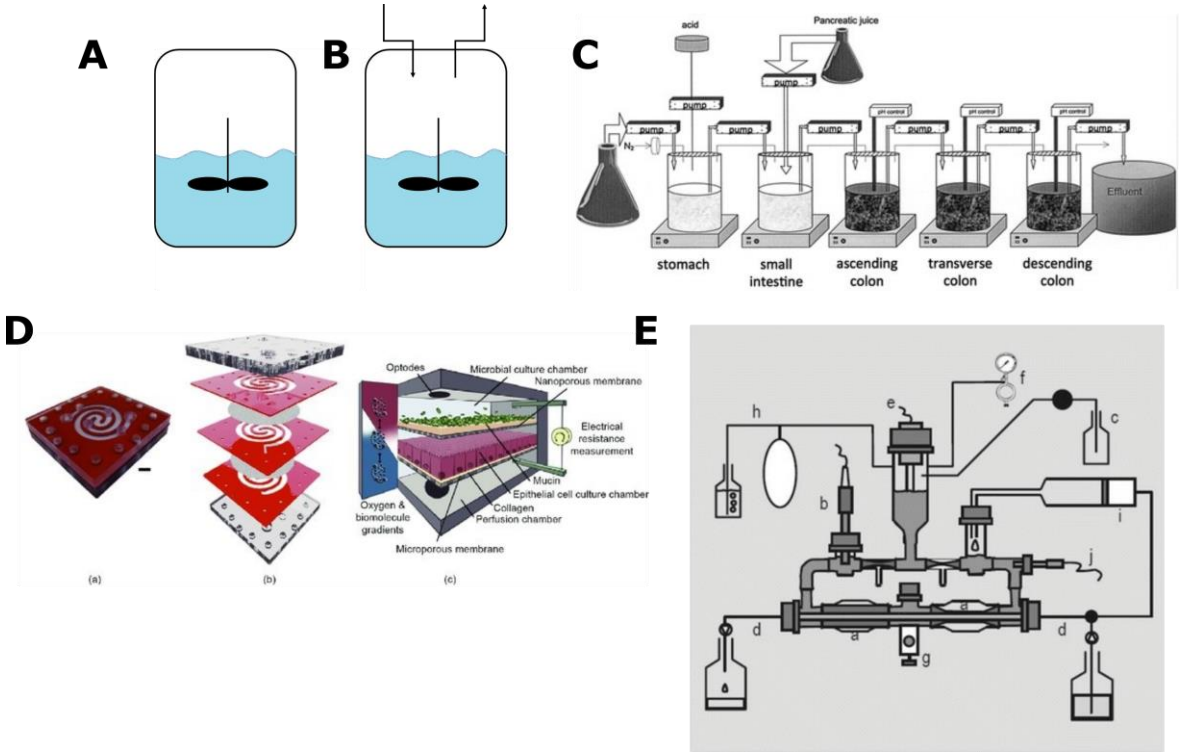


Figure 1- 6 Various types of *in vitro* simulators *Figure summarises various types of bioreactor systems:* ***A*** *Batch-type simulator;* ***B*** *Single-stage chemostat-type simulator;* ***C*** *Simulator of the Human Intestinal Microbial Ecosystem (SHIME)* (van de Wiele et al. 2015)*;* ***D*** *Microfluids-based in vitro system HuMix, where (a) assembled HuMiX platform; (b) exploded view of the HuMiX platform; (c) annotated schematic illustration of the key features in the HuMiX platform* (Shah et al. 2016)*;* ***E*** *Dynamic in vitro gastro-intestinal model of the colon TIM-2 where (a) peristaltic compartments containing faecal matter; (b) pH electrode; (c) alkali pump; (d) dialysis liquid circuit with hollow fibre membrane; (e) level sensor; (f) N2 gas inlet; (g) sampling port; (h) gas outlet; (i) 'ileal efflux' container; (j) temperature sensor* (Venema 2015)

1.4.2 Synthetic gut models as research tools to gut biology

Building models, both theoretical and practical, is a crucial step in understanding how a system operates and what the principal drivers of the system are (Godfrey-Smith 2006). *In vitro* gut systems can be used as a tool to study microbial communities in a controllable environment for mechanistic and molecular profiling without host cofounders. More advanced fermenters can be inoculated with microorganisms from different gut compartments to accurately replicate the microbial flora, such as in the SHIME model, allowing standardization of the system. Thus, these models provide results with high reproducibility and the ability to monitor microbiome composition not only in the lower intestine (van de Wiele et al. 2015). In order to improve the accuracy of the representation of the microbiome composition and lower variation between samples, tightly controlled sample handling and storage can be built into descriptive study design.

Furthermore, *in vitro* models enable collection of individual time-series data from multiple compartments to explore shifts in communities over time, identify interactions between microbial communities, and detect period signals (Coenen et al. 2020).

It is now well established that the gastrointestinal microbiome has an impact on animal physiology, both in health and disease (Kostic et al. 2013). For example, in humans, numerous links have been observed between different diseases or syndromes and altered gut microbiomes, thus understanding how the gut microbiome can be manipulated is an important task that can aid in the development of new treatments for some conditions (de Vos and De Vos 2012). The microbiota can be manipulated by different types of agents, including prebiotics, probiotics, faecal transplants, drugs, feed, and others. *In vitro* systems allow samples to be collected before the simulation of infection, disease, or the addition of a disturbing agent, allowing evaluation of a control (zero time point) before any perturbation of the system. Furthermore, these systems allow the study of different dosages of pathogens, toxic or radioactive compounds that might be toxic to animals, or introducing foreign bacteria that have unpredictable effects on gut microbiome composition without ethical constraints (Costa and Ahluwalia 2019).

Gut models can also be used to study different gut microbiome interactions. Firstly, as it was mentioned above, *in vitro* gut models allow easy access to samples that can be later analysed by fluorescent in situ hybridization. This experimental setup can highlight the interactions between different gut microbes, for instance, which microorganisms produce biofilms (Crowther et al. 2016). To analyse the host-microbiome interaction in real-time (as they communicate with each other), the HuMiX system can be used (Shah et al. 2016). This type of approach can help to study how some microorganisms, such as *Salmonella*, can survive and interact with host gut cells. Furthermore, the analysis of host-microbiome interaction can demonstrate how engineered commensal bacteria can be used to stimulate the intestinal cells to function as glucose-responsive insulin-producing beta cells (Duan et al. 2008). Moreover, it is known that the gut microbiome is responsible for several vital metabolic functions, such as fermentation of non-digestible substrates, production of vitamins, short-chain fatty acids and amino acids, and bile acid biotransformation (Putignani et al.

2015). Finally, host eukaryotic organisms have likely co-evolved with gut microbiota, leading to symbiosis between them (Backhed 2005). This symbiotic relationship has a beneficial effect on the gut epithelium by helping to maintain the physiological homeostasis of the intestinal mucosa (Holmes et al. 2011). Thus, understanding the metabolic output of the microorganisms in the gut can provide insight into the relationship between the host and gut microbiome. This can be achieved by performing a metabolomics analysis of the samples from bioreactor systems containing only gut microorganisms.

However, the lack of repeatability and reproducibility remain a major problem of current artificial gut models and are frequently criticised (Payne et al. 2012a). To overcome these problems, several steps have been taken. Firstly, stable and highly reproducible communities have to be developed to get similar results each run (Payne et al. 2012a). Secondly, to achieve within-run reproducibility, technical repeats must be run, and to achieve between-run repeatability the inoculum from the same donor has to be used. It was suggested that to achieve better biological repeatability the samples from different hosts should be pooled together, however, later this idea was rejected over concerns regarding inter-microbial interaction that could favour the growth of specific microorganisms, leading to unrepresentative host-microbiota (Rajilic-Stojanovic et al. 2010). Furthermore, the consecutive testing of all error sources (e.g. medium preparation) have to be performed to assure that microbial community composition is not affected by these factors while shifting from *in vivo* to *in vitro* systems. Lastly, it is important to measure the inter-individual differences by using alternative biological replicates to ensure that the differences or similarities between different samples are sufficient enough to allow conclusions to be made.

1.5 Techniques used while working with *in vitro* gut systems

1.5.1 Monitoring microbial composition

1.5.1.1 Phenotype-based culture-dependent methods

The main experimental goal of artificial gut models is to monitor how microbial community composition changes when exposed to different environmental inputs and biochemical stimuli (Verhoeckx et al. 2015a). Phenotype-based culture-

dependent approaches were used for tracking microbial diversity change in faecal or intestinal samples to different experimental conditions prior to the advent of tractable molecular technologies (Hiergeist et al. 2015). Commonly, faecal or intestinal samples at different time points or from different conditions were plated on selective (e.g. MacConkey agar, Phenylethyl alcohol) and non-selective (e.g. Peptone-yeast extra-glucose, Plate count agar) media, and then bacteria were classified according to the selective growth on plates containing different growth media (Finegold et al. 1974, Lagier et al. 2012). After isolation, the microbial community change in different microbial isolates was analysed by different phenotypic tests, such as fatty acid methyl ester (FAME) analysis that allows comparing different samples by producing a fatty acid profile (Wall et al. 2009). However, 40-90% of microorganisms cannot be isolated under laboratory conditions as culture-dependent methods cannot replicate the *in vivo* intestinal conditions, such as biochemical interactions between host cells and bacteria, and among the microbes themselves (Zoetendal et al. 2004, Nocker and Camper 2009).

1.5.1.2 Molecular methods

Although phenotype-based culture dependent-methods are useful approaches to study in-depth physiology of the isolated microorganisms, these type of methods are time-consuming and not reliable to distinguish microorganisms to species and strain level (Gong and Yang 2012). This problem was overcome by introducing molecular (novel) approaches that allow accurate identification of species of unknown isolate by sequence analysis of small subunit ribosomal ribonucleic acid ribosomal RNA (SSU rRNA). The method to analyse these sequences was first developed by Woese *et al*. (1987), which help to identify, classify, and establish an evolutionary relationship between different organisms. This was followed by the establishment of databases containing SSU rRNA sequences, such as GenBank and the ribosomal database project (Benson et al. 2014). By having these tools, new methods to analyse and quantify the microorganisms in the gut were developed.

1.5.1.3 PCR and associated techniques

Most molecular methods rely on amplification of target sequence (such as 16s rRNA amplicon) by using the polymerase chain reaction (PCR) to assess the occurrence

of particular taxa (by detecting presence or absence of the PCR product), or distinguish different taxa by analysing PCR product size (Gamper and Leuchtmann 2007, Rodrigues-Luiz et al. 2017). PCR alone cannot be reliably used to quantify the DNA present in the bulk sample as the only way in which it is possible to quantify the results is by measuring band intensity on the agarose gel that can only give semi-quantitative results. Thus, traditional PCR has advanced from detecting the end-product of the reaction to the ability to measure the kinetics of the reaction during the exponential phase of PCR, allowing to quantify the amount of DNA present in the sample. This type of approach, known as quantitative PCR (qPCR), requires primers that can target all bacterial phyla (to quantify total bacterial load) or specific taxon level (to quantify the presence of the specific group of microorganisms). Thus, by using these specific primers, it is possible to simultaneously detect and quantify the minor populations of bacteria within a large population (Postollec et al., 2011). The qPCR approach combined with next-generation sequencing was successfully applied to detect and quantify the composition of the microbiota in different gut sections of piglets with different diarrhoeic status to the phyla and taxonomically related subgroups level (Hermann-Bank et al. 2013). The qPCR approach offers a quick, quantitative, and sensitive approach to analyse gut microbial communities, however, it suffers from various limitations, which are described at the end of this section.

Fingerprinting is the technique which at the initial steps relies on amplification of the 16S gene by PCR and provides quick profiling of the diverse microbial community by visualising how many variants of a gene are present (assuming that each gene variant represents a different taxon in a microbial community). After the initial PCR, the products are then separated on polyacrylamide gel by applying temperature (TGGE) or chemical gradient (DGGE), leading to the separation of the sequences according to their thermal or chemical stability. The resulting 16S bands can then be eluted and analysed further by sequencing to identify which microorganism each band represents. Fingerprinting techniques include two major types: denaturing gradient gel electrophoresis (DGGE: used to analyse the quality of intestinal microbiota and monitor the progression of bacterial communities over time) and temperature gradient gel electrophoresis (TGGE: used to detect the most predominant bacterial flora) (Zoetendal et al. 1998, Favier et al. 2002). DGGE was successfully applied to analyse the microbial communities in a variety

of different samples, including human and animal intestines (Satokari et al. 2001, Kim et al. 2007). DGGE was used to examine the microbial diversity and evaluate the stability of the microbial communities in the *in vitro* gut model SHIME (Possemiers et al. 2004). The main advantages of this technique include the capability to semi-quantitatively monitor the bacterial populations (provide the information on the range of different bacterial strains and their relativity), conduct the rapid analysis of the samples, and ability to reuse samples for further analysis.

However, all PCR based techniques suffer from amplification bias (differences in the amplification efficiency of templates) and primer mismatch (Polz and Cavanaugh 1998, Acinas et al. 2005, Ruiz-Villalba et al. 2017). Furthermore, as it requires specific primers for each group of microorganisms or targets, it can only identify and quantify the known bacterial species or target sequence. Finally, general bacterial primers cannot identify all the components of the microbiome, such as viruses, archaea, and eukaryotes, thus requiring further analysis with specific primers targeting these microorganisms.

1.5.1.4 Flow cytometry

Monitoring changes in bacterial community composition in complex microbiota by using molecular techniques based on the amplification of DNA can prove tedious, time-consuming, and expensive. To overcome this problem, flow cytometry (FC) can be used. FC is a rapid technique allowing the qualitative and quantitative study of mixed populations of cells (Buranda et al. 2011, Mulroney et al. 2017). The technique can distinguish and count cell types in suspension based on the difference in light scattering or fluorescence emission properties resulting from variances in cell size and membrane granularity (De Roy et al. 2012). The technique's resolution to distinguish cell types is enhanced with the use of dyes or monoclonal antibodies binding cellular features such as surface-bound (membrane) or intracellular molecules and nuclear antigens thereby providing multiple distinguishing signals or traits (Adan et al. 2017).

Even though FC was initially employed in the study of eukaryotic cell populations and used in applications such as cell proliferation and cell cycle stage analysis, it was gradually adapted to the study of prokaryotic cells (Darzynkiewicz et al. 2011,

Wlodkowic and Darzynkiewicz 2011). It is now routinely used to determine the total cell counts, perform size measurements, determine nucleic acid content, establish cell viability and activity, and to detect specific bacterial groups or species (Hammes and Egli 2010). For example, De Roy *et al*. compared FC and DGGE to detect changes in microbial community composition of drinking water caused by changing environmental factors concluding that FC is a faster and more accurate approach to determine and monitor changes in microbial community fingerprints (De Roy et al. 2012). Notably, a fluorescence *in situ* hybridization-flow cytometry (FISH/FC)-based method was developed using traditional oligonucleotide probes targeting 16S rRNAs of type I and type II methanotrophs labelled with fluorescein or Alexa Fluor to detect and conduct enrichment experiments on type I and type II methanotroph populations from a natural sediment sample collected on the shore of Lake Washington (Kalyuzhnaya et al. 2006) – thus merging two approaches: specific DNA oligomer labelling and individual cell-based counting from microbes.

The automated (online) FC systems are also successfully used to analyse microbial community fingerprints in bioreactors. The simultaneous generation and interpretation of microbial community structure data can be used to detect early signs of contamination, or the growth of pro-biotic constituents. Furthermore, automated online FC systems are currently used in industrial settings requiring multiple successive fermentation processes, where a final product needs to meet specific quality standards, where fermenting microorganisms are responsible for the development of characteristic organoleptic properties of a product and to detect the presence of pathogenic or spoilage microorganisms (Caplice and Fitzgerald 1999, Sohier et al. 2014). For instance, in the dairy industry FC is employed to evaluate the quality of lactic acid bacteria starter cultures, raw materials, and the different stages of production processes, to monitor the fermentation processes and perform quality control during the production of beer, wine and drinking water as well as wastewater treatment (Ruszczynska et al. 2007, Manti et al. 2008, Guzzon and Larcher 2015, Liu et al. 2016, Zhao et al. 2017).

Flow cytometry is characterised by short analysis times, high accuracy, no need for DNA extraction and amplification and the availability of a wide range of differential dyes and labelling strategies (Vives-Rego et al. 2000, De Roy et al.

2012). Although automated FC setups capable of measuring 14 parameters simultaneously are currently available, data analysis remains challenging especially when trying to follow population composition shifts in complex microbial communities or subpopulations in turbid cultures varying in viability, activity, and physiological state (Hammes and Egli 2010, Wilkerson 2012). The increasing future applicability of FC to complex fermenter setups is highly dependent on the development of statistically robust and reliable automated algorithms for FC data analysis.

1.5.1.5 Biomarkers of microbial activity

The simplest and the most broadly used way to study microbial activity is by measuring gas production by microorganisms in the gut. For instance, this approach was applied in the *in vitro* batch-type system in which horse faecal inoculate was supplemented with different live yeast additives (Elghandour et al. 2016). In this study, the total fermentation gas, methane, and carbon dioxide produced was used as an indicator for the fermentative activity in the faecal samples. Another tool used to study microbial activity is the measurement of enzymes, such as ß-glycosidase (involved in the hydrolysis of plant polyphenol glycosides), ß-glucuronidase (responsible for cleavage of glucuronidated hepatic dietary metabolites), and various polysaccharide-degrading enzymes that are involved in the metabolism of different dietary and endogenous compounds (Cole et al. 1985). The major problem with both approaches is that they only focus on the overall microbial activity rather than analysing the contribution of the individual bacterial types. To overcome this limitation, a more modern molecular method, qPCR, can be used; this approach utilises gene-specific primers that can be used to target and quantify the microorganism capable to performing specific activities in the gut, such as butyrate production (Louis et al. 2010). However, this technique has some limitations; for example amplification biases, experimental variability, and variable primer specificity (Smith and Osborn 2009).

Other more recent tools for analysis of microbial activity involve the '-omics' approaches. Metagenomics, metaproteomics and metabolomics are some of the new meta-omics approaches used to study the modulation of the gut microbiota *in vivo*. However, advances in *in vitro* gut models allow the use of these types of approaches to study microbiota in artificial guts as well. The use of metagenomics

to get insights into the microbial and genetic composition of the host gut microbiota allows a more accurate replication of the host microbiome in *in vitro* systems, and the ability to track the microbial community changes due to the different treatments (Malla et al. 2019). However, even though this type of approach allows the ability to identify the coding capacity of the microbiota and the community structure, it does not allow the identification of the microbial functionality. This problem can be overcome by using several different techniques in both *in vivo* and *in vitro* systems: metatranscriptomics (allows identification of microbial functionality changes in gene expression), metaproteomics (allows identification of the "function" information by studying the proteins produced by different bacteria) and metabolomics (allows identification of the metabolites of interest) (Vernocchi et al. 2016, Petriz and Franco 2017). Combining these different approaches in *in vitro* gut models can make them very useful testbeds for investigation of the effects of environmental factors, such as diet and drugs, on the gut microbiota in a controlled setting in a host.

1.6 SalmoSim – an *in vitro* gut system for Atlantic salmon

While the aquaculture industry is expanding rapidly, our understanding of the factors that contribute to fish performance (such as optimal health and growth) is still very limited. Traditionally the impact of different food compositions of fish growth and performance are assessed using *in vivo* feed trials as a standard approach (Moldal et al. 2014, Ytrestøyl et al. 2015b, Egerton et al. 2020). These trials involve the use of thousands of fish, often fed suboptimal and untested formulations that can significantly impact fish welfare (Johansen et al. 2006, Weihe et al. 2018). Furthermore, frequent terminal sampling from the test cages leads to significant handling and associated stress (Humphrey 2007, Fast et al. 2008). In addition to ethical concerns around feed trials, cost and availability are a major barrier to bringing new feeds to market. While there are over 3000 salmon farms in Northern Europe, there are only c.10 marine test sites for trialling new ingredients (Clarke and Bostock 2011, Boyd et al. 2020).

In vitro gut model systems can be deployed as an alternative to feed trials, or a pre-screening tool to improve their efficiency and ethical propriety (Payne et al. 2012b, Kim et al. 2016). We have developed a synthetic, continuous salmon gut microbial fermentation system, called SalmoSim, simulating salmon gut

compartments representing the generalized marine lifecycle stage. The SalmoSim project aims to provide an *in vitro* platform to study the link between the gut microbiota and digestion in Atlantic salmon. Once the system is fully set up and running it can be used for many different applications, such as testing how food affects gut bacteria, predict how feed additives help with feed digestion, or used as a test bed for drug delivery.

The SalmoSim system can also deliver on all the "Three Rs" principles; to reduce harm to animals by replacing, reducing and refining animal studies (Russell and Burch 1959). SalmoSim can help with animal study Reduction by pe-screening different feed formulations or drug concentrations in SalmoSim prior to *in vivo* trials in order to minimise the number of experimental groups required in real salmon feed trials. SalmoSim can also help with animal study Refinement by conducting long term time course experiments by using samples only from a few fish rather than killing a lot of them at different time points. Finally, SalmoSim can help with relative Replacement of animal studies when testing different feeds and drugs, and it can also help with absolute Replacement when testing feed batch consistency (how each batch will affect fish). Finally, by using the SalmoSim system new diets absolutely replacing fish meal can be developed, leading to more a sustainable aquaculture sector, and fewer fish killed.

1.7 Thesis outline

The work conducted in this thesis acts as a precursor to the development and design of the artificial salmon gut system SalmoSim. The work aims to define and determine the key parameters required to accurately simulate physiochemical conditions of the Atlantic salmon gut to sustain gut microbial communities within the artificial environment, build the system, and develop methods to analyse data generated from SalmoSim runs. Further, the work aims to address some fundamental questions relating to the system, such as identify the variability between the runs, and validate the system against an *in vivo* feed trial. Finally, the system aims to be used to test an existing pre-biotic used in aquaculture setting to analyse its effects on the microbial communities within SalmoSim. All these aims are addressed in the following Chapters of the thesis:

1.7.1 Chapter 2 Developing and trialling the SalmoSim system to analyse microbial population dynamics.

The literature reveals the key physiochemical parameters within the Atlantic salmon gut. The Method Development section lays out the range of operational parameters selected for simulation of the Atlantic salmon in an *in vitro* simulator, and the reasoning behind the selections. This chapter also describes the preliminary SalmoSim replicate study performed, in which two SalmoSim runs, both inoculated with the same starting inocula, were performed side by side. This type of experiment allows testing of the variability (experimental error) of the experimental runs caused by the system itself and to determine the 'burn-in' period to enable microbial communities to acclimatise to the simulator environment. Furthermore, this preliminary run acted as a useful pilot to identify future pitfalls and improvements allowing the improvement of SalmoSim runs for future chapters.

1.7.2 Chapter 3 SalmoSim: the development of a three-compartment *in vitro* simulator of the Atlantic Salmon GI tract and associated microbial communities

Prior to deploying an *in vitro* gut microbiome simulator to perform biological experiments, it should first be validated against a parallel *in vivo* experiment, to establish the degree to which the results from the experimental protocol within the artificial gut are generalisable to the *in vivo* situation. Thus, this chapter describes the study performed to validate the system against an *in vivo* feed trial with the aim to see if bacterial community response to the change in feed within *in vitro* system is similar to the *in vivo* study.

1.7.3 Chapter 4 Deploying an *in vitro* gut model to assay the impact of a mannan-oligosaccharide prebiotic, Bio-Mos®, on the Atlantic salmon (*Salmo salar*) gut microbiome.

Mannose-oligosaccharide (MOS) pre-biotics are widely deployed in animal agriculture as immunomodulators as well as to enhance growth and gut health. Their mode of action is thought to be mediated through their impact on host microbial communities and the associated metabolism. This chapter presents a study in which Bio-Mos, a commercially available MOS pre-biotic, is assessed for potential use as a prebiotic growth promotor in salmonid aquaculture, using a

modified version of an established Atlantic salmon *in vitro* gut model, SalmoSim, and evaluates its impact on the host microbial communities.

1.7.4 Chapter 5 Contributions, Discussion and Conclusion

Main results and contributions in this thesis are summarised and are discussed in this chapter. Future work is proposed that might build upon the results conducted in this thesis. These future perspectives include adapting SalmoSim system for other fish species, introducing digestibility to the SalmoSim system, and applying the developed system to understand microbial resistance transfer.

Chapter 2 Developing and trialling the SalmoSim system: architecture and parameters to analyse microbial population dynamics.

2.1 Introduction

2.1.1 Importance of the gut microbiome

Animal guts contain dense, complex, and dynamic microbial communities that may not be merely a collection of passenger microorganisms, but rather active players in vertebrate immunity and physiology (McFall-Ngai et al. 2013, Murdoch and Rawls 2019). Microbes play many roles in the host's gut, including digestion and absorption of nutrients, energy harvest from non-digestible dietary components, the maintenance of intestinal homeostasis and preventing the propagation of pathogenic microbes by competing with them (Maslowski and MacKay 2011, Yu et al. 2012). It is also the case that microbial communities and processes can be modulated by consumption of different foods, dietary ingredients (prebiotics and probiotics) and drugs (Jernberg et al. 2010, Grootaert et al. 2011, Versalovic 2013).

The gut microbiota in fish has been shown to play a key role in health (van Kessel et al. 2011) and is dependent on fish species (Sullam et al. 2012, Li et al. 2014, Givens et al. 2015), life cycle stage (Giatsis et al. 2014, Ingerslev, von Gersdorff Jørgensen, et al. 2014, Zarkasi et al. 2014, 2016) and diet (Navarrete et al. 2013, Wong et al. 2013, Ingerslev, von Gersdorff Jørgensen, et al. 2014). It is believed that environmental sources have a much higher effect on aquatic vertebrate microbiomes compared to terrestrial vertebrates as aquatic vertebrates are in constant contact with the water (Austin 2006, Ringø, Løvmo, et al. 2010). However, a knowledge gap exists between determining the composition of the microbiome and understanding its function due to complex and variable ecology of teleost gastrointestinal tracts and unknown bacterial taxa, as well as complex interactions between the host, environment, and the gut microbiome (Nayak 2010, Perry et al. 2020).

2.1.2 SalmoSim – tool to study salmon microbiome

The aquaculture industry in Scotland is dominated by production of Atlantic salmon, which has increasing significance both in terms of economics and food sustainability. The continuing decline in wild Atlantic salmon stocks has resulted in a rising demand for farmed fish as well as constant increase in fish consumption worldwide (Costello et al. 2020). However, the expansion of the Salmonid aquaculture industry is unsustainable due to two reasons: the reliance on over-exploited wild fish stocks as the protein and lipid food source, andimpaired Salmonid gut health on alternative plant-based feed that is associated altering the microbiome (Krogdahl et al. 2003, Ytrestøyl et al. 2015a, Hemre et al. 2016). Thus, given that the fish microbiome is believed to play a pivotal role in regulating host immune system, health status and physiology (Nayak 2010, Romero et al. 2014, Egerton et al. 2018, Xiong et al. 2019) as well as an altered fish gut microbiota being associated with diseases (Tran et al. 2018, Wang et al. 2018, Rosado et al. 2019), the analysis of various feeds and feed additives on gut microbial communities is becoming more common (Bozzi et al. 2021).

To study the impact of the gut microbial diversity and composition on the digestion of the food or feed additives *in vivo* animal trials are usually performed. As described in Chapter 1 (Section 1.4.2) *in vitro* gut model systems offer an appealing alternative tool to mimic microbial activity and composition in different compartments in the gut without ethical constraints (Payne et al. 2012a, Verhoeckx et al. 2015b, Costa and Ahluwalia 2019). The advancement in technology of *in vitro* gut system simulators allows the study of complex gut microbial community composition and functionality in a simplified context, allowing well-controlled and repeatable conditions for the evaluation of microbial community response to various experimental treatments (Costa and Ahluwalia 2019). One of the important factors to consider while designing these systems is inter-individual variation in gut microbial communities. These variations are widely observed in human studies, which demonstrate more between-person variation than within-person variation, with adults having an average unique microbial signature that is largely stable over time (Costello et al. 2009, Stearns et al. 2011, Human Microbiome Project Consortium 2012, Huttenhower et al. 2012, Rajilić-Stojanović et al. 2013). This is also true in Atlantic salmon – our

previous work clearly shows high levels of interindividual variability in farmed (Heys et al. 2020a) and wild (Llewellyn et al. 2016) fish. It was reported, for example, that a single *Lactobacillales* OTU represented 96% of the microbiome of one individual Atlantic salmon which compared to a mean of only 3.5% relative abundance in the other fish from the same shoal in an aquaculture setting (Schmidt et al. 2016). Accounting for inter-individual variability is a key feature of any study that attempts to distinguish signal from noise in microbiome studies and adequate biological replication is vital in any experiment. This important consideration is overlooked by many well-established gut microbiome systems, such as the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), which uses inoculums from only one donor (Van Den Abbeele et al. 2010).

2.1.3 Requirements to sustain the gut microbial communities in an *in vitro* system

A bioreactor is a vessel in which an optimum external environment (such as dissolved oxygen concentration, pH, temperature, mixing) is provided and controlled for a biological reaction or change to take place (Wang and Zhong 2007). The *in vitro* reproduction of an accurate Atlantic salmon gastrointestinal microbiota is highly dependent on the physicochemical parameters that characterise the physiology of the three gut compartments to be simulated: the stomach, the pyloric caeca and the midgut (approximately 20 cm from the vent, which is suggested to be an analogue to the mammalian colon (Ng et al. 2005)). The choice of inoculum and the composition of growth media also represent key parameters in determining the development of a representative microbiota.

2.1.3.1 Physiochemical conditions

Temperature is an important factor in determining and shaping the composition and function of animal gut microbiomes (Sepulveda and Moeller 2020). The farmed salmon characterised and sampled for the study described in this chapter were collected from the MOWI aquaculture facility in Loch Linnhe. This Loch displays a relatively stable seasonal cycle with temperatures reaching a maximum of 14.5^{o}C in summer and dropping to a minimum of 6.6^{o}C in the autumn after a high flow event (Rabe and Hindson 2017). MOWI also provided the water temperature charts containing data for the aquaculture pens in Loch Linnhe where the salmon were

collected. The water temperature during the period in which the salmon were culled (August) had a daily fluctuation of 12-14^{o}C (Figure 2-1). Although teleost fish are considered poikilotherms, to understand whether the internal temperature of the stomach, pyloric caeca and midgut could be affected by microbial fermentation, the measurements of the internal temperature of the real salmon needs to be conducted.

Another important factor to consider while building an artificial gut system is pH as choosing the right pH for each of the bioreactors will not only provide a condition resembling the natural environment within the salmon gut, but also help to expose feed to the stomach's acidity and optimal environment for enzymatic activity (Beasley et al. 2015). Thus, measurements of the pH within different gut compartments needs to be conducted.

It is believed that the aerobic and facultative anaerobic microorganisms in the fish intestine are more abundant than obligate anaerobes (Nayak 2010, Navarrete et al. 2012, Llewellyn et al. 2014a). Thus, the measurements of dissolved oxygen concentration (mg/L) inside of each gut compartments needs to be performed to ensure that the established dissolved oxygen conditions within each simulated gut compartment are adequate to sustain microbial communities.

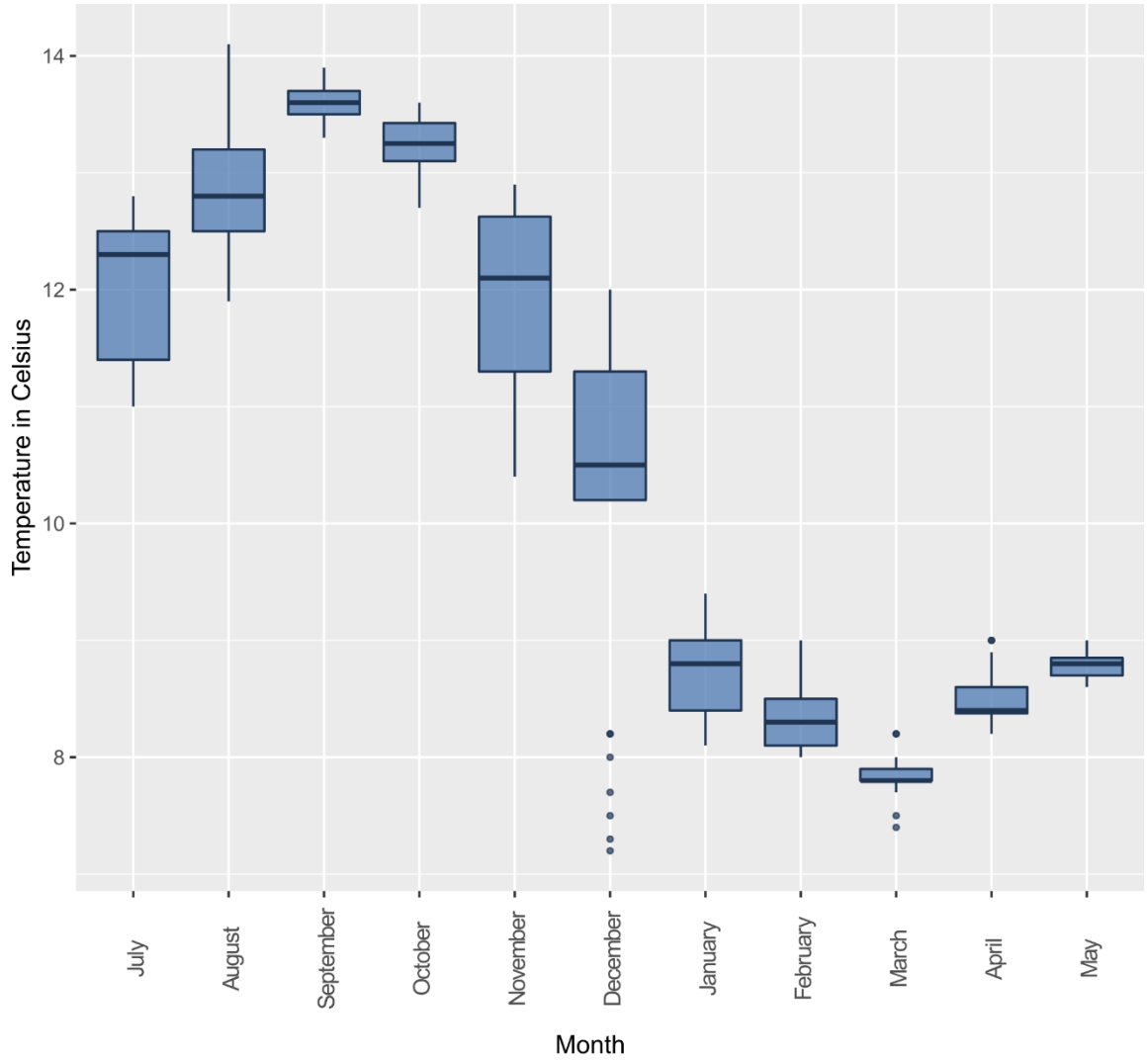


Figure 2-1 Water Temperature of Aquaculture Pens in Loch Linnhe Measured by MOWI. *Figure summarises daily water temperatures in the aquaculture pens where the salmon were collected from 01/07/2016 to 09/05/2017 provided by MOWI. The temperature measurements were taken almost every day.*

2.1.3.2 Media composition

The choice of media composition is a key factor in determining the resulting biodiversity, relative abundance, and population dynamics of the microbiota in the individual SalmoSim compartments. To avoid dehydration, salmon drink continuously in sea water, which they will swallow alongside their food bolus (Talbot et al. 1992). As such it is reasonable to assume that seawater and feed pellets should constitute the bulk of the media composition.

It is known that commensal microorganisms inhabiting fish guts can be split into two groups based on their attachment to the intestinal mucosa: autochthonous (attached to the intestinal mucosa) and allochthonous (do not adhere to the intestinal mucosa) (Llewellyn et al. 2014a, Givens et al. 2015, Gajardo et al. 2016, 2017a). The autochthonous microorganisms can, directly and indirectly, shape the teleost immune system (Chi et al. 2014, Mladineo et al. 2016). Autochthonous microorganisms were also shown to respond differently to changes in diet when compared to the allochthonous group, with mucosa-associated intestinal microbiota showing higher resilience to variations in the diet composition (Li et al. 2021). Furthermore, in the M-SHIME model of the human gut microbiome, mucin-enriched K1 media nodules were used as a surface to enable biofilm formation by the autochthonous microorganisms in the bioreactor (Van den Abbeele et al. 2012). Based on this knowledge, the mucins and surface for biofilm formation should be added to the system to ensure the retention of autochthonous microorganisms.

2.1.3.3 Bile acid supplementation

The primary function of bile acids (produced by the liver) in vertebrates is the solubilisation of dietary fats in the intestine, especially by phosphatidylcholine (predominant phospholipid present in bile at high concentration) that plays a pivotal role in the digestion of fats (Hofmann et al. 2010, Hansen, Kortner, Krasnov, et al. 2020). However, they also have additional functions that include cholesterol homeostasis, antimicrobial effects and endocrine signalling (Houten et al. 2006, Hofmann and Hagey 2008). Furthermore, insufficient dietary bile salt choline has been shown to lead to lipid malabsorption syndrome in the Atlantic salmon gut, which is characterised by a pale and foamy appearance of the enterocytes of the pyloric caeca as a result of lipid accumulation (Hansen, Kortner, Krasnov, et al. 2020). The research indicates a bile acid-gut microbiome axis, in which the host and microbiome appear to regulate bile acid pool size, which if reduced, is associated with bacterial overgrowth and inflammation, thus making bile an important part of the gut environment for microbiome formation (Ridlon et al. 2014). For instance, it was found that low levels of bile acid entering the intestine are linked to the bacterial dysbiosis observed in cirrhosis, suggesting that bile acids affect the structure of the microbiota in the human gut (Kakiyama

et al. 2013). Considering the importance of the bile salts, it should be added to the system simulating Atlantic salmon gut.

2.1.3.4 Flow rate

The final important parameter required to decide upon for the SalmoSim system setup is the flow rate in between the reactors, as it determines how fast the bacteria are transferred from one reactor vessel (representing a gut compartment) to the other. It is known that temperature is a key parameter in determining the gut evacuation time (the time needed to remove the feed from the stomach and gut completely), whichin adult salmon is at least 5-7 days long if fish are grown at 4ºC. This time is two times longer than fish grown at 13ºC (Waagbø et al. 2017). Thus, depending on the gut temperature of the Atlantic salmon, the flow rate can be calculated.

2.1.4 Aims and objectives

As covered in the previous section, an *in vitro* system, designed to study gut microbial communities, needs well-controlled and repeatable conditions, in order to sustain the microbial communities added to it. Motivated by this, the main aim of this chapter was to measure the physiochemical conditions (temperatures, pH, dissolved oxygen) within the real salmon gut compartments. As a preliminary test of the system, we also performed a trial SalmoSim run to assess the impacts of selected conditions and aims to explore three different objectives. Firstly, this study aims to identify **differences between different replicate runs within SalmoSim system**. Prior to accounting for inter-individual variation between microbiome samples in an experimental design (e.g. Chapters 3&4), the system needs to be set up and the potential for technical variation within the SalmoSim system must be addressed. Stochastic colonisation patterns in early life are thought to be an important driver of microbial community assembly in vertebrates (e.g. Furman et al., 2020; Martínez et al., 2018). Similarly, community assembly in SalmoSim may follow different routes despite a largely identical founding population, if, for example, nutritional niches are colonised in a different order between replicates (Pereira and Berry 2017). As such, this replicate run experiment aims to indicate if the SalmoSim system generates results which are robust, or noisy (Naegle et al. 2015). Secondly, this study aims to inspect **bacterial**

dynamics within SalmoSim system over time with respect to inoculum: Prior to deploying an *in vitro* gut microbiome simulator to test the effect of experimental procedure on the microbial communities within the simulator, steady-state microbial communities need to be established to ensure that results due to experimental treatments are not confounded with bacterial adaptation to an *in vitro* environment (Possemiers et al. 2004). Thus, this trial replicate run experiment aims to determine the time it takes for bacterial communities to reach steady-state conditions in our *in vitro* gut model. Finally, Firstly, this study aims to identify **differences or similarities between different microbial communities inhabiting the different gut compartments simulated in SalmoSim:** Previous attempts to map compositional differences between the microbial communities of salmon gut compartments indicate significant divergences (Gajardo et al. 2017b, Heys et al. 2020b). Thus, we aim to identify if any of these differences are observed within the SalmoSim system.

2.2 SalmoSim method development

2.2.1 Measuring physiochemical conditions in real salmon

To determine the physiochemical conditions (temperature, dissolved oxygen, pH) within three different gut compartments (stomach, pyloric caecum, and mid gut), five starved farmed adult Atlantic salmon from the MOWI aquaculture facility in Loch Linnhe were fished out and euthanized. Average temperature measurements were taken over two minutes (in 20 second intervals) in all of the fish (n=5 for stomach, n=3 for pyloric caeca, and n=3 for mid gut compartments – n=3 rather than n=5 was used for the latter two due to probe breakage). The pH was measured using a semi-solid pH probe in corresponding gut compartments (n=5 fish per gut compartment). Finally, average measurements of dissolved oxygen concentration (mg/L) inside of each gut compartment (n=3 fish per gut compartment – n=3 rather than n=5 was used due to probe breakage) was performed using a FireSting monitor and probe (PyroScience, Aechen, Germany) over two minutes (in 20 second intervals) in all the fish.

2.2.2 Performing trial replicate runs

In order to test running conditions in practice, three identical trial replicate reactor runs were planned to be performed, however due to system malfunction only two runs were completed successfully. Materials constraints at the Environmental Microbiology Laboratory, School of Engineering, necessitates that replicates within this three-compartment had to be undertaken in series. As such, two identical back-to-back SalmoSim runs were performed, using the inoculum sampled from a single adult Atlantic salmon from marine cages at an aquaculture farm site at Corran Ferry, near Fort William, Scotland, in autumn 2017. The SalmoSim system was fully cleaned up, autoclaved, and reassembled in-between runs.

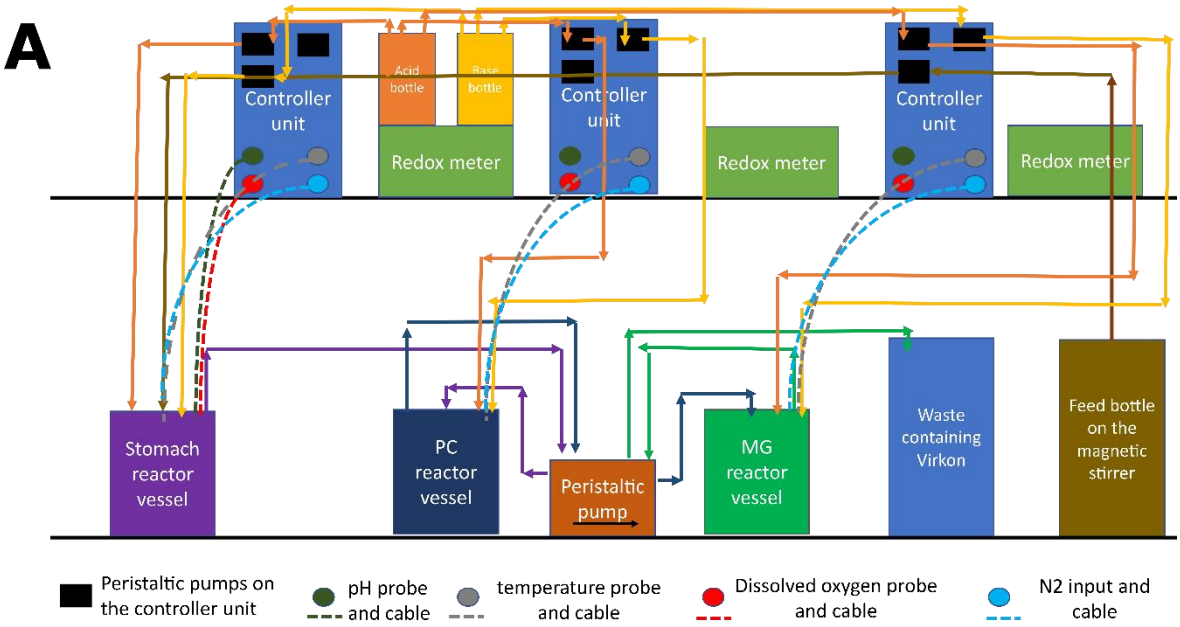
2.2.2.1 Media preparation

SalmoSim feed media was prepared by adding the following into a 2 litre Duran bottle; 35 g/L of Instant Ocean® Sea Salt (Loch Linnie salinity 35 ppt as reported by Rabe and Hindson, 2017), 10 g/L of the Fish meal feed (Table 3-1) used in MOWI feed trial in Averøy, Norway, ground using a coffee grinder (150W Andrew James Electric Coffee Grinder) and 2 L of deionised water. Furthermore, 1 g/L freeze-dried mucous was added, which was collected by squeezing from the pyloric caecum compartments of multiple adult starved Atlantic salmon at the marine cages at an aquaculture farm site at Corran Ferry (different individuals from SalmoSim inoculum). This mucous was processed in a vacuum freeze-drier overnight to produce a powdery mixture (contains mucous, pancreatic secretions, mucous, bile, blood, epithelial cells, bacteria, etc.) that was stored at -20^{o}C. This media was then autoclaved, followed by sieving of the bulky flocculates, and finally subjected to a second round of autoclaving. Once the feed bottle had cooled, the feed bottle cap was exchanged inside the laminar cabinet to keep feed media sterile.

2.2.2.2 SalmoSim system preparation

Three Applikon MiniBio 500 reactors were filled with four cubes (dimension: 1 cm x 1 cm x 1 cm made from aquarium sponge filters (Liannmarketing Aquarium Biochemical Cotton Filter Foam Fish Tank Sponge)) used as a surface for biofilm

formation. The Bioreactors were then assembled by attaching appropriate tubing and probes (pH (8 mm classic pH sensor), temperature (Pt-100 sensor), and dissolved oxygen (8 mm classic polarographic DO_2 sensor)), and autoclaved. Autoclaving was followed by attaching reactor vessels to the stands and MyControl system, connecting feed, acid and base bottles, and filling the reactors with 400 ml of prepared feed media. The SalmoSim reactors were connected in series running anaerobically, representing three Atlantic salmon gut compartments: stomach, pyloric caeca, and mid gut (Figure 2-2). Once the system was set up, it was left to run for about 24 hours to adjust the temperature, pH, and oxygen concentration (all physiochemical conditions were adjusted to represent values measured in real salmon in section 2.2.1) without the feed transfer.



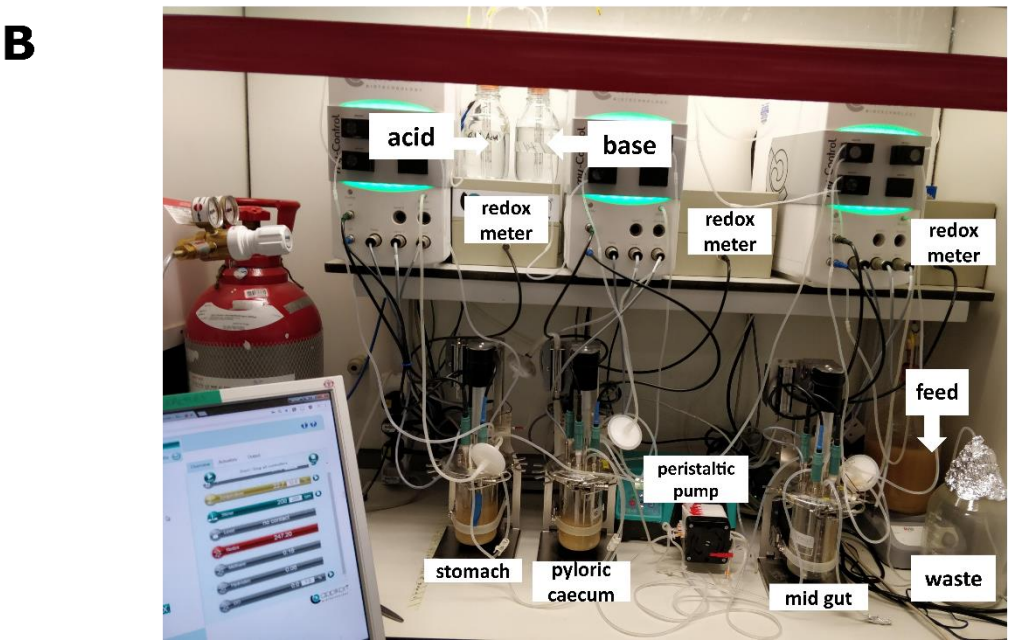


Figure 2-2 SalmoSim system set up using Applikon MiniBio 500. *Figure illustrates: A schematic representation of SalmoSim; B photograph of constructed SalmoSim system with labels.*

2.2.2.3 SalmoSim inoculation

A single adult starved farmed Atlantic salmon (3 to 5 kg) from a marine cage at an aquaculture farm site at Corran Ferry, near Fort William, Scotland, in autumn 2017 was dissected using aseptic technique aerobically, and samples of gut content from three different gut compartments (stomach, pyloric caecum, mid-intestine) were opened, scraped, and collected into 15 ml falcon tubes containing 15% glycerol, following long term storage in a -80°C freezer. The SalmoSim system was

inoculated using a 1 ml homogenate of the scrapings of the corresponding individual compartments, suspended in 1 ml of 35 g/L Instant Ocean® Sea Salt solution.

2.2.2.4 Initial pre-growth

To establish the inoculum for the different trial replicate reactor runs, the microbial communities within inoculum from real salmon were pre-grown inside the SalmoSim system for four days without any media transfer (to increase the quantity/concentration of bacterial populations, prevent sampling bias, and reduce the likelihood of 'washout'). After this period, three 50 ml falcon tubes for each reactor vessel (nine tubes in total), were filled with 30 ml of the contents from each bioreactor and centrifuged for 10 minutes at 5000 rpm. Centrifugation was followed by the removal of the supernatant from each falcon tube, snap freeze of the pellet in liquid nitrogen for 5 minutes, and long-term storage in a -80^{o}C freezer.

2.2.2.5 Trial replicate runs

Each technical replicate the experiment was inoculated with the 30 ml pre-grown inocula (described in section 2.2.2.4) and run continuously for a 20-day period. During this experiment the reactor vessels were maintained at the temperature determined in section 2.2.1, controlled using an electrical cooling jacket via bioreactor wall; dissolved oxygen content was kept at the concentration determined in section 2.2.1 via a 20-minute daily purge with nitrogen gas, and pH was kept stable in each bioreactor by the addition of 0.01 M NaOH and 0.01 M HCl (specific for each bioreactor representing different gut compartments, as determined in section 2.2.1). Temperature, dissolved oxygen, and pH were all continuously monitored using Applikon probes and the bioreactor content was mixed constantly at 300 rpm using marine type impellers. The transfer rate of slurry in between reactor vessels during the experiment was 238 ml per day. Finally, every day 0.5 ml of filtered bile and 1 ml of autoclaved 5% mucous solution were added into the reactor, simulating pyloric caecum compartment (different batches of bile and mucous were used for different runs).

2.2.2.6 Sampling for DNA-based analyses

Samples from initial inoculums (before pre-growth) were collected to provide the baseline: one sample per gut compartment. Once the SalmoSim main experiment was started, sampling from each bioreactor vessel was performed every second day throughout the 20-day run period (10 samplings in total). The SalmoSim samplings were achieved by collecting 30 ml of the bioreactor contents (less than 10% of the total bioreactor volume - the maximum volume of sampling without disturbing microbial community structure (Obom et al. 2013)) into 50 ml falcon tubes, centrifuging them for 10 minutes at 5000 rpm speed, and freezing the pellets in a -20^{o}C freezer.

2.2.3 Measuring bacterial population dynamics

2.2.3.1 Genomic DNA extraction

Pellets of all samples, stored in -20^{o}C after each sampling point were defrosted on ice and combined with 1/4" Ceramic Sphere and Lysing Matrix A Bulk (Garnet), and vortexed for 10 minutes (including the initial inoculums). Later, the protocol in the "QIAamp® DNA Stool Handbook June 2012" was followed from step 4 (Qiagen, UK), finishing in DNA elution in 200 µl of the elution buffer.

2.2.3.2 NGS library preparation

The diluted DNA samples were amplified by PCR with 27F and 338R primers targeted at the V1 region of the 16S rRNA gene for 20 cycles at 55^{o}C annealing temperature (McGovern et al. 2018) in triplicates to correct to PCR bias (Polz and Cavanaugh 1998). After the triplicate reactions were pooled into one, their concentration was measured by using Qubit® (Life Technologies, 2015), and all of them were diluted to 5 ng/µl by using microbial-free water. After, barcoding PCR was performed at 60^{o}C annealing temperature for eight cycles. The DNA clean-up followed this using magnetic beads according to the "Agencourt AMPure XP PCR Purification" protocol (Beckman Coulter, 2016). The cleaned-up DNA was eluted in 40 µl of the elution buffer and then quantified by using Qubit® (Life Technologies, 2015). All the PCR products were diluted to 10 nM concentration, pooled together, and sent for HiSeq 2500 sequencing carried out by Novogene (Beijing, China) .

2.2.4 Analysis of NGS data using bioinformatics

NGS data was analysed as described in Chapter 3 (Kazlauskaite et al. 2020). In short, two alpha diversity metrics, effective microbial richness and effective Shannon diversity, were calculated by using Rhea pipeline (Lagkouvardos et al. 2017a) and visualised by using microbiomeSeq package based on phyloseq package (McMurdie and Holmes 2013, Ssekagiri et al. 2017).

Principle Coordinates Analysis (PCoA) was performed by using microbiomeSeq package based on phyloseq package (Love et al. 2017, Ssekagiri et al. 2017) with Bray-Curtis dissimilarity measures calculated by using the vegdist() function from the vegan v2.4-2 package (Oksanen et al. 2013). Microbial composition (25 most common genera) amongst different SalmoSim runs and compartments over replicate experiments were determined using the phyloseq package and plotted in bar plots using the ggplot2 package.

These datasets were then used to compute ecological distances (a measure of distance between OTUs based on different dimensions determined by the method) by using Bray-Curtis (based on abundance or read count data) and Jaccards (based on presence/absence of OTUs between samples) methods using the vegdist() function from the vegan v2.4-2 package (Oksanen et al. 2013), and phylogenetical distances were computed for each dataset using the GUniFrac() function (generalised UniFrac) from the Rhea package at 0% (unweighted), 50% (balanced) and 100% (weighted) weights within the phylogenetic tree (weight identifies the level of abundance of each OTU taken into account) (Lagkouvardos et al. 2017a). Finally, a permutational multivariate analysis of variance (PERMANOVA) using calculated ecological and phylogenetic distances was performed to determine if the separation of selected groups is significant as a whole and in pairs (Anderson 2001).

2.3 Results

2.3.1 Physiochemical conditions within the real salmon gut

The results suggested that the internal temperature of all measured gut compartments (stomach, pyloric caeca, and midgut) in the real adult salmon was

approximately 12ºC (Figure 2-3), and dissolved oxygen inside all gut compartments was anaerobic (dissolved oxygen concentration less than 0.05 mg/L) (Figure 2-4). The measured pH in each gut compartment is summarised in Figure 2-5, which indicates that the stomach had an acidic environment (pH 4.0), the mid intestine had a slightly basic environment (pH 7.6) whereas the pyloric caecum compartment displayed a neutral pH (pH 7.0).

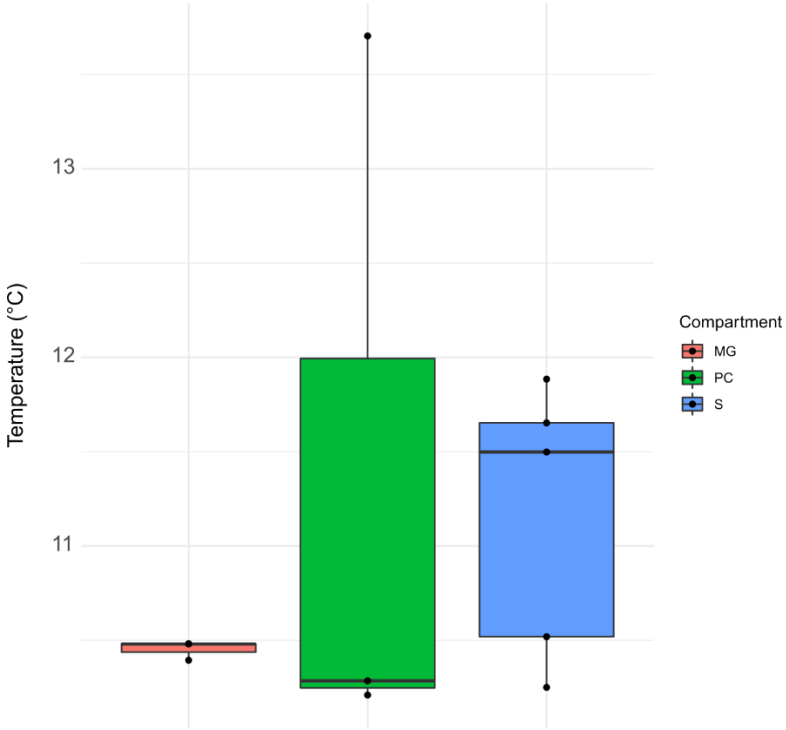


Figure 2-3 Measured Temperature of separate gut compartments within real Atlantic salmon. *Figure summarises the measured temperatures of the distinct gut compartments: stomach (n=5), pyloric caeca (n=3), and midgut (n=3). The average temperature measurements were taken over two minutes every 20 seconds in all of the fish.*

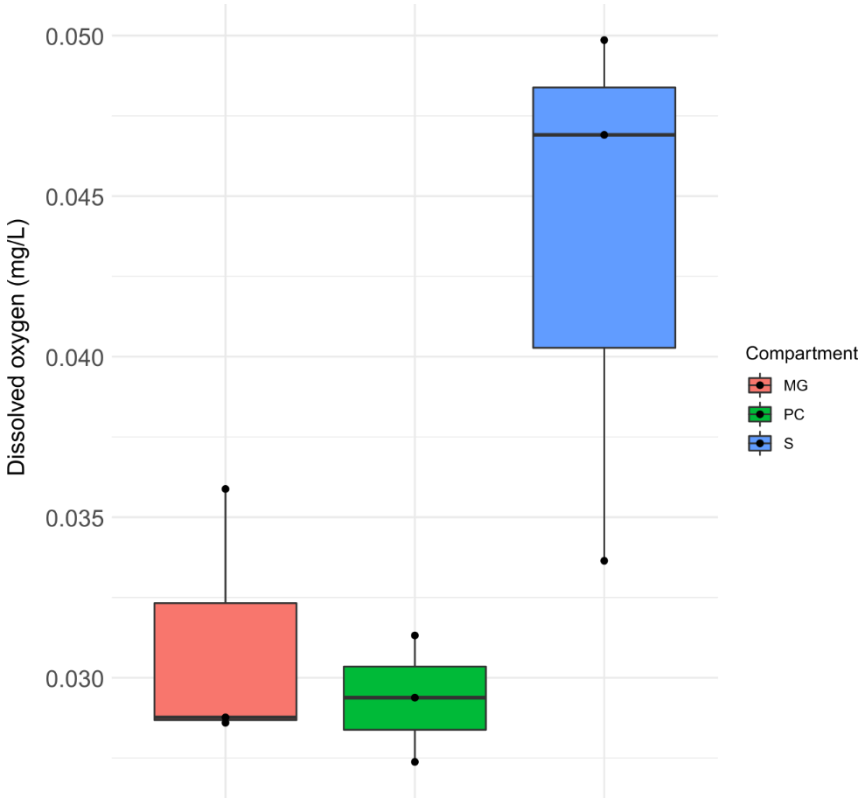


Figure 2-4 Measured Dissolved Oxygen of separate gut compartments within real Atlantic salmon. *Figure summarises the measured dissolved oxygen of the distinct gut compartments. The average dissolved oxygen for all gut compartments was measured in three fish. The dissolved oxygen measurements were taken over two minutes (in 20 second intervals) in all the fish.*

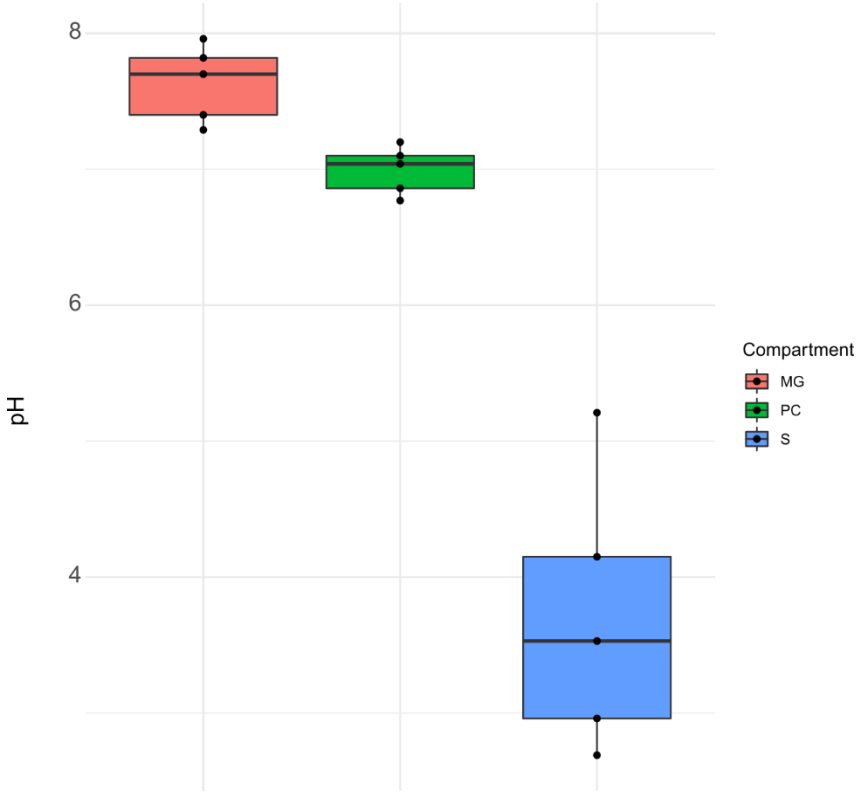


Figure 2-5 Measured pH of separate gut compartments within real Atlantic salmon. *Figure summarises the measured pH of the distinct gut compartments in 5 different fish.*

2.3.2 Differences between replicate runs within the SalmoSim system

A PCoA plot (Figure 2-6) visualising bacterial communities within different gut compartments (stomach, pyloric caeca, and midgut) and samples (replicate runs and inoculums) indicates that the major driver for microbial differences is the SalmoSim gut compartment (stomach being the most dissimilar from the other gut compartments) and not the replicate run (indicating similar bacterial composition patterns within the same SalmoSim gut compartment). This was visually confirmed by the taxa plot in Figure 2-7, whichshows that inter-gut compartment differences are more visually observable than between the two replicate runs. However, the alpha diversity metrics indicated that the second replicate run (run 2) showed a visually higher number of OTUs and bacterial diversity compared to the first replicate run (run 1) in all three different SalmoSim compartments (Figure 2-8). Furthermore, over the experimental run, both alpha diversity metrics increase in the pyloric caecum compartment within both runs, however, a decrease in these metrics is seen in the mid gut compartment within run 1, and no change within run 2. Finally, within the stomach compartment the effective richness increases in both runs over the course of the experiment (more gradual increase in run 1), while the effective Shannon diversity increases in run 1 and decreases in run 2. Finally, Table 2-1 indicates that when using the full dataset (data from days 2-20) in total 254 OTUs were shared between both runs. These OTUs account for >99.9% of the total reads in both runs. Furthermore, Table 2-1 also indicates that when using the data from only the last three time points (days 16-20 – once communities had over 2 weeks to adapt to the SalmoSim system), 128 OTUs were found to be shared between both runs, which account for >99% of the total reads in both runs. These results suggest that any differences between the runs are driven by rare OTUs accounting for less than 1% of total reads in both SalmoSim replicate runs.

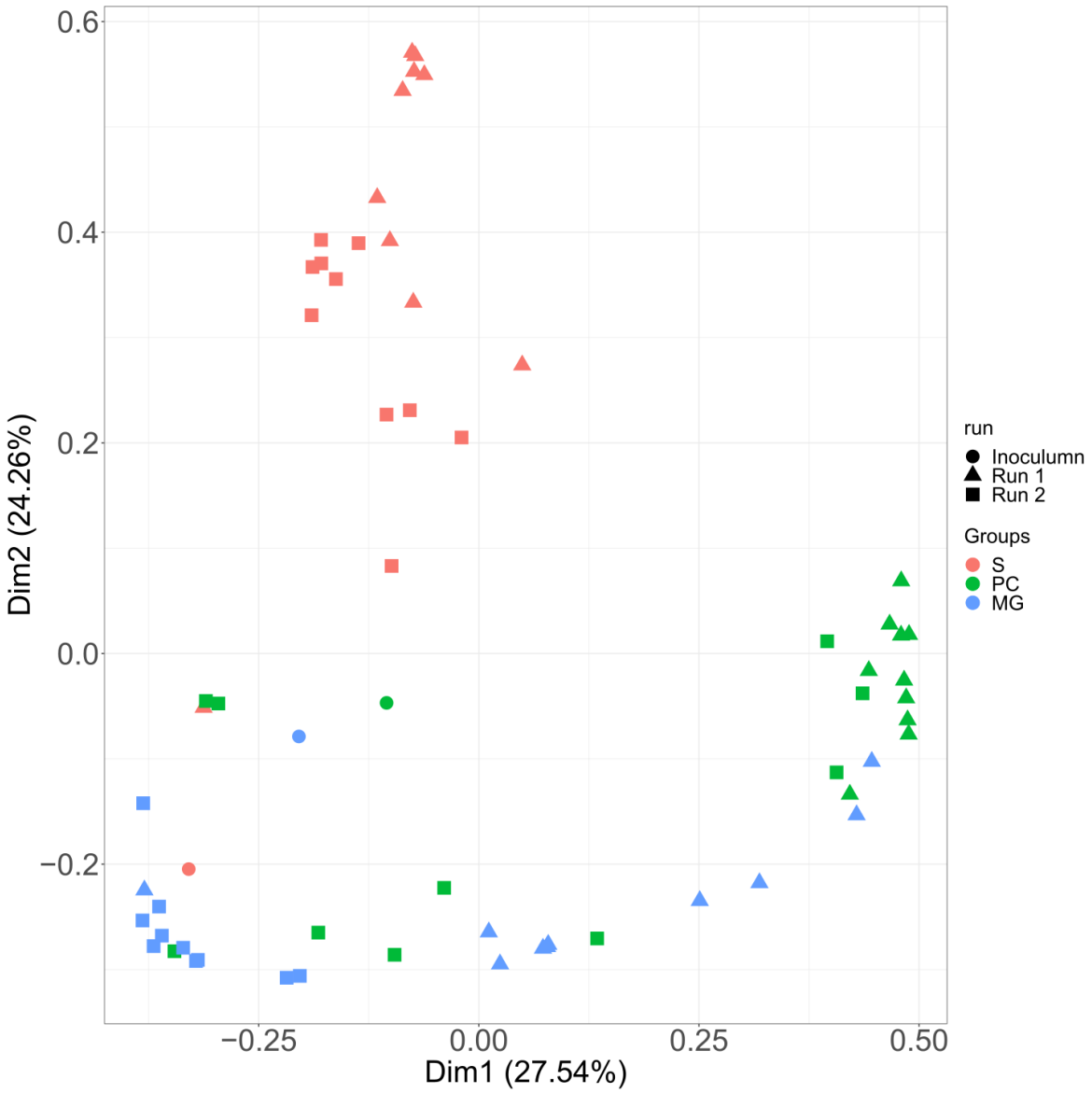


Figure 2-6 PCoA plot visualising bacterial communities within different gut compartments (stomach, pyloric caeca, and midgut) and samples (replicate runs and inoculums). *Figure visualises principal-coordinate analysis (PCoA) plot for Bray-Curtis dissimilarity measures for different samples (different shapes: Inoculum, replicate runs 1 and 2) and different SalmoSim gut compartments (different colours: S: stomach (red), PC: pyloric caeca (green), MG: midgut (blue)). Dim 1 is principal coordinate 1 and Dim 2 is principle coordinate 2.*

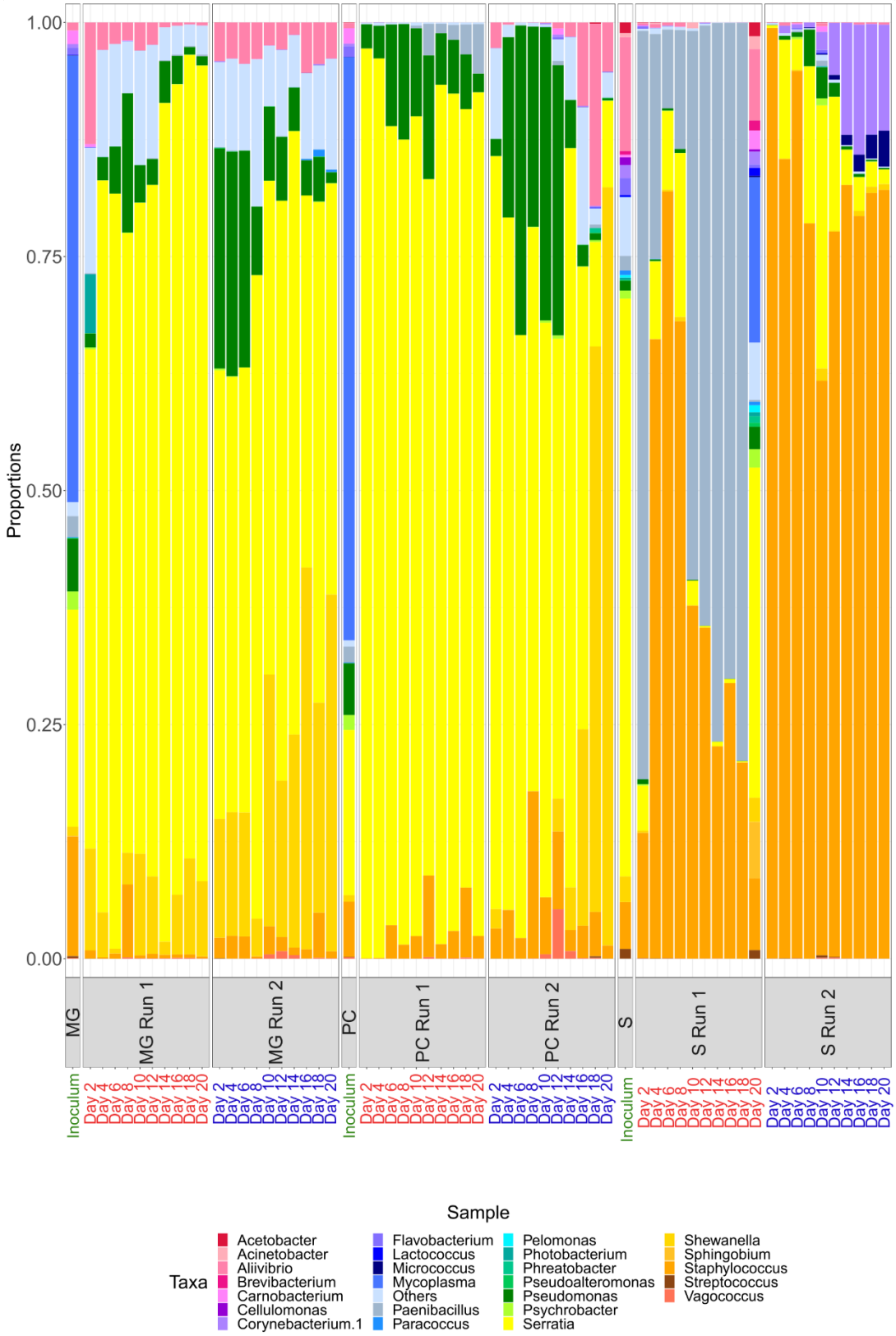


Figure 2-7 Microbial composition (25 most common genus) amongst different SalmoSim runs and compartments over replicate experiments. *Figure visualises microbial composition within 3 different SalmoSim and real salmon (Inoculum) compartments over time in 2 different replicate runs. The different sample types are represented by the labels on the x-axis: MG – midgut, PC – pyloric caeca and S – stomach. Labels in green represent initial inoculum from the real salmon, labels in red and blue - samples from replicate runs 1 and 2, respectively.*

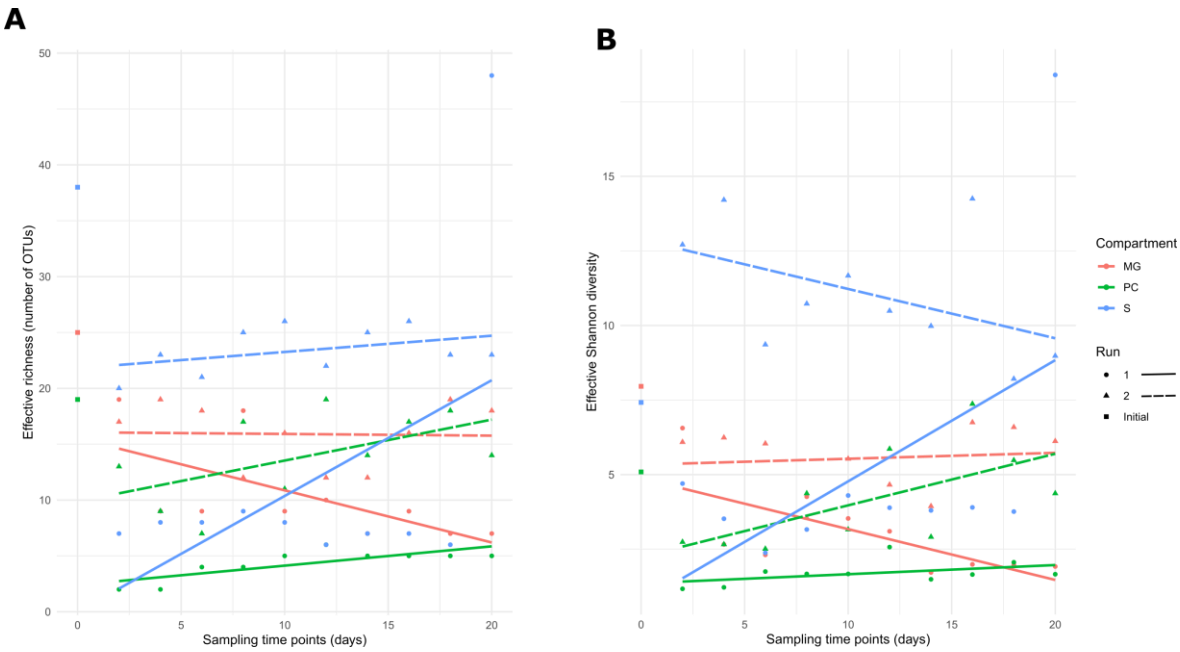


Figure 2-8 Calculated alpha-diversity metrics within SalmoSim system over time in different gut compartments and SalmoSim replicate runs. *The figure represents different alpha diversity outputs at different sampling time points (days) from SalmoSim system at different SalmoSim gut compartments (stomach (S) – blue, pyloric caecum (PC) – green, mid gut (MG) – red) and different replicate SalmoSim runs (circle/solid line – first SalmoSim run, triangle/dashed line – second SalmoSim run). Time point 0 represents microbial community composition within initial SalmoSim inoculum from the real salmon, time points 2-20 identifies samples from SalmoSim system during replicate experiment.* ***A*** *represents effective richness (number of OTUs), and* ***B*** *represents effective Shannon diversity.*

Table 2-1 Shared number of OTUs and their corresponding proportion of total reads between two SalmoSim replicate runs in all time points and final time points alone. *Table compares number of samples, OTUs, reads for each SalmoSim replicate run, as well as number of shared OTUs and their reads within each SalmoSim replicate run within two datasets: full dataset (containing all sampling time points: days 2-20) and final timepoints dataset (containing data from time points 16-20). It also summarises what percentage of a given group of samples' total reads came from the shared OTUs (OTUs present in both runs).*

	Full dataset		Final timepoints dataset	
	Run 1	**Run 2**	**Run 1**	**Run 2**
Number of OTUs	370	342	232	179
Number of reads	3,348,092	1,586,200	998,581	221,938
Number of shared OTUs	254	254	128	128
Shared reads	3,346,090	1,584,990	992422	220891
% shared reads	99.94%	99.92%	99.38%	99.53%

2.3.3 Bacterial dynamics within the SalmoSim system over time

Figure 2-9 indicates that effective richness (number of OTUs) and effective Shannon diversity remained largely static throughout each replicate run regardless of gut compartment, with no observed visual pattern. However, Figure 2-9 indicates that in the majority of cases (apart from Effective Shannon diversity in stomach compartment) the effective richness and Shannon diversity dropped after inoculum introduction (time point 0) to the SalmoSim system. This was visually confirmed by taxa plots in Figure 2-7 that identifies loss of taxa from inoculum to SalmoSim system, such as *Mycoplasma* in the pyloric caeca and mid gut compartments.

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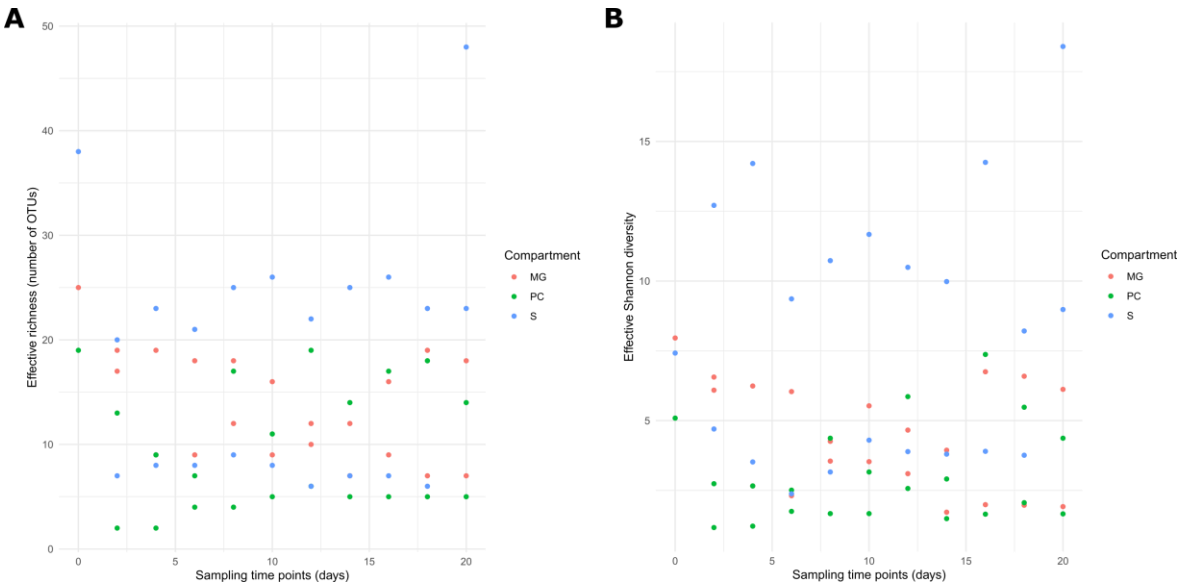

Figure 2-9 Calculated alpha-diversity metrics in different SalmoSim compartments over time. ***A*** *represents effective richness (number of OTUs), and* ***B*** *represents effective Shannon diversity. S: stomach (blue), PC: pyloric caeca (green), MG: midgut (red). Time point 0 represents microbial community composition within the initial SalmoSim inoculum from the real salmon, time points 2-20 identify samples from the SalmoSim system during replicate experiments.* ***A*** *represents effective richness (number of OTUs), and* ***B*** *represents effective Shannon diversity.*

2.3.4 Comparison between different SalmoSim compartments

Figure 2-8 indicates that in both SalmoSim replicate runs the effective richness and effect Shannon diversity was higher within the stomach compartment

compared to pyloric caeca and mid gut compartments, especially at the later time points. Figure 2-7 also indicates that in both runs the pyloric caecum and mid gut compartments were dominated by *Serratia*, but the stomach compartment in the first and second SalmoSim replicate runs were dominated by *Paenibacillus* and *Vagococcus* genera, respectively. Beta diversity analysis using both ecological and phylogenetic distances identified statistically significant differences between all three SalmoSim compartments using the full dataset (Table 2-2). However, once the final dataset (containing only stable time points: once bacterial communities had over two weeks to adapt and grow within the SalmoSim system) was used for the analysis, the difference between pyloric caeca and midgut compartments were not statistically different in both ecological and phylogenetic terms, but the statistical difference between stomach and the rest of gut compartments remained (Table 2-2).

Table 2-2 Beta diversity comparisons of microbial composition between different SalmoSim compartment. *The table summarises different beta-diversity analysis outputs calculated by using different distances: phylogenetic (unweighted (0%), balanced (50%) and weighted (100%) UniFrac) and ecological (Bray-Curtis and Jaccard's), between different SalmoSim compartments. The analysis was performed for 2 different datasets:* ***Full*** *that contained all the data collected, and* ***Final*** *– containing data only from last time points 16, 18 and 20. A permutational multivariate analysis of variance (PERMANOVA) by using phylogenetic and ecological distances was performed to determine if the separation of selected groups is significant as a whole and in pairs. Numbers represent p-values, with p-values <0.05 identifying statistically significant differences between compared groups.*

Test		Dataset	S vs PC	S vs MG	PC vs MG
UniFrac	unweighted (0%)	Full	0.001	0.001	0.001
		Final	0.012	0.002	0.086
	balanced (50%)	Full	0.001	0.001	0.001
		Final	0.006	0.001	0.219
	weighted (100%)	Full	0.001	0.001	0.002
		Final	0.003	0.002	0.611
Bray-Curtis		Full	0.001	0.001	0.038
		Final	0.003	0.003	0.605
Jaccard's		Full	0.001	0.001	0.007
		Final	0.003	0.001	0.363

2.4 Discussion

2.4.1 Physiochemical conditions within real salmon gut

The temperature within each of the real salmon gut compartments was determined to be around 12^{o}C, which can be explained by the fact that teleost

fish are poikilotherms, thus their temperature is determined by the surrounding environment temperature, which in Loch Linnhe was around 12-14^{o}C during the period in which samples were taken, as summarised in Figure 2-1 (Sigholt and Finstad 1990, Rabe and Hindson 2017). Thus, the SalmoSim system should be operated at 12^{o}C.

In the real salmon the stomach was found to have an acidic environment (pH 4.0), the mid intestine was found to have a slightly basic environment (pH 7.6) whereas the pyloric caecum compartment displayed a neutral pH (pH 7.0), which corresponds to previously described values (Bravo et al. 2018). Thus, the corresponding SalmoSim gut compartments should be kept at the determined pH values by the addition of acid and base.

Finally, we identified that the environment inside all gut compartments is anaerobic (dissolved oxygen concentration less than 0.05 mg/L). Based on these data, the entire SalmoSim system was kept anaerobic by daily 20-minute flushing of the headspace of the respective compartments with N_2 gas mixture and tracking dissolved oxygen levels within bioreactors to make sure that oxygen levels do not rise.

2.4.2 Differences between different replicate runs are driven by rare OTUs

While statistical analysis could not be performed on the current experiment due to insufficient replication as a result of a failure during one of the planned triplicate runs, visual analysis of the results indicates some differences between the two replicate runs. However, the major drivers of differences are rare OTUs, which comprise less than 1% of total reads in both runs. One of the sources of these OTUs may be due to the variability in sampling times. Even though sampling was performed every two days during both runs, they were sometimes performed at different times of the day, and it is known that at the initial days of a bioreactor run, bacterial dynamics fluctuate not only daily, but hourly as well (Silverman et al. 2018). Furthermore, during each sampling, most of the bacteria captured are planktonic with just a few biofilms collected randomly, meaning that during each sampling round different bacterial populations might be captured due to spatial

heterogeneity of microorganism sampling, especially during early biofilm formation (Christensen et al. 2018). Finally, the added bile and mucous were collected in batches from several different fish, meaning that their origin (fish from which they came) was different during both runs in this study. These variabilities between runs are not uncommon, for instance while using the same pool of biological replicates to inoculate several runs of the *in vitro* Chicken Gut Model, microbiomes still show variability between runs (Card et al. 2017). These limitations can be overcome by adapting SalmoSim protocols (such as producing a pool of bile and mucous, and sampling at the same time of the day), taking run as a random effect during statistical analysis, or running duplicate experiments in parallel at the same time, such as the TWIN-SHIME version of the SHIME system (García-Villalba et al. 2017).

2.4.3 Bacterial dynamics within the SalmoSim system over time with respect to inoculum

In the findings from the simulator of the human intestinal microbial ecosystem (SHIME) in which it was found that it takes around 2 weeks (14 days) for bacterial communities to stabilise and around 3 weeks to reach functional stability (Possemiers et al. 2004). This means that in order for SalmoSim to have stable microbial communities the system has to run for at least 2 weeks prior to any experimental variables being changed. However, due to insufficient replication, the statistical confirmation of this observation is absent in this study, with only visual observation of largely static alpha diversity metrics measurements throughout the replicate runs regardless of gut compartment, with no observed visual pattern.

We also visually determined that in the majority of cases (apart from Effective Shannon diversity in the stomach compartment) the effective richness and Shannon diversity dropped after inoculum introduction to the SalmoSim system. Our taxa plots confirm this, identifying a loss of some taxa when comparing inocula to the SalmoSim system, such as *Mycoplasma* in the pyloric caeca and mid gut compartments. This could be explained by the fact that inocula from real salmon were collected under aerobic conditions, while the fish intestine is abundant in microorganisms that are facultative anaerobes, such as *Mycoplasma* (Holben et al.

2002, Nayak 2010, Navarrete et al. 2012, Llewellyn et al. 2014a). Furthermore, while glycerol was used in this study to store initial inocula as cryoprotectant to preserve cell viability following defrosting (Hubálek 2003, Waite et al. 2013), it has been shown that once bacterial samples stored in glycerol are thawed, the stored bacteria rapidly use the glycerol as a source of energy, modifying their community structure (Prates et al. 2010). Moreover, in another study it was shown that once the rumen digesta from cows was exposed to glycerol, the microbial adaptation was immediate; increasing *n*-butyrate proportions at the expense of acetate (Rémond et al. 1993). Thus, even though storing microbial samples in glycerol preserves the diversity of bacterial communities, this method is considered inadequate for fermentation studies (Prates et al. 2010). To overcome these limitations, inoculum samples should be collected and processed under anaerobic conditions and a snap freezing method in liquid nitrogen should be used as an alternative before long term storage at -80^{o}C, as this method has been shown to contribute towards a maximum recovery of cells after defrosting (Dan et al. 1989).

2.4.4 Microbial communities differ between stomach and other compartments of the SalmoSim system

Our previous microbiome study found that microbial composition within stomach and mid gut compartments of farmed Atlantic salmon were not statistically different, while the pyloric caecum composition was statistically different due to higher enrichment in the *Mycoplasma* genus compared to other gut compartments (Heys et al. 2020b). However, in this study we found that while the pyloric caecum and mid gut compartments are not statistically different, the microbial composition of the stomach compartment is significantly different from both the pyloric caecum and mid gut. This could be explained by the fact that physiochemical conditions within the stomach are vastly different from conditions in the pyloric caecum and mid gut compartments (pH within the stomach is 4, pyloric caecum and mid gut are 7 and 7.6, respectively). While some bacteria, such as *Bacteroides*, can grow over a wide range of pH values (Duncan et al. 2009), thus being able to grow in different gut compartments, others, such as some species of *Veillonella* and *Streptococcus*, are inhibited by low pH (Bradshaw and Marsh 1998). Furthermore, the differences in pH determine the distribution of

major bacterial fermentation end product production, such as butyrogenic reactions, which occur at pH 5.5 (Walker et al. 2005), propionate-production, which usually occurs at around pH 7 (Belenguer et al. 2007), and acetogenic reactions, which occur at a wide range of pH depending on the microbial species producing them (Belenguer et al. 2007). Thus, these differences in pH requirements for bacterial growth and fermentation lead to differences in microbial composition within the stomach compartment compared to the pyloric caecum and mid gut compartments in SalmoSim.

2.5 Summary

In this methodological development chapter, via experimentation, literature review, and data from industry partners, physiochemical conditions in the SalmoSim system were estimated and established, including temperature, pH, dissolved oxygen, flow rates and more. Furthermore, crude extract preparations are described. This chapter lays the foundation for experimentation in SalmoSim described in the remainder of the thesis. We also describe a preliminary SalmoSim replicate run study in which two identical SalmoSim replicate runs were performed. Unfortunately, statistical analysis could not be performed on the current experiment due to insufficient replication as a result of a failure during one of the triplicate runs. We assayed the variability (experimental error) between two identical twenty-day experimental runs of the system via the next generation sequence profiling of the 16S rRNA gene. Our results suggest visual differences between the SalmoSim system replicate runs using alpha diversity metrics. However, shared OTU analysis indicated that these differences were driven by rare OTUs, which comprise less than 1% of the total reads in both runs. The results indicated that microbial communities remained largely static throughout each replicate run regardless of gut compartment, with no observed visual pattern over time based on visual alpha diversity analysis. Furthermore, this study identified a visual difference between the initial inoculum and SalmoSim system, in particular, the loss of the *Mycoplasma* genus in pyloric caecum and mid gut compartments. Finally, our results suggest that the microbial composition within the stomach compartment is statistically different from the other two compartments.

This preliminary run acted as a useful pilot to identify future pitfalls and improvements allowing the improvement of SalmoSim runs for future chapters. In order to improve the replicability between different replicate runs, the SalmoSim protocols need to be adapted (such as producing a pool of bile and mucous, and sampling at the same time of the day), as well as, taking run as a random effect during statistical analysis, or running duplicate experiments in parallel at the same time. In order to prevent the loss of taxa from founding inoculum to SalmoSim, the initial inoculum samples from real salmon should be collected and processed under anaerobic conditions and a snap freezing method in liquid nitrogen should be utilised instead of addition of glycerol before long term storage at -80^{o}C. Overall, although limited in scope, our data suggests that SalmoSim can achieve a robust level of accuracy in technical replication, especially if the improvements identified during our trial runs are incorporated.

Chapter 3 SalmoSim: the development of a three-compartment *in vitro* simulator of the Atlantic Salmon GI tract and associated microbial communities

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3.1 Abstract

3.1.1 Background

Atlantic salmon are a fish species of major economic importance. Innovative strategies are being sought to improve salmon feeds and feed additives to enhance fish performance, welfare, and the environmental sustainability of the aquaculture industry. There is still a lack of knowledge surrounding the importance and functionality of the salmon gut microbiome in fish nutrition. *In vitro* gut model systems might prove a valuable tool to study the effect of feed, and additives, on the host's microbial communities. Several *in vitro* gut models targeted at monogastric vertebrates are now in operation. Here, we report the development of an Atlantic salmon gut model, SalmoSim, to simulate three gut compartments (stomach, pyloric caecum, and midgut) and associated microbial communities.

3.1.2 Results

The gut model was established in a series of linked bioreactors seeded with biological material derived from farmed adult marine phase salmon. We first aimed to achieve a stable microbiome composition representative of founding microbial communities derived from Atlantic salmon. Then, in biological triplicate, the response of the *in vitro* system to two distinct dietary formulations (fish meal and fish meal free) was compared to a parallel *in vivo* trial over forty days. Metabarcoding based 16S rDNA sequencing, qPCR, ammoniacal nitrogen and

volatile fatty acid measurements were undertaken to survey the microbial community dynamics and function. SalmoSim microbiomes were indistinguishable (p=0.230) from their founding inocula at 20 days and the most abundant genera (e.g. *Psycrobacter, Staphylococcus, Pseudomonas*) proliferated within SalmoSim (OTUs accounting for 98% of all reads shared with founding communities). Real salmon and SalmoSim responded similarly to the introduction of novel feed, with majority of the taxa (96% Salmon, 97% SalmoSim) unaffected, while a subset of taxa (e.g. a small fraction of *Psychrobacter*) were differentially affected across both systems. Consistent with a low impact of the novel feed on microbial fermentative activity, volatile fatty acids profiles were not significantly different in SalmoSim pre- and post-feed switch.

3.1.3 Conclusion

By establishing stable and representative salmon gut communities, this study represents an important step in the development of an *in vitro* gut system as a tool for the improvement of salmon nutrition and welfare. This system aims to be utilised as a pre-screening tool for new feed ingredients and additives, as well as being used to study antimicrobial resistance and transfer, and fundamental ecological processes that underpin microbiome dynamics and assembly.

3.2 Introduction

In the last 50 years, per capita fish consumption has almost doubled from 10 kilograms in the 1960s to over 19 kilograms in 2012 (FAO 2018). This increase in the demand for fish protein has put wild fish stocks under pressure. The aquaculture sector now accounts for almost 50% of all fish for human consumption and is anticipated to provide 62% by 2030 (Moffitt and Cajas-Cano 2014). The Atlantic salmon (*Salmo salar*) is the leading farmed marine finfish and, in economic terms, the ninth most important aquaculture fish species farmed globally (FAO 2018). Atlantic salmon are carnivores and wild pelagic fish stocks from reduction fisheries are an important protein source (fish meal (FM)), as well as the principal lipid source (fish oil FO), exploited to feed farmed salmon. Reduction fisheries negatively impact the marine ecosystem, and feeding farmed salmon on FM/FO ingredients is unsustainable as well as expensive (Worm et al.

2006, Cashion et al. 2017). To address these issues, farmed salmon feed composition has changed considerably during the relatively short history of intensive salmon farming, for example in Norway, reducing the ratio of the marine origin components within salmon feed from around 90% in 1990 to 30% in 2013 (Ytrestøyl et al. 2015a). However, there is evidence that non-marine dietary ingredients can result in poor fish growth, altered gut health alongside a modified fish gut microbial community composition and activity (Ingerslev, Strube, et al. 2014, Gajardo et al. 2017a, Beheshti Foroutani et al. 2018). For instance, Atlantic salmon feed supplementation with dietary soybean protein concentrate can induce intestinal disorder (Green et al. 2013). Concomitant alterations in gut microbiota can result in the undesirable fermentation of various feed components (Green et al. 2013, Michl et al. 2019). In view of all this, considerable interest lies around the development of novel ingredients that have comparable performance to marine ingredient-based feeds in terms of their impact of the host and its associated microbes.

To study the impact of novel feed ingredients on gut microbial communities (e.g. Gajardo et al., 2017), as well as the supplements (e.g. pre-biotics, pro-biotics) tailored to modify microbial community diversity and function (e.g. Gupta et al., 2019), *in vivo* trials are widely deployed in salmonid aquaculture. Although physiologically relevant, *in vivo* trials have several scientific, ethical, and practical disadvantages. In salmonids, for example, gut sampling is terminal, preventing the generation of time series data from individual animals/microbial communities. Furthermore, microbial impacts on feed ingredients cannot be subtractively isolated from host enzymatic/cellular activity. From an ethical perspective, *in vitro* models offer the opportunity to reduce harm via replacement of *in vivo* models (Payne et al. 2012a). Practically, *in vivo,* testing of novel feed ingredients is both time consuming and costly. A reliable *in vitro* model could offer advantages in this respect. To the best of our knowledge, there is only one other gut system in place simulating a generalised teleost gut, (‘fish-gut-on-chip’ (Drieschner et al. 2019)) The ‘fish-gut-on-chip’ system exploits microfluidic technology and is based on the reconstruction of the rainbow trout's intestinal barrier by culturing only intestinal cell lines in an artificial microenvironment and currently does not involve microbial communities isolated from the fish's gut.

Prior to deploying an *in vitro* gut microbiome simulator to perform biological experiments, several criteria must be met. Firstly, steady-state microbial communities need to be established prior to the experimental procedure to ensure that results due to experimental treatments are not confounded with bacterial adaptation to the *in vitro* environment (Possemiers et al. 2004). Secondly, physicochemical conditions within the artificial gut simulator and the gut of the target species should be similar. Thirdly, the bacterial communities need to be gut compartment-specific and representative of (if not identical to) the *in vivo* situation (Van Den Abbeele et al. 2010). Finally, the *in vitro* gut simulator should be validated against a parallel *in vivo* experiment, to establish the degree to which the results from the experimental protocol within the artificial gut are generalisable to the *in vivo* situation (Molly et al. 1994). Towards this end, several molecular techniques can then be deployed to analyse microbial populations within the gut. Multiplex quantitative PCR (qPCR) coupled with taxon-specific primers can rapidly detect and quantify the bacterial consortia within a large population (Postollec et al. 2011). Whilst shotgun metagenomics and amplicon sequencing approaches can provide a detailed taxonomic assessment of the microbial composition of the gut, they may be less useful for day-to-day monitoring of specific taxa (Malla et al. 2019).

In view of the above criteria, the aim of the current study is to develop a synthetic, continuous salmon gut microbial fermentation simulator, representative of generalised marine lifecycle stages of the Atlantic salmon. Salmonids are gastric fish (Aas et al. 2017), with their guts characterised by a clearly defined stomach followed by a pylorus with attached blind vesicles called pyloric caeca, as well as a relatively short and non-convoluted posterior (mid and distal) intestine leading to the anus (Lkka et al. 2013). Our experimental gut system simulates the stomach, the pyloric caeca, and the midgut regions of the gastrointestinal tract of farmed Atlantic salmon. In this context, we first aimed to establish a stable gut community, representative of the salmon gut communities used to found it. Secondly, we validated the system as a potential means of testing the impact of feeds on salmon gut microbial communities by comparing the performance and response of the *in vitro* simulator during a feed trial with parallel *in vivo* modulation of the gut microbial community in a cohort of marine phase Atlantic salmon.

3.3 Methods

3.3.1 Experimental set-up and sample collection in an aquaculture setting

The Atlantic salmon (*Salmo salar*) *in vivo* feed trial was performed by MOWI ASA at their research site in Averøy, Norway. Prior to commencement of the feed trial, salmon were fed on a fish meal diet (FMD) until they reached *circa* 750 grams in mass. Fish were separated into 5x5 meter marine pens (150 randomly distributed fish per pen) in 4x4 modular design. Four pens were randomly assigned to each of the trial diets. This study focused on eight pens housing fish fed on FMD and fish meal free diet (FMF) (Table 3-1, Figure 3-1D). The feed trial was conducted over five months (November 2017 - March 2018). For *in vivo* samples recovered from actual salmon, three randomly selected fish were collected at the end of the feed trial for two different feeds (N=3 fish/feed: Fish 1, 2, and 3 for FMD and Fish 4, 5, and 6 for FMF) and sacrificed by MOWI employees (Figure 3-1E). After, 1 cm in length samples from three salmon gut compartments were collected (stomach (N=3/feed), pyloric caeca (N=3/feed) and mid gut (N=3/feed) (approximately 20 cm from the vent)), placed into 1.5 ml cryovials and kept on ice before long term storage in -80^{o}C conditions. For *in vitro* initial inoculum samples (the founding community for SalmoSim runs), a further three fish fed on FMD were sacrificed (Fish 7, 8 and 9) and 5 cm in length samples from three distinct gut compartments were collected (Stomach (N=3), pyloric caecum (N=3) and midgut (N=3)), transferred to 15 ml Falcon tubes and kept on ice before long term storage in -80^{o}C conditions (Figure 3-1E). Details of samples collected from farmed Atlantic salmon have been described previously (Heys et al. 2020b).

Table 3-1 Fish meal and Fish meal free diets composition. *Table summarises Fish meal and Fish meal free diets composition in percentage of the feed.*

Ingredient (% of the feed)	**Fish meal**	**Fish meal free**
Fish meal	17.5	0
Soya protein concentrate	12	27.8
Corn gluten	7	7.35
Wheat gluten	10	14.34
Sunflower expeller	3.41	0
Wheat	4.81	11.22
Beans dehulled	10	0
Fish oil	15.68	16.99
Rapeseed oil	11.78	11.79
Linseed oil	3.05	3.2
Mannooligosaccharide	0.4	0.4
Astaxanthin	0.04	0.04
Crystalline amino acids	1.35	1.99
Mineral premixes	1.83	2.66
Vitamin premixes	0.6	0.73
Macronutrients (% of the feed)	**Fish meal**	**Fish meal free**
Moisture	5.9	6.13
Crude Protein	39.1	40.1
Crude Fat	33.4	33.3
Ash	5.47	4.2
Starch	9.4	11
Crude Fibre	2	2.7

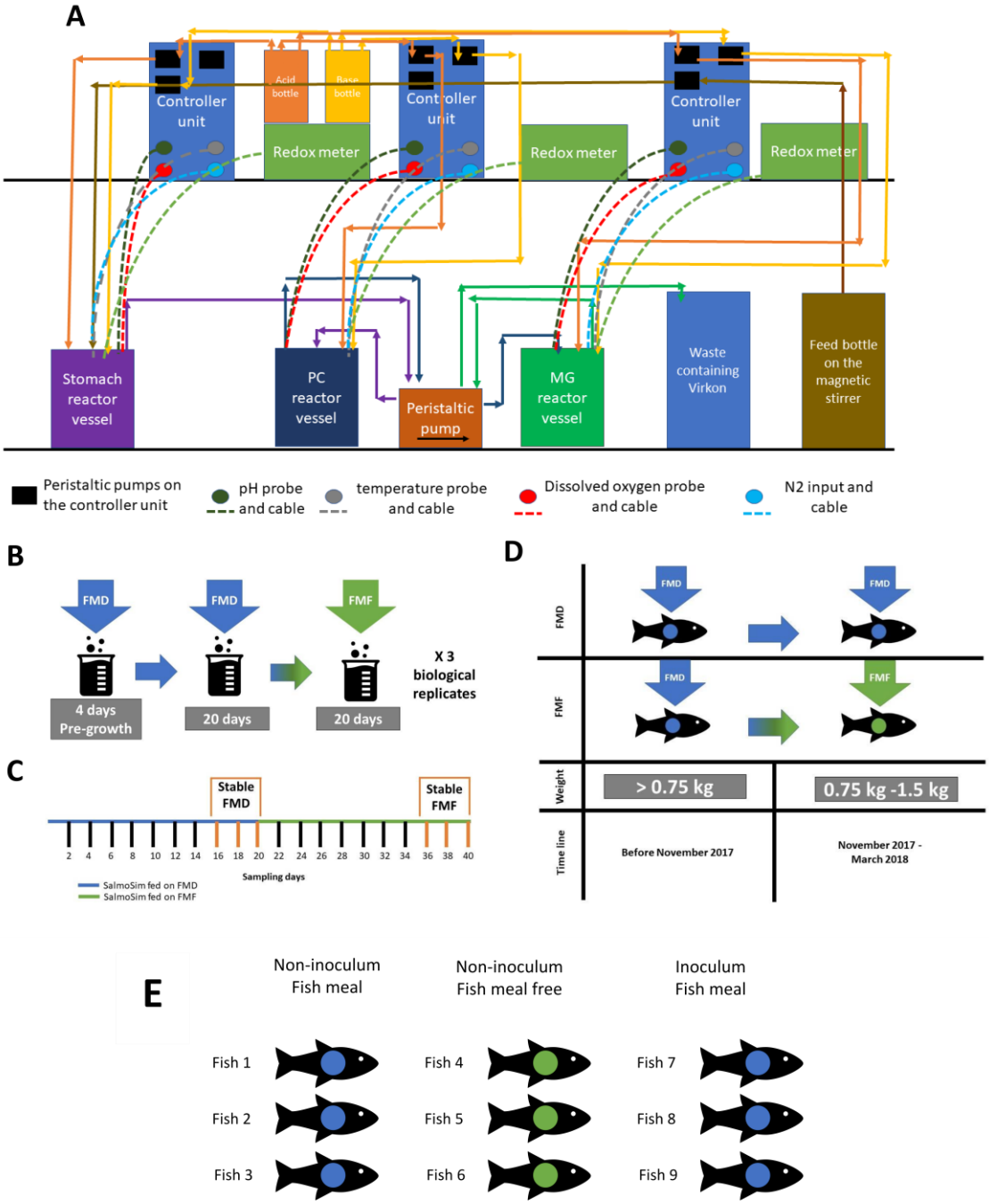


Figure 3-1 Salmon gut *in vitro* simulator. *Schematic encompasses the artificial gut model system set-up, in vivo and in vitro feed trial set up.* ***1A*** *is a schematic representation of SalmoSim system with transfer rate of 238 ml per day for each bioreactor;* ***1B*** *SalmoSim feed trial design;* ***1C*** *SalmoSim sampling time points, which include definition of stable time points (days 16, 18, and 20 for Fish meal (once bacterial communities had time to adapt to SalmoSim system), and days 36, 38, and 40 for Fish meal free diet (once bacterial communities had time to adapt to change in feed);* ***1D*** *in vivo feed trial design. FMD – Fish meal diet and FMF – Fish meal free diet;* ***1E*** *real salmon sacrificed for non-inoculum and inoculum samples (9 fish in total).*

3.3.2 Establishment of stable, representative gut communities in SalmoSim and subsequent feed trial.

3.3.2.1 Physicochemical conditions within Atlantic salmon gastrointestinal tract and microbiome sampling

Physicochemical conditions (temperature, pH, dissolved oxygen) were directly measured in adult Atlantic salmon from a MOWI salmon farm in Loch Linnhe, Scotland (Figure A3-1A-C). Bacterial inoculums were prepared for the *in vitro* trial from the different gut compartments sampled from individual fish (three biological replicates, three gut compartments per fish – stomach, pyloric caecum, and midgut) collected at the start of the *in vivo* feed trial in Averøy, Norway. Prior to SalmoSim inoculation, inoculums that were stored in 15 ml falcon tubes in -80^{o}C freezer were dissolved in 1 ml of autoclaved 35 g/L Instant Ocean® Sea Salt solution. Distinct individual fish collected in Averøy formed the founder community for each distinct replicate of the *in vitro* trial (i.e., a true biological replicate (Figure 3-1)).

3.3.2.2 *In vitro* system 'feed' preparation

In vitro system feed media was prepared by combining the following for a total of 2 litres: 35 g/L of Instant Ocean® Sea Salt, 10 g/L of the FMD or FMF used in the MOWI feed trial (Table 3-1: the concentration of feed added was optimized to run through the system without clogging the narrow tubing), 1 g/L freeze-dried mucous collected from the pyloric caecum of Scottish marine phase Atlantic salmon and 2 litres of deionised water. This feed was then autoclave-sterilised, followed by sieving of the bulky flocculate, and finally subjected to the second round of autoclaving.

3.3.2.3 *In vitro* system preparation

Three 500 ml Applikon Mini Bioreactors (one for each gut compartment: stomach, pyloric caecum, and mid gut) were filled with four 1 cm^3 cubes made from sterile aquarium sponge filters used as a surface for biofilm formation, assembled by attaching appropriate tubing and probes (redox, temperature, and dissolved oxygen, Figure 3-1A), and autoclaved. Bioreactor preparation was followed by attachment of reactor vessels to the Applikon electronic control module,

connection of feed and acid and base bottles (0.01 M hydrochloric acid and 0.01 M sodium hydroxide solutions filtered through 0.22 µm polyethersulfone membrane filter unit (Millipore, USA)). Nitrogen gas was periodically bubbled through each vessel to maintain anaerobic conditions and dissolved oxygen continually monitored. The bioreactors were then allowed to fill with 400 ml of feed media. Once the system was set up, media transfer, gas flow and acid/base addition occurred for 24 hours in sterile conditions (without microorganisms present in the system) in order to stabilise the temperature, pH, and oxygen concentration with respect to levels measured from adult salmon.

3.3.2.4 Initial pre-growth period during *in vitro* trial

In order to allow bacterial communities to proliferate in the *in vitro* environment without washing through the system, the microbial populations within the inoculum from real salmon were pre-grown inside the SalmoSim system for four days. During this phase, the system was filled with FMD media preparation and inoculum, and no media transfer occurred.

3.3.2.5 Performing feed trial within SalmoSim system

After the initial pre-growth period, each validation experiment was run for 20 days while supplying SalmoSim system with FMD. After 20 days, SalmoSim was run for 20 additional days while supplying FMF food. During the full 44-day experiment (4-day pre-growth period, 20-day system fed on FMD, and 20-day system fed on FMF) physiochemical conditions within three SalmoSim gut compartments were kept similar to the values measured in real salmon: temperature inside the reactor vessels were maintained at 12°C, dissolved oxygen contents were kept at 0% by daily flushing with N_2 gas for 20 minutes, and pH was kept stable in each bioreactor by the addition of 0.01 M NaOH and 0.01 M HCl (stomach pH 4.0, pyloric caecum pH 7.0, and mid intestine pH 7.6). During this experiment (apart from the pre-growth period) the transfer rate of slurry between reactor vessels was 238 ml per day as described *in vivo* (Aas et al. 2017, Waagbø et al. 2017). Finally, 1 ml of filtered salmon bile and 0.5 ml of sterile 5% mucous solution (both collected from Scottish marine phase Atlantic salmon) were added daily to the reactor simulating the pyloric caecum compartment. The schematic representation of SalmoSim

system is visualised in Figure 3-1A and full feed trial within SalmoSim is visually summarised in Figure 3-1B and C.

Sampling was performed in several steps. First, samples from initial inoculums were collected for each gut compartment. Once SalmoSim main experiment started, the sampling from each bioreactor vessel was performed every second day throughout the 40-day run period (20 samplings in total). The SalmoSim samplings entailed collecting 30 ml of the bioreactor contents (less than 10% of the total bioreactor volume - the maximum volume of sampling without disturbing microbial community structure) into 50 ml falcon tubes, centrifuging them for 10 minutes at 5000 rpm speed, and freezing the pellets at -20^{o}C for storage. The pellets were frozen to perform DNA extraction all together with the same kit (to prevent batch effect) and supernatant was used for VFA analysis.

3.3.2.6 Measuring nitrogen metabolism within the SalmoSim system

At each sampling point, the protein concentration in each chamber of the system was measured using Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and the ammonia concentration using Sigma-Aldrich® Ammonia Assay Kit (Sigma-Aldrich, USA) to assay the bacterial community activity. Both methods were performed according to manufacturer protocol by using The Jenway 6305 UV/Visible Spectrophotometer (Jenway, USA). The same samples were used for both of these analyses immediately after sampling (no freezing or intermediate steps required).

3.3.2.7 Measuring Volatile Fatty Acid (VFA) production in SalmoSim

The last two time points for each diet were selected from the SalmoSim system (for all three gut compartments) for VFA analysis: 18 and 20 for FMD; and time points 38 and 40 for FMF, respectively, to ensure that the bacterial communities had enough time to adapt to SalmoSim system (for FMD time points) and the feed change (for FMF time points). During runs, 1 ml of supernatant from SalmoSim bioreactors was frozen in -80°C which, was then used for VFA extraction. The protocol involved combining 1ml of supernatant with 400 µl of sterile Phosphate-buffered saline (PBS) solution (Sigma Aldrich, USA) and vortexing the mixture for 1 minute. The sample was then centrifuged at 16,000 *g* for 30 minutes, followed

by two rounds of supernatant removal, before centrifuging at 16,000 *g* for 30 minutes. Finally, the supernatant was then filtered through 0.2 µm Costar SpinX centrifuge tube filters (Corning, USA) at 15,000 *g* for 2 minutes until clear. The extracted VFAs were sent for gas chromatographic analysis at MS-Omics (Denmark). In order to determine if the VFA concentrations were statistically different between SalmoSim fed on FMD and FMF diets, measured VFA values dataset were subjected to statistical analysis using linear mixed effect models (See Supplementary methods 2). Results are shown in Figure A3-9.

3.3.2.8 *In vivo* phenotypic fish performance fed on two different feeds

Phenotypic performance data (fish length, weight, gutted weight, carcass yield, gonad, and liver weights) was collected and provided at the end of the *in vivo* feed experiment by MOWI. The differences between each feed group (n=32 fish per feed) for each phenotype were visualised and statistical analysis was applied (independent two-sample t-test) to identify statistically significant differences between the two feed groups.

3.3.3 Measuring bacterial population dynamics in SalmoSim

3.3.3.1 Genomic DNA extraction

The DNA extraction protocol followed was previously described by (Heys et al. 2020b). In short, samples were subjected to a bead-beating step for 60 seconds by combining samples with MP Biomedicals™ 1/4" ceramic sphere (Thermo Fisher Scientific, USA) and Lysing Matrix A Bulk (MP Biomedicals, USA). Later, DNA was extracted by using the QIAamp® DNA Stool kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol (Claassen et al. 2013).

3.3.3.2 NGS library preparation and sequencing

In the first instance, microbial population dynamics in SalmoSim were measured in near real-time using a set of qPCR primers including published and custom sequences to enable the stability of the system to be monitored (See supplementary Methods 1 and data Figure A3-4). Subsequently 16S rRNA sequencing was deployed to provide a fuller picture community dynamics. The commonly used 16S ribosomal hypervariable region 4 primers were shown to cross-

amplify *Salmo salar* 12S ribosomal gene sequences (Werner et al. 2012, Heys et al. 2020b) and hence were not used in this study. Rather, amplification of the 16S V1 hypervariable region was adopted as an alternate approach (Gajardo et al. 2016). Amplification of 16S V1 hypervariable region from diluted DNA samples was achieved using redundant tagged barcode 27F and 338R at final concentration of 1 pM of each primer. Primer sequences are summarised in Table A3-2. First-round PCR was performed in triplicate on each sample and reaction conditions were 95°C for ten minutes, followed by 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by a final elongation step of 72°C for 10 minutes. After the triplicate reactions were pulled together into one, their concentration was measured using a Qubit® fluorometer (Thermo Fisher Scientific, USA), and all of them were diluted to 5 ng/µl using Microbial DNA-Free Water (Qiagen, Valencia, CA, USA). The second-round PCR, which enabled the addition of the external multiplex identifiers (barcodes), involved only six cycles with otherwise identical reaction conditions to the first. The detailed composition of second-round PCR primers is summarised in Table A3-3. This was followed by the DNA clean-up using Agencourt AMPure XP beads (Beckman Coulter, USA) according to the manufacturers' protocol. The cleaned DNA was then gel-purified by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and then quantified by using Qubit® (Thermo Fisher Scientific, USA). All the PCR products were pulled together at 10 nM concentration and sent for sequencing using HiSeq 2500.

3.3.3.3 Bioinformatic analysis of 16S rRNA gene amplicon sequencing data

Sequence analysis was performed with our bioinformatic pipeline as described previously with slight modifications (Heys et al. 2020b). First, quality filtering and trimming (>Q30 quality score) was performed on all the reads of the 16S rRNA V1 hypervariable region using Sickle version V1.2 software (Joshi and Fass 2011). Second, read error correction was performed using the BayesHammer module within SPAdes V2.5.0 software to obtain high-quality assemblies (Nikolenko et al. 2013). Third, paired-end reads were merged (overlap length 50bp) using PANDAseq v2.11 software with simple_bayesian read merging algorithm (Masella et al. 2012, Schirmer et al. 2016). After overlapping, paired end reads merged reads were dereplicated, sorted, and filtered chimaeras using GOLD SILVA reference dataset

(Mukherjee et al. 2019) and singletons were removed by using VSEARCH version 2.3.4 tool (Rognes et al. 2016). Merged pair-end filtered reads were clustered in operational taxonomic units (OTUs) using VSEARCH software at 97% identity followed by a decontamination step by mapping OTUs against the host (*Salmo salar*) reference genome (available on NCBI) DNA using bwa aligner implemented in DeconSeq v0.4.3 tool (Schmieder and Edwards 2011). Taxonomic assignment of OTUs was achieved using the Naïve Bayesian Classifier (Pedregosa et al. 2011) implemented in the QIIME 2 platform using SILVA 132 database (Quast et al. 2013, Bolyen et al. 2019). Phylogenetic trees of OTUs were generated using FastTree software after using MAFFT for multiple sequence alignment (Katoh and Standley 2013). The resultant OTU table was converted to a biological observation matrix (BIOM) format for the post-OTUs statistical analysis (Douglas et al. 2019).

3.3.3.4 Post-OTUs statistical analysis: diversity metrics and community structure and composition analysis

All statistical analysis of the OTU table was performed by using R v 4.0.1 and RStudio v 1.3.959 (Rstudio Team 2019). Alpha diversity analysis was performed using Rhea pipeline (Lagkouvardos et al. 2017b), supplemented by microbiomeSeq (Ssekagiri et al. 2017), and PhyloSeq (McMurdie and Holmes 2013) for ANOVA and visualisation steps. Two alpha diversity metrics were calculated: microbial richness (estimated number of observed OTUs) and Shannon diversity (an estimate of balance of the community using effective Shannon index (Jost 2006, 2007)). Before calculating effective microbial richness, proportional filtering was performed at a relative abundance of 0.25% in each community to minimise the inflation in microbial richness caused by the very low abundant OTUs. Afterwards, a one-way ANOVA of diversity between groups was calculated with the p-value threshold for significance (p-value <0.05) represented using boxplots.

To investigate the effect of time on the bacterial community stability, beta diversity analysis was performed using different phylogenetical distances metrics to assay phylogenetic similarities between samples (weighted, generalised, and unweighted UniFrac). To compare communities isolated from various sources (SalmoSim, inoculum, and real salmon), only samples fed on FMD were included as initial inoculum were collected from fish fed on FMD alone. Furthermore, only SalmoSim samples from the last 3 time points fed on FMD were selected as they

are considered stable time points (once bacterial communities had over two weeks to adapt and grow within SalmoSim system). In short, the resulting dataset contained: real salmon samples fed on FMD, all inoculum samples and stable SalmoSim time points fed on FMD (days 16, 18, and 20). This dataset was then subdivided into several smaller datasets that included OTUs, shared by various percentage of samples (60%, 50%, 40% and 30% of samples), with the aim of minimising the impact of rare OTUs (low prevalence) on comparisons and only focusing on prevalent OTUs between samples (see details in Table A3-4).

To analyse the response of microbes to the diet change (see Table 3-1 for feed formulation) in real salmon and SalmoSim, in addition to the full dataset (*in vivo* and *in vitro* samples); three different full dataset subsets were used to perform beta diversity analysis: samples from the *in vivo* study, all samples from SalmoSim (all data points), and samples only from SalmoSim once it had achieved stability (the last 3 time points fed on FMD (days: 16, 18, and 20) and FMF (days 36, 38, and 40). These datasets were used to compute ecological (Bray-Curtis and Jaccard) and phylogenetic (unweighted, weighted, and generalised UniFrac) distances with vegdist function from the vegan v2.4-2 package and GUniFrac function (generalised UniFrac) from the Rhea package (Oksanen et al. 2013, Lagkouvardos et al. 2017b). Both ecological and phylogenetical distances were then visualised in two dimensions by Multi-Dimensional Scaling (MDS) and non-metric MDS (NMDS) (Anderson 2001). Finally, a permutational multivariate analysis of variance (PERMANOVA) by using both calculated distances was performed using the adonis function to determine if the separation of selected groups was significant as a whole and in pairs (Anderson 2001). The full beta-diversity workflow is summarised in Supplementary Methods 3.

To provide an overall visualisation of microbial composition across all samples, a principal Coordinates Analysis (PCoA) was performed using the microbiomeSeq (Ssekagiri et al. 2017) package based on phyloseq package (Love et al. 2017) with Bray-Curtis dissimilarity measures calculated and visualised for four different subset-datasets: the full dataset (real salmon, inoculum, and all SalmoSim samples), and, three different subsets each containing only one of the three biological replicate samples from SalmoSim (Fish 1, 2, or 3), along with all real salmon and inoculum samples.

Differential abundance was calculated by using microbiomeSeq based on DESeq2 package (Love et al. 2017, Ssekagiri et al. 2017). BIOM generated OTU table was used as an input to calculate differentially abundant OTUs between selected groups based on the Negative Binomial (Gamma-Poisson) distribution.

3.4 Results

3.4.1 Stabilisation of representative microbial communities within the SalmoSim system

Effective richness (Figure 3-2A) indicates that within the stomach and midgut compartments the initial inoculum contained the highest number of OTUs compared to later sampling time points from the SalmoSim system: in the stomach compartment, effective richness was statistically different between time point 0 (initial inoculum derived from salmon guts) and time points 18, 30, 36 and 38, and within midgut compartment number of OTUs within initial inoculum (time point 0) was statistically different from time points 2, 4, 6, 16, 34, 36, 38, and 40. However, within the pyloric caeca compartment, only one-time point (time point 34) had a significantly different number of OTUs from initial inoculum (time point 0).

Our results reveal that within the stomach compartment over time (including initial inoculum), the effective Shannon diversity was stable with a downwards but non-significant trend over the course of the experiment (Figure 3-2B). A similar downwards trend was observed in the pyloric caecum, with significant differences between later time points, but no significant differences were noticed between the inoculum and SalmoSim. Within the midgut compartment Shannon diversity was statistically lower than the inoculum (time point 0) over most time points (sampling days 2-40).

Taken together, diversity and richness metrics suggest some loss of microbial taxa as a result of transfer of salmon gut communities into SalmoSim in the pyloric caecum and midgut, but not in the stomach. Subsequently, richness and evenness were then stable over the time course of the experiment in stomach and mid gut compartments (some instabilities seen only between initial inoculum and later

time points), whilst much more instability within alpha diversity metrics were detected in the pyloric caecum compartment.

To assess the compositional stability of the system, comparisons over time were undertaken with reference to pairwise beta-diversity metrics. Significant differences in composition between time points represent instability in the system. Figure 3-3 visually summarises between time point comparison of beta-diversity metrics within the SalmoSim system across all replicates using generalised UniFrac (visual representations of the results using unweighted and weighted UniFrac are summarised in Figure A3-3). Irrespective of the metric used, microbial community composition appeared to stabilise in all gut compartments by approximately 20 days, with little-observed impact of introducing the different feed at day 20. This trend was supported by our qPCR data, suggesting increasing stability over the course of the 40-day experiment (Figure A3-4). Prevailing stability was also observed when each biological replicate's individual gut compartment was examined separately (stomach, pyloric caecum, and mid gut) (Figure A3-5). Importantly, stabilisation over twenty days was a feature of two previous pilot runs of the system using unrelated marine salmon gut communities (Figure A3-6).

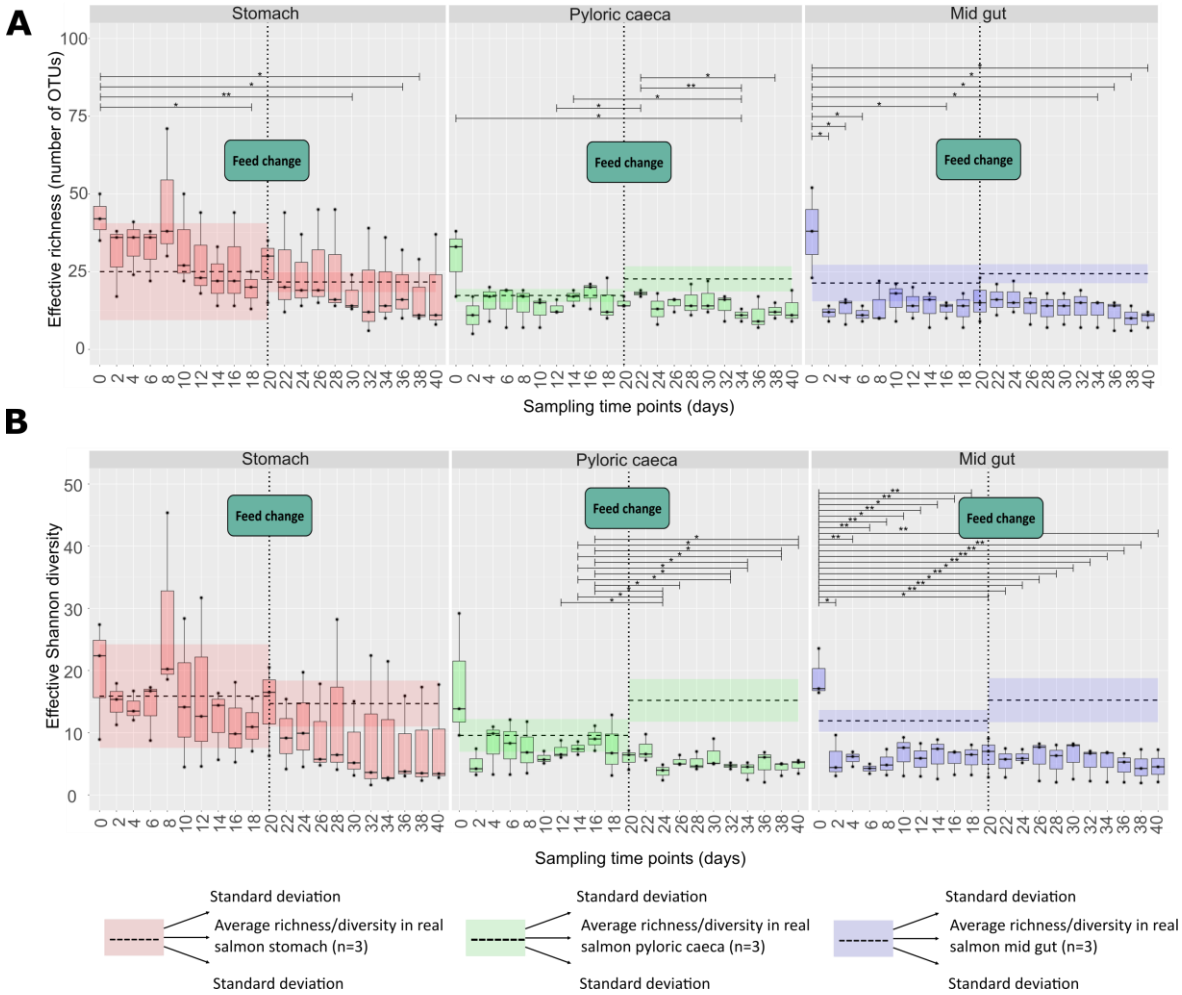


Figure 3-2 Calculated alpha-diversity metrics within SalmoSim system over time. *The figure represents different alpha diversity outputs at different sampling time points (days) from SalmoSim system. Time point 0 represents microbial community composition within initial SalmoSim inoculum from the real salmon, time points 2-20 identifies samples from SalmoSim system fed on Fish meal diet, and time points 22-40 identifies samples from SalmoSim system fed on Fish meal free diet. The dotted vertical line between days 0-20 represents average alpha diversity values measured in real salmon fed on Fish meal diet and dotted vertical line between days 22-40 represents average alpha diversity values measured in real salmon fed on Fish meal free diet. Finally, the horizontal dashed lined represent average effective richness (**A**) and effective Shannon diversity (**B**) in real salmon individual gut compartments fed on different diets (n=3 fish/feed and gut compartment) and shaded region around the horizontal dashed line represents the standard deviation of the values measured within real salmon samples fed on the different diets. A visually represents effective richness (number of OTUs) and B represents effective Shannon diversity. The lines above bar plots represent statistically significant differences between different time points. The stars flag the levels of significance: one star (*) for p-values between 0.05 and 0.01, two stars (**) for p-values between 0.01 and 0.001, and three stars (***) for p-values below 0.001.*

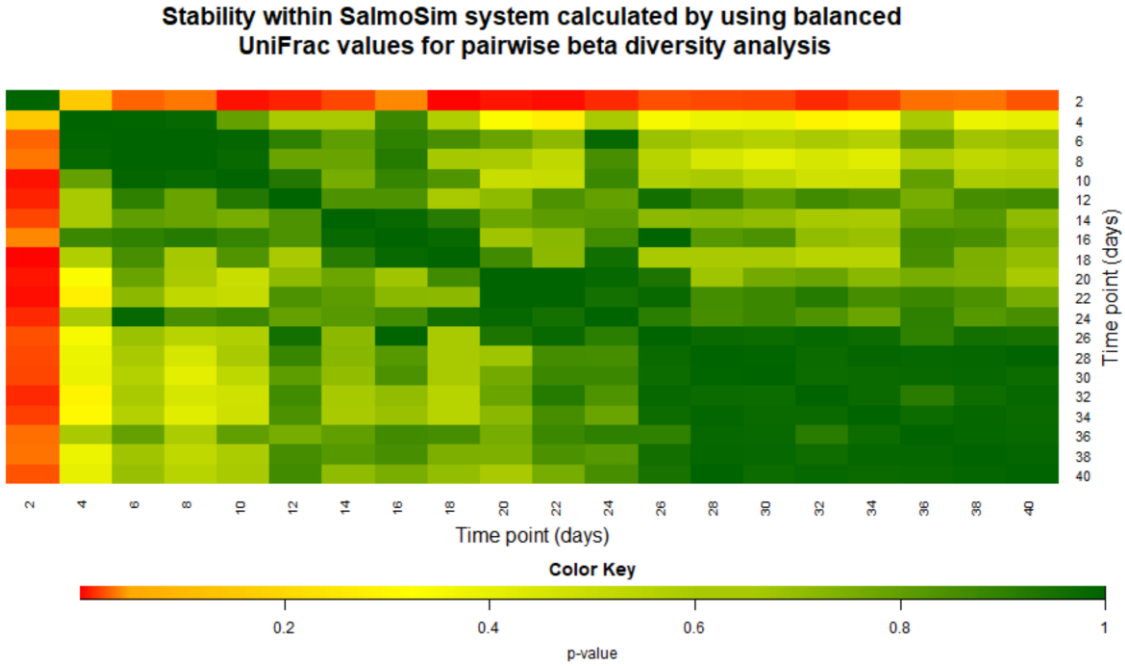


Figure 3-3 Stability within SalmoSim system calculated by using generalised UniFrac values for pairwise beta diversity analysis. *The figure represents microbial stability within the SalmoSim system (data from all gut compartments combined) as the pairwise beta diversity comparison between different sampling time points (days), calculated by using generalised (50%) UniFrac as a distance measure. A small p-value indicates that the two time points are statistically different, and p>0.05 indicates that two time points are not statistically different. The colour key illustrates the p-value: red end of spectrum denoting low p values (distinct compositions between time points) and dark green indicating high p values (similar compositions between timepoints).*

3.4.2 Microbial identity and diversity compared between SalmoSim and salmon.

To compare microbial identity and microbiome composition between salmon and SalmoSim sample sizes were first balanced by examining a reduced dataset that contained: real salmon samples (three fish individuals used to inoculate and three others, all fed FMD) and stable SalmoSim time points fed on FMD (days 16, 18, and 20). Alpha diversity comparisons between inoculum, real salmon and SalmoSim are shown in Figure 3-2.

Compositional comparisons between different sample types (inoculum salmon, three other individuals and SalmoSim) were made using several pairwise beta-diversity metrics (phylogenetic (unweighted, generalised, and weighted UniFrac and ecological distances (Bray Curtis and Jaccard)) (Table 3-2). Ecological metrics could not distinguish between SalmoSim (days 16, 18, and 20) from the individual fish used to inoculate the system, suggesting that microbiome composition was very similar between salmon and SalmoSim (Table 3-2). In contrast, metrics that

incorporate phylogenetic differences between taxa (i.e., Unifrac) did identify significant differences, indicating that there is variability between the inoculum and SalmoSim, but the taxa involved are closely related. To explore the impact of rare OTUs when accounting for observed differences between sample types, the dataset was partitioned and analysed. Partitioned datasets indicated that progressive removal of rare OTUs increased the compositional similarity between SalmoSim and the fish gut communities used to inoculate the system (Table 3-2). Inoculum samples (18 samples) generated 388 OTUs and SalmoSim stable samples (54 samples in total: SalmoSim time points fed on FMD (days 16, 18, and 20) and SalmoSim time points fed on FMF (days 36, 38, and 40)) generated 508 OTUs. 291 OTUs were present in both sample types. Importantly, the shared 291 OTUs accounted for >97% of total reads in inoculum samples and >98% in stable SalmoSim samples (Table 3-2), again suggesting that the majority of abundant microbes in real salmon were maintained in the SalmoSim system.

Between real salmon that were not the direct source of inocula and SalmoSim, and between salmon used as inocula and other individual salmon, however, statistically significant differences were found in using all metrics regardless of inclusion of rare OTUs. These observations are consistent with inter-individual variation - SalmoSim and inoculum samples originated from the same individuals, while other salmon samples were, by necessity, collected from different individuals during the *in vivo* trial. Furthermore, while the number of OTUs between salmon not used to inoculate (192 OTUs) and inoculum salmon samples (388 OTUs) are different (Table 3-2), these non-inoculum salmon share 131 OTUs out of 192 OTUs with inoculums and these 131 OTUs account for around 98% of the total reads. Thus, extra OTUs found only in inoculum salmon and not in others are relatively rare in abundance terms. Differences in OTU numbers and composition is not unexpected as a slightly larger amount of inoculum sample were collected (5 cm of intestine length vs 1 cm for other salmon).

Table 3-2 Beta diversity comparisons of microbial composition between different samples (real salmon, inoculum and SalmoSim). *The table summarises different beta-diversity analysis outputs calculated by using different distances: phylogenetic (unweighted, balanced and weighted UniFrac) and ecological (Bray-Curtis and Jaccard's), between different samples (data from all gut compartments combined): real salmon (Salmon), SalmoSim inoculum from the real salmon (Inoculum), and SalmoSim (only stable time points: 16, 18, and 20 fed on Fish meal diet, and 36, 38 and 40 fed on Fish meal free diet). A permutational multivariate analysis of variance (PERMANOVA) by using phylogenetic and ecological distances was performed to determine if the*

separation of selected groups is significant as a whole and in pairs. Numbers represent p-values, with p-values <0.05 identifying statistically significant differences between compared groups. The comparisons are shown for 3 different datasets: All (completed data set containing all the OTUs sequenced: 978 OTUs in total), Subset (containing OTUs that appear only in more than 3 samples and contribute to 99.9% of abundance within each sample: 374 OTUs in total), and core OTUs (containing OTUs that appear in 60% (6 OTUs in total), 50% (13 OTUs in total), 40% (34 OTUs in total) and 30% (65 OTUs in total) of the samples). The stars flag the levels of significance: one star (*) for p-values between 0.05 and 0.01, two stars (**) for p-values between 0.01 and 0.001, and three stars (***) for p-values below 0.001. Finally, the bottom of the table compares number of samples, OTUs, reads for each sample group, as well as number of shared OTUs and their reads within each sample within compared groups. It also summarises what percentage of a given group of samples' total reads came from the shared OTUs. The SalmoSim samples used for this test consist of stable SalmoSim time points: days 16, 18, and 20 (Fish meal diet – once bacterial communities adapted to the SalmoSim environment), and days 36, 38, and 40 (Fish meal free diet – once bacterial communities adapted to feed change). For non-inoculum real salmon all samples were included (fed on both Fish meal and Fish meal free diets), and for inoculum real salmon all samples were included (fed on Fish meal diet).

Test		Data		Salmon vs SalmoSim	Salmon vs Inoculum	SalmoSim vs Inoculum
UniFrac	Unweighted (0%)	All		0.001 ***	0.002 **	0.002 **
		Subset		0.001 ***	0.002 **	0.001 ***
		Core OTUs	60%	0.04 *	0.032 *	0.143
			50%	0.001 ***	0.001 ***	0.033 *
			40%	0.001 ***	0.003 **	0.244
			30%	0.001 ***	0.001 ***	0.005 **
	Balanced (50%)	All		0.001 ***	0.003 **	0.001 ***
		Subset		0.001 ***	0.001 ***	0.003 **
		Core OTUs	60%	0.138	0.059	0.12
			50%	0.002 **	0.019 *	0.041 *
			40%	0.002 **	0.062	0.132
			30%	0.001 ***	0.005 **	0.008 **
	Weighted (100%)	All		0.012 *	0.007 **	0.003 **
		Subset		0.012 *	0.007 **	0.004 **
		Core OTUs	60%	0.381	0.063	0.125
			50%	0.008 **	0.217	0.078
			40%	0.023 *	0.467	0.122
			30%	0.021 *	0.014 *	0.06
Bray - Curtis		All		0.001 ***	0.001 ***	0.23

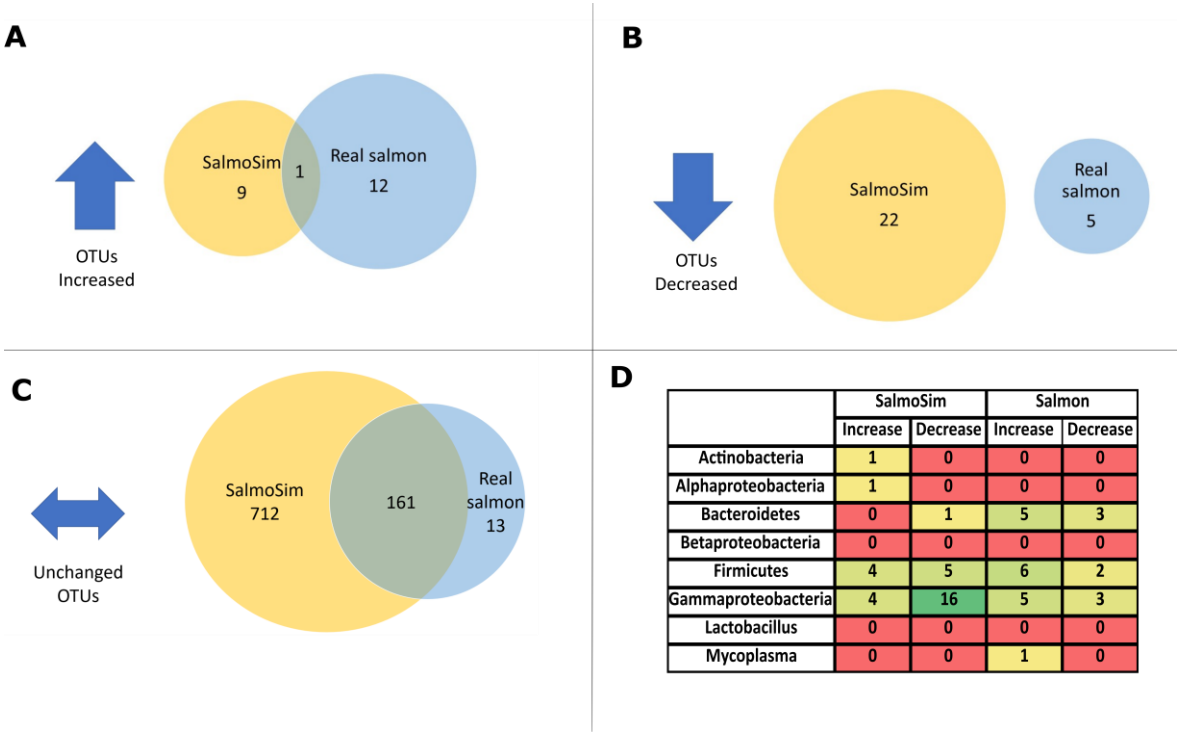
	Subset		0.001 ***	0.001 ***	0.273
	Core OTUs	**60%**	0.009 **	0.004 **	0.079
		50%	0.001 ***	0.008 **	0.394
		40%	0.001 ***	0.002 **	0.327
		30%	0.001 ***	0.001 ***	0.388
Jaccards	**All**		0.001 ***	0.001 ***	0.147
	Subset		0.001 ***	0.001 ***	0.161
	Core OTUs	**60%**	0.002 **	0.003 **	0.073
		50%	0.001 ***	0.002 **	0.386
		40%	0.001 ***	0.002 **	0.22
		30%	0.001 ***	0.001 ***	0.254

	Salmon	**SalmoSim**	**Salmon**	**Inoculum**	**SalmoSim**	**Inoculum**
Number of samples	18	54	18	9	54	9
Number of OTUs	192	508	192	388	508	388
Number of shared OTUs	139	139	131	131	291	291
Number of reads	78,400	1,004,494	78,400	192,429	1,004,494	192,429
Shared OTU reads	77,123	707,199	76,963	134,984	989,884	187,569
% shared OTU reads	98.37%	70.40%	98.17%	70.15%	98.55%	97.47%

3.4.3 Effect of changing diet on the microbiome of real salmon (*in vivo*) and SalmoSim (*in vitro*).

The impact of diet on the abundance of individual taxa: In response to the change of diet, the relative abundances of individual taxa in salmon vs SalmoSim also revealed some differences, as well as multiple similarities in response of the two systems (Figure 3-4). In this respect, the abundance of the vast majority of OTUs (SalmoSim – 97%; Salmon – 95%; Figure 3-4C) were unaffected by the change in feed; these included 161 OTUs shared by SalmoSim and the real salmon assayed. For OTUs whose individual abundance was impacted by feed across the two systems, only a single common OTU changed in the same way in both Salmon and SalmoSim (Figure 3-4A). qPCR-based estimates of taxon abundance variation in

response to diet (Table A3-5), and corresponding data for the same taxa from 16S OTU profiles (Figure 3-4D) show several similarities and differences between SalmoSim and real salmon. Again, however, the overall pattern is that of limited change in both *in vivo* and *in vitro* systems in response to the change in diet. Invariance observed in the microbiome in response to feed were reflected in estimates of physical attributes of fish in response to the change in feed formulation. As such, no statistically significant differences in various phenotypic measurements (fish length, weight, gutted weight, carcass yield, gonad, and liver weights) were noted in salmon fed on the two different diets used in the experiment (see Figure A3-8). Invariance was also observed in VFA production data, in which no significant differences were observed in SalmoSim between the FMD and FMF diets (see Figure A3-9).



	SalmoSim		Salmon	
	Increase	Decrease	Increase	Decrease
Actinobacteria	1	0	0	0
Alphaproteobacteria	1	0	0	0
Bacteroidetes	0	1	5	3
Betaproteobacteria	0	0	0	0
Firmicutes	4	5	6	2
Gammaproteobacteria	4	16	5	3
Lactobacillus	0	0	0	0
Mycoplasma	0	0	1	0

Figure 3-4 Differential abundance of OTUs within the real salmon and SalmoSim samples fed on Fish meal and Fish meal free diets ***A****: Venn diagram representing number of OTUs that were upregulated in both SalmoSim and real Salmon samples once the feed was switched,* ***B****: Venn diagram representing number of OTUs that were downregulated in both sample after the feed change,* ***C****: Venn diagram representing number of OTUs that did not change (relative abundance did not change) within SalmoSim and real salmon samples despite feed switch,* ***D****: table summarising number of OTUs that increased/decreased after feed change in real salmon and SalmoSim samples within different bacterial groups (the same that were analysed by using qPCR approach). Red colour indicates values that are 0, transitioning to greener colours representing higher values.*

Microbial composition in SalmoSim and real salmon fed different feeds: Most gut compartments for real salmon, SalmoSim, and the salmon used to inoculate

SalmoSim were abundant in *Pseudomonas*, *Psychrobacter,* and *Staphylococcus* genera, suggesting that genera present in the marine phase salmon are generally maintained in SalmoSim (Figure 3-5), with these three genera accounting for 42%, 39%, and 34% of all OTUs in non-inocula salmon, inoculum salmon, and SalmoSim samples, respectively. In terms of change in alpha diversity, the only statistically significant difference in response to the switch in feed was observed in the pyloric caeca compartment within SalmoSim based on the Shannon diversity metric (Figure A3-6), where a slight decrease alongside the FMF occurred. Otherwise, the change in feed formulation did not impact alpha diversity in any gut compartment, either in real salmon, or in SalmoSim. Furthermore, no differences were indicated between real salmon and SalmoSim within each gut compartment (Figure A3-7).

To provide an overview of microbial composition and variation in the experiment, a PCoA (Principal coordinates Analysis) based on Bray-Curtis distance was performed and plotted (Figure 3-6A-D). Biological replicate (the fish providing the founding inoculum of each SalmoSim run) appears to be a major driver of community composition in the experiment (Figure 3-6A). Taxonomic composition represented in stacked bar plots in Figure 3-5 also supports this observation. Once individual SalmoSim runs (biological replicates) are visualised separately, changes to microbial communities in response to the feed become apparent (Figure 3-6B-D). Statistical comparisons based on PERMANOVA show there is an effect of feed on microbial composition in both salmon and SalmoSim (Table 3-3), however, based on OTU differential abundance data (above) the effect seems to be small (Figure 3-4). Samples from real salmon fed on the different diets also diverge from one and other (supported by Table 3-3, Figure 3-5), however, not necessarily along the same axes as each SalmoSim replicate. This divergence is potentially indicative of an effect of the biological replicate (i.e., inter-individual variation). Consistent with Figure 3-5, inoculum for the respective SalmoSim replicates cluster among SalmoSim samples for the FMD in each case.

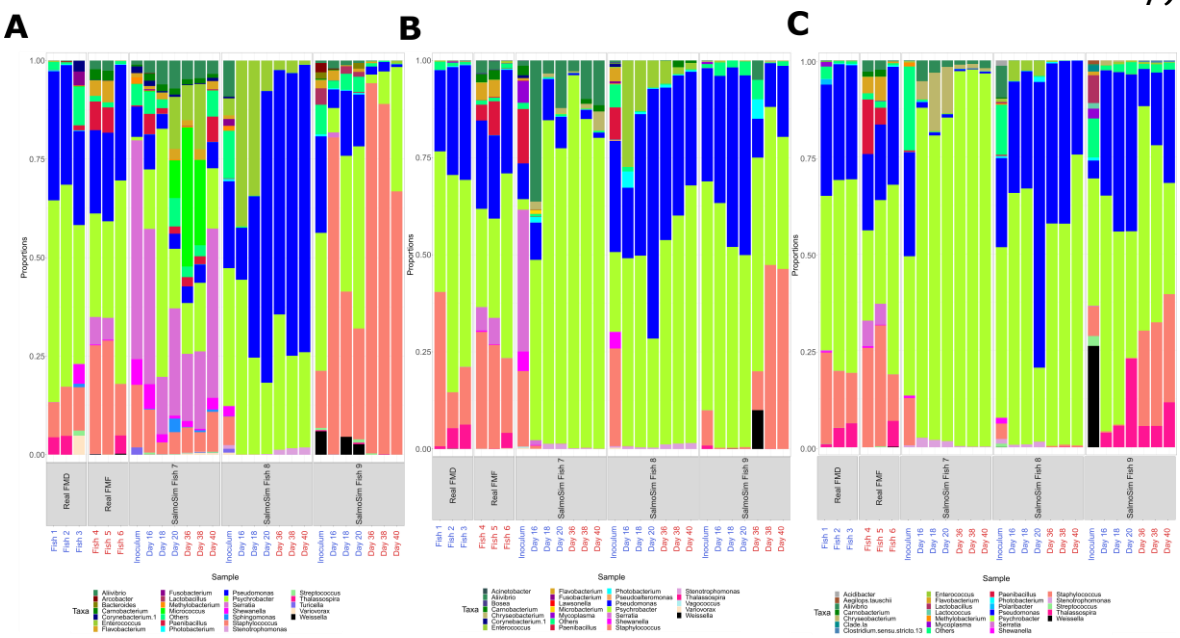


Figure 3-5 Microbial composition (25 most common genus + others) amongst sample types and feeds. ***A****: microbial composition within stomach compartment,* ***B****: microbial composition within pyloric caeca compartment, and* ***C****: microbial composition within midgut compartment. The different sample types are represented by the labels on the x-axis: Real FMD (real salmon fed on Fish meal: Fish 1, 2, and 3), Real FMF (real salmon fed on Fish meal free diet: Fish 4, 5, and 6), SalmoSim Fish 7-9 (SalmoSim biological replicate runs 1-3). Labels in blue represent samples fed on Fish meal diet and in red samples fed on Fish meal free diet. For SalmoSim only stable time points for each feed were selected: time points 16-20 for Fish meal diet, and time points 36-40 for Fish meal free diet.*

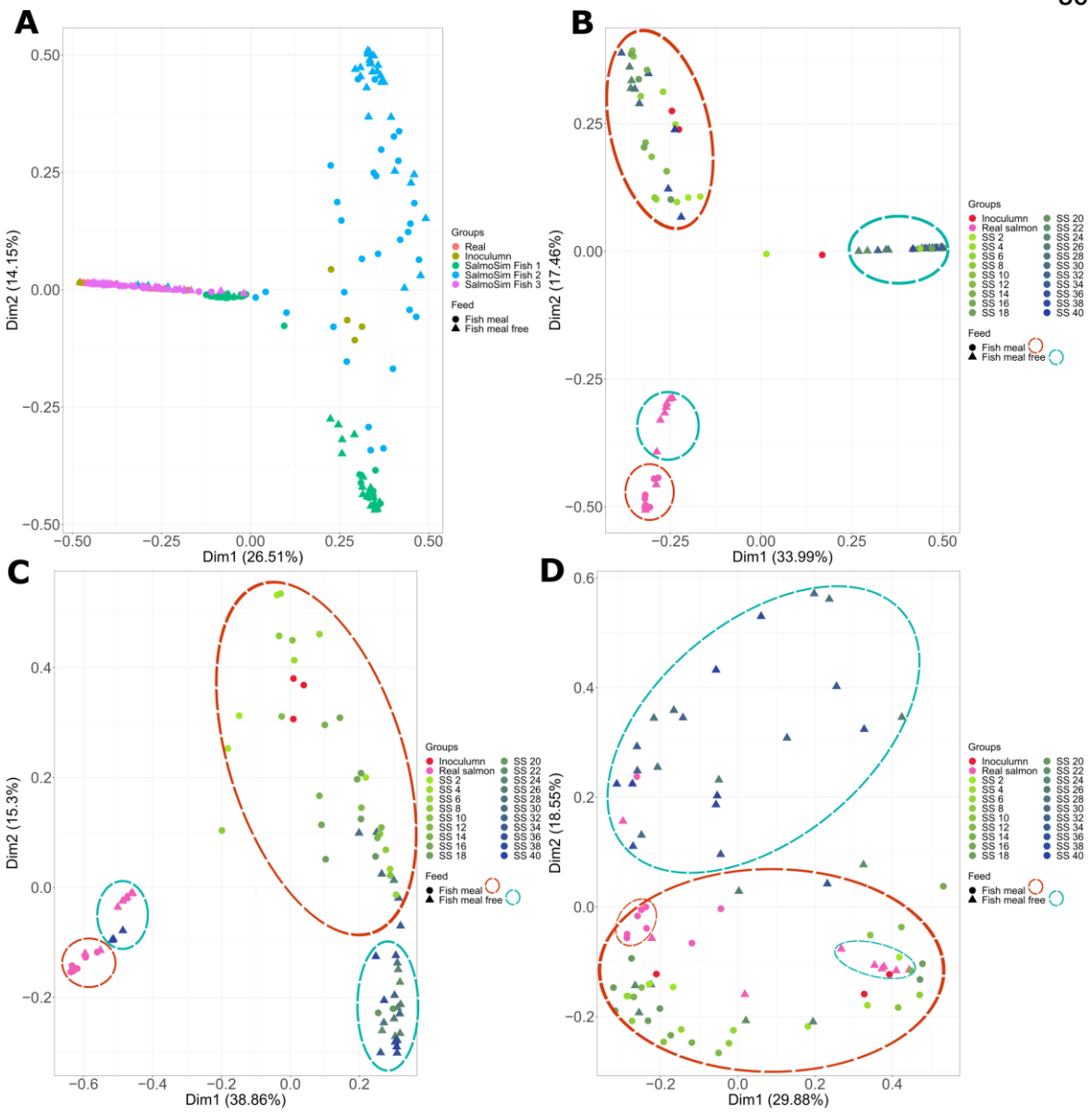


Figure 3-6 PCoA analysis for various samples fed on different feeds. *Figure visualises four principal-coordinate analysis (PCoA) plots for Bray-Curtis dissimilarity measures for different samples (Inoculum, real salmon and SalmoSim), different sampling time points from SalmoSim system, different biological replicates, and different feeds.* ***A*** *represents all sequenced data together (all real salmon, inoculum and all 3 biological SalmoSim runs) in which different colours represent different samples (real salmon, inoculum and 3 different SalmoSim biological replicates) and different shapes represent different feeds; while* ***B-D*** *(subsets of Figure 6A) represent sequenced data together for real salmon, inoculum and different biological replicates of SalmoSim (B: Fish 1, C: Fish 2, D: Fish 3). In figures B-D different colours represent different samples (inoculum, real salmon, and different sampling points of SalmoSim), different shapes represent samples fed on two different feeds, and samples fed on same feeds were circled manually in dotted circles. Dim 1 is principal coordinate 1, and Dim 2 is principle coordinate 2.*

Table 3-3 Beta diversity analysis for various samples fed on different feeds. *Table summarises different beta-diversity analysis outputs calculated by using different distances: phylogenetic (unweighted, balanced, and weighted UniFrac) and ecological (Bray-Curtis and Jaccard's), between samples fed on Fish meal or Fish meal free diets. Numbers represent p-values, with p-values <0.05 identifying statistically significant differences between compared groups. The comparisons are shown for three different subset-datasets:* ***Salmon*** *(containing sequenced samples from real salmon),* ***All SalmoSim*** *(containing all samples from SalmoSim system excluding inoculum), and* ***Stable SalmoSim*** *(containing samples only from stable time points: 16, 18 and 20 fed on Fish meal (once bacterial communities adapted to SalmoSim system), and 36, 38 and 40 fed on Fish meal free diet (once bacterial communities adapted to feed change). The stars flag the levels of significance: one star (*) for p-values between 0.05 and 0.01, two stars (**) for p-values between 0.01 and 0.001, and three stars (***) for p-values below 0.001.*

		Fish meal vs Fish meal free diets		
		Salmon	**SalmoSim**	**Stable SalmoSim**
UniFrac	**Unweighted (0%)**	0.001 ***	0.002 **	0.062
	Generalised (50%)	0.001 ***	0.001 ***	0.251
	Weighted (100%)	0.016 *	0.011 *	0.288
Bray-Curtis		0.008 **	0.001 ***	0.126
Jaccards		0.01 **	0.001 ***	0.053
Number of differentially abundant OTUs		18	32	28

3.5 Discussion

Our findings suggest a loss of microbial taxa diversity and richness as a result of transferring initial inoculums from real salmon into the SalmoSim system in the pyloric caeca and mid gut compartments. Several lines of evidence from our core OTU analysis suggest that low prevalence (rare) OTUs make up most of the taxa lost, and progressive removal of rare OTUs increased the compositional similarity between inoculum and SalmoSim samples using both phylogenetic and ecological distances. Furthermore, shared OTUs between inoculum salmon and SalmoSim samples accounted for around 97% and 98% of total reads respectively, and as such the microbiota in the model are highly representative of those from the fish that founded them. A general trend was observed, in which all gut compartments became increasingly stable throughout the 40-day experiment, with little-observed impact of introducing the different feed at day 20. Comparison of non-inoculum salmon and SalmoSim samples at the microbial level showed significant differences using both ecological and phylogenetic metrics as well as a different number of OTUs (SalmoSim 508 OTUs and real salmon samples 192 OTUs). These differences may be explained by the fact that samples used for non-inoculum salmon and SalmoSim originated from different individuals, whereas initial inoculum and SalmoSim samples for a given run originated from the same fish.

Furthermore, the non-inoculum salmon and inoculum samples were derived slightly differently - with a longer section of the gut sampled for the inoculum samples, which could explain the higher diversity of bacteria (number of OTUs) which in turn can affect beta diversity output. However, despite this, shared OTUs between non-inoculum salmon and inoculum samples accounted for around 98% of total reads generated in non-inoculum real salmon and around 70% within inoculum real salmon samples. Correspondingly, we observed that the biological replicate (the founding inoculum of each SalmoSim run that originated from different individuals) was the major driver of community composition in the experiment. Once the individual runs were separated, phylogenetic and ecological distances suggested that changing feed was a statistically significant driver of community composition in both real salmon and SalmoSim. However, the vast majority of OTUs remained unchanged by the switch in feed in both systems and no changes were noticeable in the bacterial activity (VFA production) within the system after the introduction of the plant-based feed, nor in phenotypic performance of Atlantic salmon fed on two different feeds (fish length, weight, gutted weight, carcass yield, gonad, and liver weights) in the *in vivo* trial.

Many of the microbes we detected, and cultured, from the salmon gut microbiome have been reported previously in this species. For example, gram-negative *Pseudomonas* and *Psychrobacter*, the most abundant genera we observed, are among the core bacterial taxa known to reside within the real salmon gut (Navarrete et al. 2009, Gajardo et al. 2016, Webster et al. 2018). *Staphylococcus* genera have also been reported widely in fresh-water and marine farmed salmon (Dehler et al. 2017). SalmoSim was able to maintain these species in culture throughout the experimental run, and although some diversity was lost, no statistical differences could be detected between the composition of SalmoSim and that of the fish gut communities used to found the different biological replicates via ecological metrics. Notable by their scarcity were *Mycoplasma* OTUs, which occurred at relatively low abundance in both the *in vivo* and *in vitro* systems in this study. *Mycoplasma* OTUs were recovered from most SalmoSim gut compartments at low abundances (see Table A3-5), suggesting that these fastidious microbes can survive in the bioreactors. Our group and several others have widely reported *Mycoplasma* species from marine and freshwater stage of wild and farmed Atlantic salmon (higher abundance in farmed salmon), where

many proliferate intracellularly in the gut epithelial lining (Holben et al. 2002, Llewellyn et al. 2014b, Cheaib et al. 2020, Heys et al. 2020b). Establishing whether *Mycoplasma* can actively proliferate in SalmoSim would require the use of founding communities rich in these organisms in a future experiment. One means of achieving this could be by using mock communities to better understand the temporal development of the observed microbial compositions from inocula to SalmoSim community (Amos et al. 2020).

We identified that a change in feed resulted in an overall shift in microbial community structure in both real salmon and SalmoSim system, as has been found to be the case in many previous studies (Gajardo et al. 2017a, Michl et al. 2017, Egerton et al. 2020), but the vast majority of OTUs within both real salmon and SalmoSim were not affected by the feed change. The direction of this shift, and the microbial taxa involved, were not equivalent in SalmoSim and real salmon, although no overall trend was observed at higher taxonomic levels in either system. Importantly, it is also the case that the vast majority of OTUs within both real salmon and SalmoSim were not affected by the switch in feed. Furthermore, it was found that change in feed did not affect VFA production in the SalmoSim system. As such, it is not clear whether any relevant functional shifts occurred in the microbiome of SalmoSim or real salmon as a result of the treatment. Furthermore, we did not identify any phenotypic changes (fish length, weight, gutted weight, carcass yield, gonad, and liver weights) within *in vivo* trial of Atlantic salmon fed on two different feeds. This lack of change is not unexpected, considering the plant-based feed was developed to have similar macronutrient composition to a Fish meal-based feed. One difference is a slightly higher crude fibre (fermentable substrate) proportion in Fish meal free diet, which could explain higher microbial diversity in *in vivo* samples fed on plant-based feed (Gajardo et al. 2017b).

The use of *in vitro* systems to study and model the microbial communities of monogastric vertebrates is becoming increasingly widespread, with systems simulating: *Sus scrofa* (pig) (Tanner et al. 2014), *Gallus gallus* (chicken) (Card et al. 2017), *Canis lupus* (dog) (Duysburgh et al. 2020) and other vertebrate guts. Using *in vitro* gut simulators is also a widely accepted approach to study the human gut microbiome (Déat et al. 2009, Van Den Abbeele et al. 2010, Kim et al. 2016).

One of the most established systems is the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) that mimics the entire gastrointestinal tract incorporating the stomach, small intestine and different colon regions (Molly et al. 1994). This system was used to study the effects of many different dietary additives on human microbiome (Sánchez-Patán et al. 2015, Giuliani et al. 2016). The value of *in vitro* simulators in providing genuine insights is limited only by the research question and the corresponding level of sophistication required. The host component of the system, for example, is often poorly modelled, although cell lines, artificial mucosae and digestion / absorbance systems can be included, which can provide specific physiological and metabolic insights (Déat et al. 2009, Van den Abbeele et al. 2012). For instance, the growth medium in vessels representing stomach and small intestine of SHIME system is enriched with pancreatic enzymes and bile, while a further upgrade of SHIME incorporates the mucosal environment allowing a portion of the microbiota present in the vessels to adhere to a gut mucus layer (Van Den Abbeele et al. 2010, Van den Abbeele et al. 2012). Furthermore, more sophisticated *in vitro* gut models, such as TIM-2 (the TNO computer-controlled, dynamic *in vitro* gastro-Intestinal Model of the colon), incorporates a dialysis system, which helps to maintain a physiological concentration of metabolites and prevents inhibition of the microbial growth by microbial metabolite accumulates (Minekus et al. 1999, Lefebvre et al. 2015). Finally, microfluid-based *in vitro* systems, such as HuMiX, allow co-culturing of the host gut cells and microbial cells under conditions and processes mimicking gastrointestinal host-microbiome interface (Shah et al. 2016).

As we found, inter-individual variability may be an important consideration, and adequate biological replication is necessary to enable reliable interpretation of results, a consideration that can be overlooked by even the most sophisticated systems. Inter-individual variation in gut microbial communities is widely observed in human studies, that demonstrate more between-person variation than within-person variation with adults having an average unique microbial signature that is largely stable over time (Costello et al. 2009, Stearns et al. 2011, Human Microbiome Project Consortium 2012, Huttenhower et al. 2012, Rajilić-Stojanović et al. 2013). This is also true in Atlantic salmon – our previous work clearly shows high levels of interindividual variability in farmed (Heys et al. 2020a) and wild (Llewellyn et al. 2016) fish, as does the work of all others. It was reported, for

example, that a single *Lactobacillales* OTU represented 96% of the microbiome of one fish which compared to a mean of only 3.5% relative abundance in the other fish from the same shoal in an aquaculture setting (Schmidt et al. 2016). Well-established gut microbiome systems, such as SHIME, use inoculums from only one donor (Van Den Abbeele et al. 2010) or in recent studies 2 donors in which differences were found in microbiota distribution even when using control diets (Rovalino-Córdova et al. 2020). Furthermore, some artificial gut systems pool biological replicates together to produce a "representative microbiome inoculum", such as in a recent *in vitro* Chicken Gut Model, and even in these systems microbiomes still show variability (Card et al. 2017). To our knowledge in this study, we are the first to run a gut microbiome model in biological triplicate and to highlight the importance of accounting for inter-individual differences before drawing conclusions. Prior to the current study, only one other attempt was made to study the effect of diet on Atlantic salmon gut microbial composition *in vitro* (Zarkasi et al. 2017). In this preliminary study a simple *in vitro* system was used to assess the impact of different feed formulations on the microbial communities of faecal slurries prepared from live salmon. However, no direct comparison was made with a true *in vivo* trial; nor were the different gut compartments present in salmon modelled in any detail and the predictive value for such simple *in vitro* systems in not immediately clear. Nonetheless, the work provided an important catalyst for the development of more sophisticated systems.

3.6 Conclusions

Our results indicate that SalmoSim can not only stably maintain the most abundant microbial communities from real salmon, but also demonstrates similar responses to experimental feed treatments as those seen in real salmon. These results are encouraging, however, the nature of the treatment applied in this study: a switch between two similar feeds that had little effect on the gut microbiota *in vivo,* suggests that further experimentation with SalmoSim would be beneficial. For example, the survival and influence of probiotics within the system or the influence of known prebiotics could also be assessed, as has been previously studied in other *in vitro* gut systems (Duysburgh et al. 2020). Gut models such as SalmoSim could provide a powerful tool for aquaculture, where there is

considerable interest associated with the development of feed and feed additives (Kristiansen et al. 2011, Hartviksen et al. 2014, Encarnação 2016), but where the capacity for *in vivo* trials is limited. The aim of such systems could be to provide a pre-screening tool for new feed ingredients and additives with the aim of reducing the cost and scale of *in vivo* testing. In parallel, an *in vitro* gut model for salmon could also be exploited to understand questions of public health importance (e.g. antimicrobial resistance and transfer (Card et al. 2017)), as well as the fundamental ecological processes that underpin microbiome dynamics and assembly.

Chapter 4 Deploying an *in vitro* gut model to assay the impact of a mannan-oligosaccharide prebiotic, Bio-Mos® on the Atlantic salmon (*Salmo salar*) gut microbiome.

4.1 Abstract

Mannose-oligosaccharide (MOS) pre-biotics are widely deployed in animal agriculture as immunomodulators as well as to enhance growth and gut health. Their mode of action is thought to be mediated through their impact on host microbial communities and the associated metabolism. Bio-Mos is a commercially available pre-biotic currently used in the agri-feed industry. To assess Bio-Mos for potential use as a prebiotic growth promotor in salmonid aquaculture, we have modified an established Atlantic salmon *in vitro* gut model, SalmoSim, to evaluate its impact on the host microbial communities. Inoculated from biological triplicates of adult farmed salmon pyloric caeca compartments, the microbial communities were stabilised in SalmoSim followed by a twenty-day exposure to the prebiotic and in turn followed by an eight day 'wash out' period. Dietary inclusion of MOS resulted in a significant increase in formate ($p=0.001$), propionate ($p=0.037$) and isovalerate ($p=0.024$) levels, correlated with increased abundances of several, principally, anaerobic microbial genera (*Fusobacterium, Agarivorans, Pseudoalteromonas*). DNA metabarcoding with 16S rDNA marker confirmed a significant shift in microbial community composition in response to MOS supplementation with observed increase in lactic acid producing *Carnobacterium*. In conjunction with previous *in vivo* studies linking enhanced volatile fatty acid production alongside MOS supplementation to host growth and performance, our data suggests that Bio-Mos may be of value in salmonid production. Furthermore, our data highlights the potential role of *in vitro* gut models to augment *in vivo* trials of microbiome modulators.

4.2 Introduction

Since the late 1970s, the salmon aquaculture sector has grown significantly, currently exceeding 1 million tonnes of salmon produced per year (FAO 2018). In aquaculture environments, fish are exposed to abiotic conditions and biotic

interactions that are extensively different from the wild, such as changes in temperature and salinity, and close contact between animals that can favour potential disease outbreaks by proliferating pathogenic agents present from the surrounding environment from one animal to the other (Kennedy et al. 2016), as well as long term stress through physical aggression and overcrowding (Turnbull et al. 2005, Adams et al. 2007). Thus, the rapid expansion of the aquaculture sector requires a means to promote feed performance, reduce the need for medical treatments and reduce waste discharges whilst also improving farmed fish quality and boosting profitability.

In order to mitigate disease outbreaks, as a preventative nutritional strategy, and improve feed performance, prebiotics are widely deployed in agriculture and aquaculture settings (Patterson and Burkholder 2003, Ringø, Olsen, et al. 2010, Markowiak and Ślizewska 2018). Prebiotics are defined as non-digestible food additives that have a beneficial effect on the host by stimulating growth and activity of bacterial communities within the gut that improve animal health (Gibson, Glenn R. and Roberfroid 1995). One prebiotic type found in aquaculture is mannooligosaccharides (MOS), which are made of glucomannoprotein-complexes derived from the outer layer of yeast cell walls (*Saccharomyces cerevisiae*) (Merrifield et al. 2010). MOS compounds were shown to improve gut function and health by increasing villi height, evenness and integrity in chickens (Iji et al. 2001, Hooge 2004), cattle (Castillo et al. 2008) and fish (Dimitroglou et al. 2009). MOS supplementation in monogastrics has been reported to drive changes in host associated microbial communities (Sims et al. 2004, Halas and Nochta 2012). Associated increase of volatile fatty acid (VFA) production was reported which can have beneficial knock-on effects in terms of host metabolism and gut health in various mammalian species (Besten et al. 2013).

There are a limited number of studies investigating the effect of MOS on the fish microbiome (Dimitroglou et al. 2009, Ringø et al. 2016a), with disparities in the observed results that could be partially explained by the duration of MOS supplementation, fish species, age or environmental conditions. For example, it was found that MOS supplemented diets improved growth and/or feed utilization in some studies (Staykov et al. 2007, Yilmaz et al. 2007, Buentello et al. 2010, Gültepe et al. 2011, Torrecillas et al. 2013), but others found that MOS

supplementation did not affect fish performance or feed efficiency (Pryor et al. 2003, Peterson et al. 2010, Razeghi Mansour et al. 2012). Detailed studies are needed to investigate the effect of MOS supplements on the fish microbiome to enhance our understanding of the link between MOS and gut health. *In vitro* gut models offer the advantage of doing so in a replicated and controlled environment.

SalmoSim is a salmon gut simulation system that continuously maintains the microbial communities present in the intestine of marine phase Atlantic Salmon (*Salmo salar*) (Kazlauskaite et al. 2020). The current study deploys a modified version of SalmoSim designed to evaluate the effect of Bio-Mos (Alltech), a commercially available MOS product, on the microbial communities of the Atlantic salmon pyloric caecum compartment in biological triplicate. We assayed microbial composition and fermentation in the SalmoSim system and show a significant impact of Bio-Mos supplementation on both.

4.3 Materials and Methods

4.3.1 *In vivo* sample collection and *in vitro* system inoculation

Three starved adult Atlantic salmon gut samples were collected from the MOWI processing plant in Fort William, Scotland and transferred to the laboratory in an anaerobic box on ice. Samples were placed in an anaerobic hood and contents from the pyloric caeca compartment were scraped and collected into separate sterile tubes. Half of each sample was stored in a -80^{o}C freezer, whilst the other half was used as an inoculum for the SalmoSim system. Fresh bacterial inocula were prepared for the *in vitro* trial from the pyloric caeca compartment sampled from individual fish (three biological replicates) by dissolving them in 1 ml of autoclaved 35 g/L Instant Ocean® Sea Salt solution.

4.3.2 SalmoSim *in vitro* system preparation

In vitro system feed media was prepared by combining the following for a total of 2 litres: 35 g/L of Instant Ocean® Sea Salt, 10 g/L of the Fish meal (see Table 3-1), 1 g/L freeze-dried mucous collected from the pyloric caecum, 2 litres of deionised water and 0.4% of Bio-Mos (derived from the outer cell wall of

Saccharomyces cerevisiae strain 1026) for the prebiotic supplemented feed. A supplementation level of 0.4% was chosen based on previous studies (Dimitroglou et al. 2011, Torrecillas et al. 2015). This feed was then autoclave-sterilised, followed by sieving of the bulky flocculate, and finally subjected to a second round of autoclaving. System architecture was prepared as described previously with some modifications (Kazlauskaite et al. 2020). In short, appropriate tubes and probes were attached to a two-litre double-jacketed bioreactor, and three 500 ml Applikon Mini Bioreactors. Four 1 cm^3 aquarium sponge filters were added to each Mini Bioreactor vessel which were then autoclaved, sterilised, and connected as in Figure 4-1. Nitrogen gas was periodically bubbled through each vessel to maintain anaerobic conditions. The two-litre double jacketed bioreactor and three 500 ml bioreactors were filled with 1.5 litres and 400 ml of feed media, respectively. Once the system was set up, media transfer, gas flow and acid/base addition were undertaken for twenty-four hours axenically in order to stabilise the temperature, pH, and oxygen concentration with respect to levels measured from adult salmon. SalmoSim system diagram is visualised in Figure 4-1. Physiochemical conditions within the three 500 ml SalmoSim gut compartments were kept similar to the values measured *in vivo* (Kazlauskaite et al. 2020): temperature inside the reactor vessels was maintained at 12°C, dissolved oxygen contents were kept at 0% by daily flushing with N_2 gas for 20 minutes, and pH 7.0 by the addition of 0.01 M NaOH and 0.01 M HCl. The 2-litre double jacketed bioreactor (represents a sterile stomach compartment) was kept at 12°C and pH at 4.0 by the addition of 0.01 M HCl. During this experiment (apart from the initial pre-growth period), the transfer rate of slurry between reactor vessels was 238 ml per day. Finally, on a daily basis, 1 ml of filtered salmon bile and 0.5 ml of autoclaved 5% mucous solution were added to the three bioreactors simulating pyloric caecum compartments.

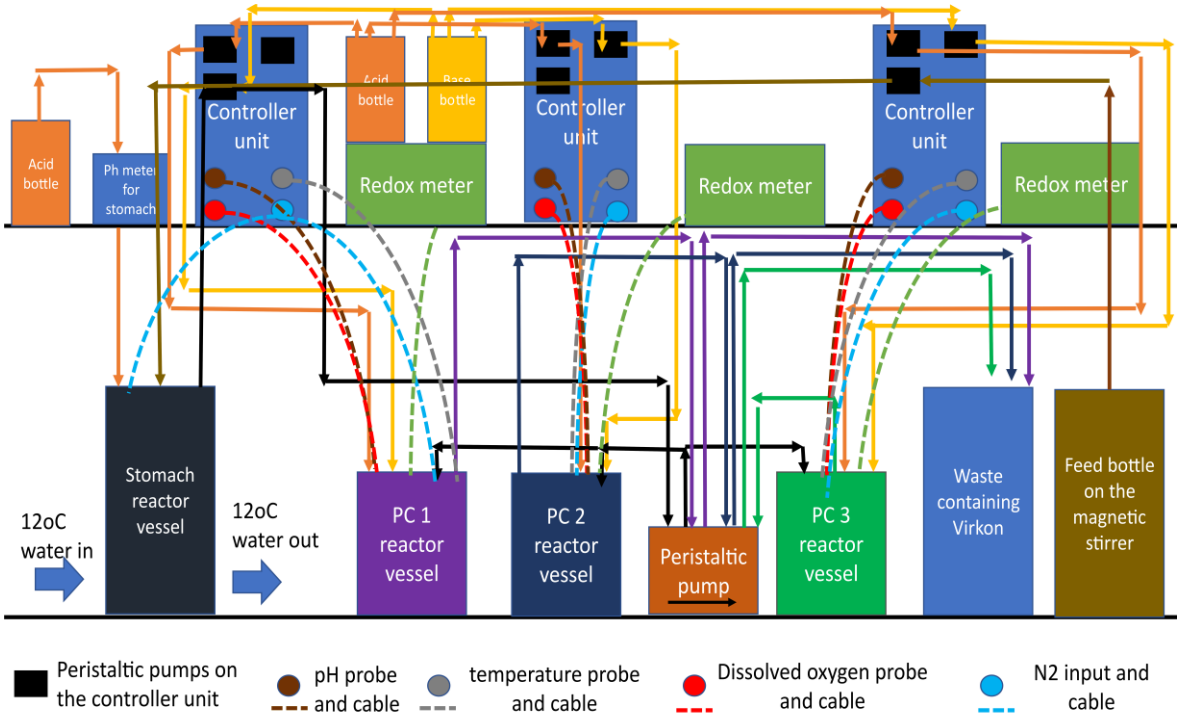


Figure 4-1 Artificial gut model system set-up and in vitro trial set up. *A SalmoSim system designed to run in biological triplicate.*

4.3.3 SalmoSim inoculation and microbial growth

To generate stable and representative microbial communities for experimentation (Kazlauskaite et al. 2020), microbial communities were grown within the SalmoSim system during a separate twenty-four day run prior to the main experimental run (Figure 4-2A). This was achieved by adding fresh inoculum from pyloric caeca to three 500 ml bioreactors which was then pre-grown for 4 days without media transfer, followed by 20 days feeding the system at a 238 ml per day feed transfer rate. A volume of 15 ml of the stable communities were collected at the end of this pre-growth period, centrifuged at 3000 *g* for 10 minutes and supernatant removed. The pellet was then dissolved in 1 ml of autoclaved 35 g/L Instant Ocean® Sea Salt solution, flash frozen in liquid nitrogen for 5 minutes and stored long term in -80ºC freezer for later microbial DNA analysis.

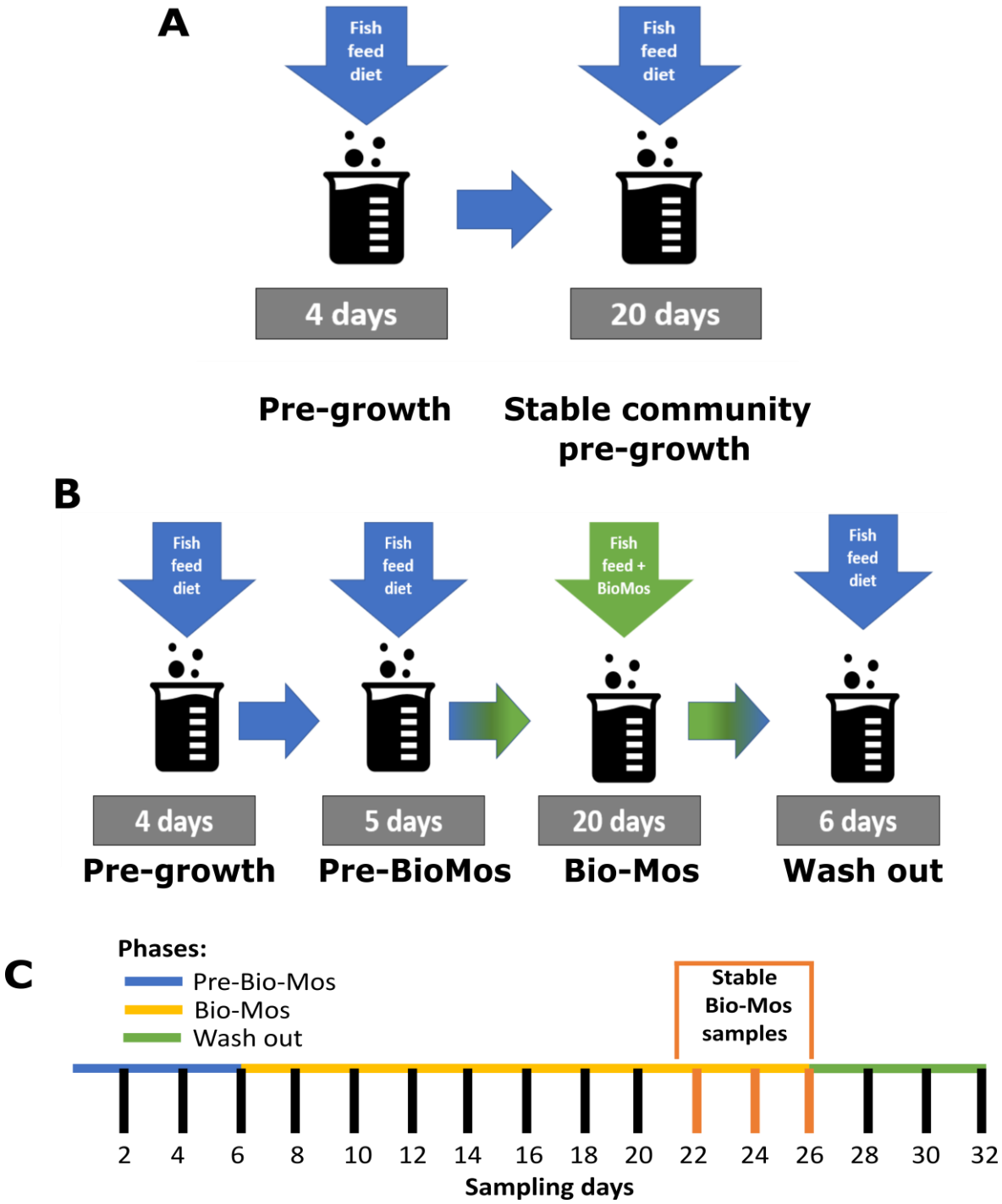


Figure 4-2 *In vitro* trial setup. ***A*** *Stable community pre-growth run within the SalmoSim system;* ***B*** *Main experimental run that involved four stages: (i) pre-growth (without feed transfer for 4 days), (ii) feeding system with Fish meal (Pre-Bio-Mos: 5 days), (iii) feeding system with Fish meal diet supplemented with Bio-Mos (Bio-Mos: 20 days), (iv) wash out period during which system was fed Fish meal without the addition of prebiotic (Wash out: 6 days);* ***C*** *SalmoSim sampling time points, which include definition of stable time points for Bio-Mos phase (days 22, 24, and 26 - once bacterial communities had time to adapt to Bio-Mos addition).*

4.3.4 Assaying Bio-Mos impact on microbial communities in the SalmoSim *in vitro* system

Frozen pre-grown stable pyloric caeca samples were thawed on ice and added to the SalmoSim system with each 500 ml bioreactor inoculated using bacterial communities pre-grown from a different fish. The system was run in several stages: (i) 4-day initial pre-growth period without feed transfer (Pre-growth), (ii) 5-day period during which SalmoSim was fed without prebiotic (Pre-Bio-Mos), (iii) 20-day period during which SalmoSim was fed on feed supplemented with Bio-Mos (Bio-Mos), (iv) 6-day wash out period during which SalmoSim was fed on Fish meal diet without addition of prebiotic (Wash out). The schematic representation of the experimental design is visually represented in Figure 4-2B. Sixteen samples were collected throughout the experimental run as described previously (one per each biological replicate at each sampling time point) (Figure 4-2C) (Kazlauskaite et al. 2020).

4.3.5 Genomic DNA extraction and NGS library preparation

DNA extraction and NGS library preparation protocols were previously described (Heys et al. 2020a, Kazlauskaite et al. 2020). Briefly, the samples collected from SalmoSim system and stable pre-grown inoculums were thawed on ice and exposed to bead-beating step for 60 seconds by combining samples with MP Biomedicals™ 1/4" ceramic sphere (Thermo Fisher Scientific, USA) and Lysing Matrix A Bulk (MP Biomedicals, USA). DNA was then extracted by using the QIAamp® DNA Stool kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol (Claassen et al. 2013). After, extracted DNA was amplified using primers targeting the V1 bacterial rDNA 16S region under the following PCR conditions: 95°C for ten minutes, followed by 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by a final elongation step of 72°C for 10 minutes. The second-round PCR, which enabled the addition of the external multiplex identifiers (barcodes), involved six cycles only and otherwise had identical reaction conditions to the first round of PCR. This was followed by the PCR product clean-up using Agencourt AMPure XP beads (Beckman Coulter, USA) according to the manufacturers' protocol and gel-purification using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA). Finally, the PCR products were pooled together

at 10 nM concentration and sent for sequencing using a HiSeq 2500 sequencer by Novogene.

4.3.6 NGS data analysis

NGS data analysis was undertaken as described previously (Kazlauskaite et al. 2020). In short, to determine microbial community stability within the SalmoSim system over time, two alpha diversity metrics (effective microbial richness and evenness (effective Shannon)) were calculated using the Rhea package (Lagkouvardos et al. 2017a) and visualised by using the microbiomeSeq package based on the phyloseq package (McMurdie and Holmes 2013, Ssekagiri et al. 2017).

To provide an overall visualisation of microbial composition across all samples, Principal Coordinates Analysis (PCoA) was performed by using the phyloseq package (Love et al. 2017, Ssekagiri et al. 2017) with Bray-Curtis dissimilarity measures calculated using the vegdist function from the vegan v2.4-2 package (Oksanen et al., 2013). Bray-Curtis distances were calculated for four different datasets: the full dataset (containing all biological replicates together), and three different subsets each containing only one of the three biological replicate samples from SalmoSim: Fish inoculum 1, 2, or 3.

To further compare microbial structure between various experimental phases, beta diversity was calculated for two different datasets: (i) all (complete data set containing all the samples sequenced) and (ii) subset (containing all samples for Pre-Bio-Mos and Wash out period, but only stable samplings from Bio-Mos period (time points 22, 12 and 26)). From these datasets ecological distances were computed using Bray-Curtis and Jaccard distances with the vegdist function from the vegan v2.4-2 package (Oksanen et al., 2013). Furthermore, the phylogenetic distances were computed for each dataset using GUniFrac distances (generalised UniFrac) at the 0% (unweighted), 50% (balanced) and 100% (weighted) using the Rhea package (Lagkouvardos et al. 2017b). Both ecological and phylogenetic distances were then visualised in two dimensions by Multi-Dimensional Scaling (MDS) and non-metric MDS (NMDS) (Anderson 2001). Finally, a permutational multivariate analysis of variance (PERMANOVA) was performed using distance matrices (including phylogenetic distance) to explain sources of variability in the

bacterial community structure as result of changes in recorded parameters (Anderson 2001).

To identify differentially abundant OTUs between various experimental phases (Pre-Bio-Mos, Bio-Mos and Wash out), differential abundance was calculated using the microbiomeSeq package based on the DESeq2 package (Love et al. 2017, Ssekagiri et al. 2017). Results were then summarised using bar plots at the genus level, identifying a number of OTUs belonging to a specific genus level that increase or decrease between various experimental phases.

To identify OTUs that correlated with measured VFAs, the Pearson correlation coefficient ($r>0.8$) was calculated between taxonomic variables (OTUs) and VFA values measured, and visualised using tools supplied by the Rhea package within different experimental phases (Pre-Bio-Mos, Bio-Mos, and Wash out) (Lagkouvardos et al. 2017b).

Finally, in order to analyse microbial community structure within different experimental phases, network analysis using Spearman correlation ($r>0.8$) was performed on three datasets: (i) all Pre-Bio-Mos samples, (ii) stable Bio-Mos samples (samples from days 22, 24, and 26), and (iii) all Wash out samples. Key network characteristics (degree and centrality betweenness) were compared between the three experimental phases: Pre-Bio-Mos, Bio-Mos, and Wash out. All these comparisons were analysed and visualised using the "ggstatsplot" package.

4.3.7 Protein fermentation and Volatile Fatty Acid (VFA) analysis

At each sampling point, microbial protein fermentation was assessed by measuring the protein concentration using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and the ammonia concentration using Sigma-Aldrich® Ammonia Assay Kit (Sigma-Aldrich, USA). Both methods were performed according to manufacturer protocol by using a Jenway 6305 UV/Visible Spectrophotometer (Jenway, USA). For VFA analysis, nine samples from each pyloric caecum compartment were collected (from 3 biological replicates): 3 samples from the Pre-Bio-Mos period (days 2-6), 3 samples from stable time points from the period while SalmoSim was fed on feed supplemented with Bio-Mos (days

22-26), and 3 samples from the Wash out period (days 28-32). VFA sampling was performed as described previously (Kazlauskaite et al. 2020). Extracted VFAs were sent for gas chromatographic analysis at the MS-Omics (Denmark).

In order to establish whether VFA concentrations were statistically different between different experimental phases (Pre-Bio-Mos, Bio-Mos and Wash out), a linear mixed effect model was deployed (Model 1) considering time point (sampling time point) and run (biological replicate of SalmoSim system) as random effects.

Model 1 = lmer(VFA~ Phase+(1|Time point)+(1|Run))

Finally, in order to establish whether ammonia production changed throughout the experimental run, a linear mixed effect model was deployed (Model 2) treating run biological replicate (of SalmoSim system) as random effect.

Model 2 = lmer(ammonia concentration~ Time point+(1|Run))

4.4 Results

In order to explore the impact of the Bio-Mos prebiotic on microbial communities in SalmoSim, microbial amplicons in different experimental phases (Pre-Bio-Mos, Bio-Mos and wash out) were surveyed using Illumina NovaSeq amplicon sequencing of the 16S V1 rDNA locus. In total 11.5 million sequence reads were obtained after quality filtering. Alpha diversity metrics (effective richness in Figure 4-3A and effective Shannon diversity in Figure 4-3B) indicated that the initial inoculum contained the lowest number of OTUs and had the lowest bacterial richness compared to later sampling time points from the SalmoSim system, but these differences were not statistically significant. Furthermore, this figure indicates no statistically significant differences between different experimental phases (Pre-Bio-Mos, Bio-Mos and Wash out) both in terms of effective richness and Shannon diversity. Taken together, diversity and richness estimates suggest non-statistically significant increase in the number of detectable microbial taxa as a result of transfer into SalmoSim system, but overwise stable diversity and richness over the different experimental phases.

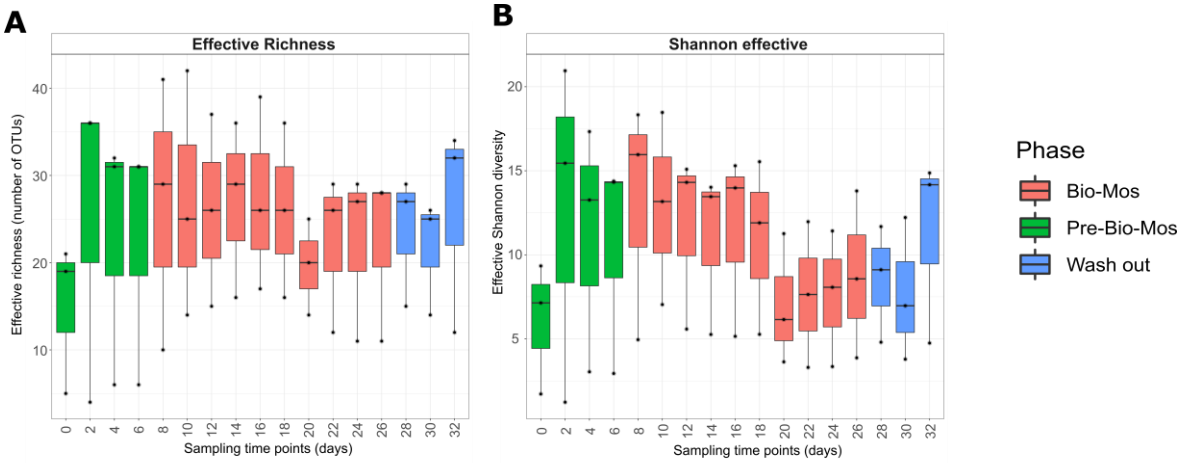


Figure 4-3 Alpha-diversity dynamics within the SalmoSim system during exposure to Bio-Mos prebiotics. *The figure represents different alpha diversity outputs at different sampling time points (days) from the SalmoSim system. Time point 0 represents microbial community composition within the initial SalmoSim inoculum from the pre-grown stable bacterial communities, time points 2-6 identify samples from the SalmoSim system fed on the Fish meal diet alone (Pre-Bio-Mos: green), time points 8-26 identify samples from the SalmoSim system fed on a Fish meal diet with addition of Bio-Mos (Bio-Mos: red), and time points 28-32 identify samples from the wash out period while SalmoSim was fed on feed without addition of the prebiotic (Wash out: blue).* ***A*** *visually represents effective richness (number of OTUs),* ***B*** *represents effective Shannon diversity.*

Biological replicate (the founding inoculum of each SalmoSim run) appears to be a major driver of community composition in the experiment (Figure 4-4A). This is supported by Figure 4-5 that visually represents varying microbial composition within different fish. Only when individual SalmoSim replicates were visualised separately in PCoA plots, do the changes to microbial communities in response to the different experimental phases become apparent (Figure 4-4B-D). These results indicate that bacterial communities shift from Pre-Bio-Mos to Bio-Mos, but they remain fairly stable (statistically similar, $p>0.05$ in majority of cases) between Bio-Mos and Wash out periods as reflected by beta diversity results summarised in Table A4-1. However, community shifts do not necessarily occur along the same axes in each SalmoSim replicate indicative, perhaps, of a different microbiological basis for that change. This trend is confirmed in Figure 4-5 that indicates a more substantial shift in microbial community profile between Pre-Bio-Mos and Bio-Mos phases for Fish 2 and 3, but to a lesser extent for Fish 1. These results were confirmed by performing beta-diversity analysis using both phylogenetic and ecological distances, both of which indicated statistically significant differences between Pre-Bio-Mos and Bio-Mos phases, but not between Bio-Mos and Wash out periods (Table A4-1). Furthermore, Table A4-1 indicates that 149 OTUs were found

to be differentially abundant between Pre-Bio-Mos and Bio-Mos phases, while only 5 OTUs were differentially abundant between Bio-Mos and Wash out phases.

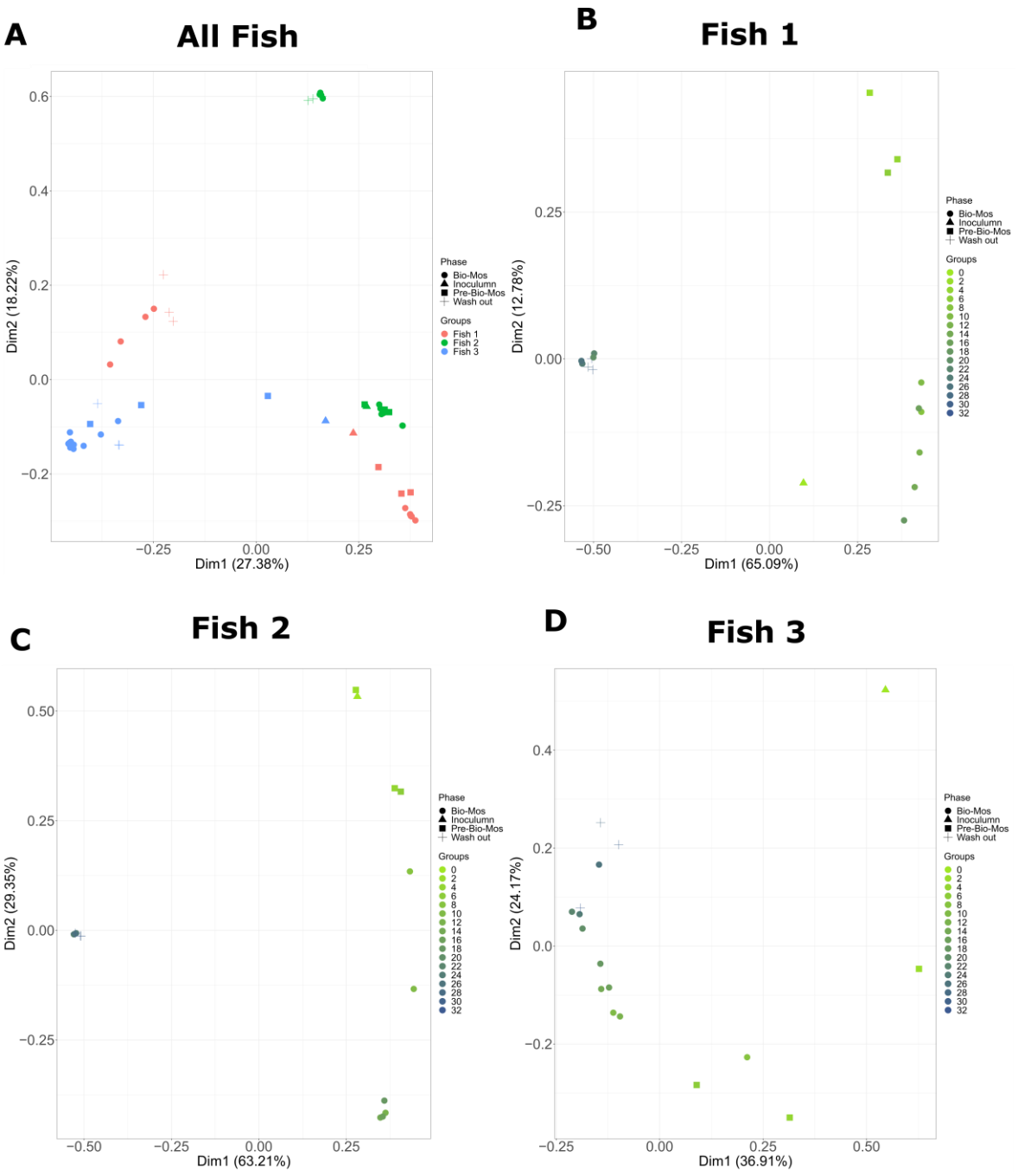


Figure 4-4 Beta diversity plots visualising bacterial communities dissimilarities within the SalmoSim bioreactors during exposure to Bio-Mos prebiotic. *In the PCoA plots, Bray-Curtis distance was used between samples originating from different experimental phases (Inoculum, Pre-Bio-Mos, Bio-Mos and Wash out), annotated with sampling time points and biological replicates.* ***A*** *represents all sequenced data together for all 3 biological replicates in which different colours represent different biological replicates (samples from pyloric caecum from 3 different fish) and different shapes represent different experimental phases (Inoculum, Pre-Bio-Mos, Bio-Mos and Wash out);* ***B-D*** *represent sequenced data for each individual biological replicate (****B****: Fish 1,* ***C****: Fish 2,* ***D****: Fish 3). In figures B-D different colours represent different sampling time points and different shapes represent different experimental phases (Inoculum, Pre-Bio-Mos, Bio-Mos and Wash out). Dim 1 is principal coordinate 1 and Dim 2 is principle coordinate 2.*

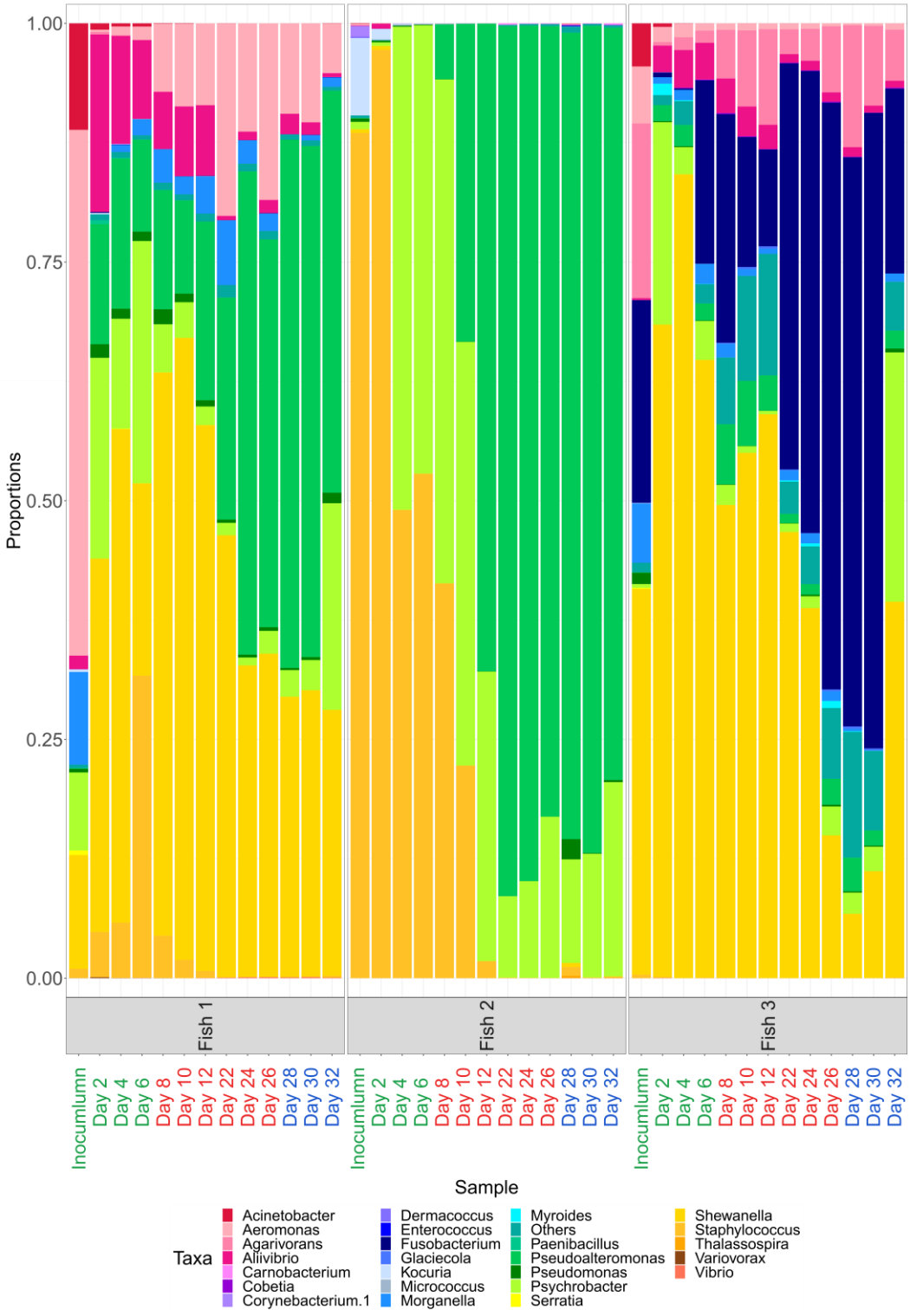


Figure 4-5 Microbial composition (25 most common genus + others) amongst different biological replicates and experimental phases. *Labels on X axis in green represent samples from Pre-Bio-Mos phases, in red samples fed on Bio-Mos phase and in blue samples from Wash out period. Only a subset of time points is visualised for each phase: time points 2- 6 for Pre-Bio-Mos, 8-12 and 22-26 for Bio-Mos, and 28-32 for Wash out.*

To compare experimental phases in more detail, differentially abundant OTUs between various experimental phases were summarised in bar plots at a genus level in Figure 4-6. Figure 4-6A indicates that between Pre-Bio-Mos and Bio-Mos phases, more OTUs decreased in abundance, rather than increased. The OTUs that differentially increased from Pre-Bio-Mos to Bio-Mos phase were identified to belong to: *Aeromonas* (higher proportion increased (50%) rather than decreased (12.5%)), *Agarivorans, Aliivibrio, Carnobacterium* (only showed increase and no decrease), *Fusobacterium, Pseudoalteromonas, Pseudomonas, Psychobacter,* and *Shewanella*. Figure 4-6B indicates the increase of OTUs belonging to *Enterococcus* and *Thalassospira* genera between Bio-Mos and Wash out, while OTUs belonging to *Micrococcus*, *Myroides,* and *Shewanella* genera have decreased.

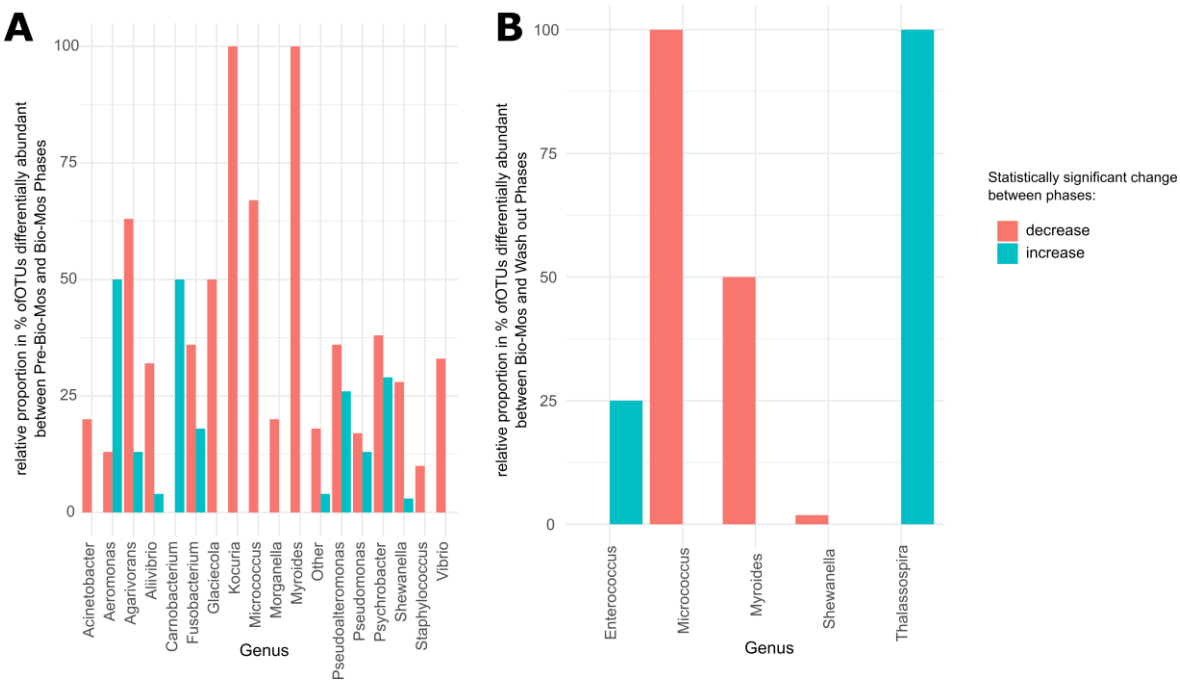


Figure 4-6 Differential abundance of OTUs grouped at genus level between different experimental phases (Pre-Bio-Mos, Bio-Mos and Wash out). *Differentially abundant OTUs grouped at genus level between different experimental phases: Pre-Bio-Mos vs Bio-Mos (**A**), Bio-Mos vs Wash out (**B**). Red and blue represents statistically significant (p<0.05) decrease and increase respectively between the experimental phases compared.*

For the analysis of the microbial community structure throughout the experiment, three OTU co-occurrence networks were analysed for each phase (Pre-Bio-Mos, Bio-Mos, and Wash out), and the main network characteristics were compared: the degree, and centrality betweenness (Figure A4-1). Pre-Bio-Mos phase (Figure A4-1A) indicates a higher average degree (number of edges per node) than in the

Bio-Mos or Wash out phases. However, the median of degrees is much higher in the Bio-Mos phase compared to the Pre-Bio-Mos, suggesting that during the Pre-Bio-Mos phase there were clusters of interacting OTUs (one cluster with a high degree and another with lower degree). As such, the distribution of connectivity is more uniform in the Bio-Mos phase, compared to the Pre-Bio-Mos. Moreover, the average of betweenness centralities (centrality measure based on the shortest paths between nodes) is higher in Bio-Mos and Wash out phases compared to the Pre-Bio-Mos phase (Figure A4-1B).

These results are visually represented in Figure 4-7, which indicates that statistically significant increases were found between Pre-Bio-Mos and Bio-Mos phases in formic, propanoic and 3-methylbutanoic acid concentrations. No significant differences in any VFA production by the system was noted between Bio-Mos and Wash out periods.

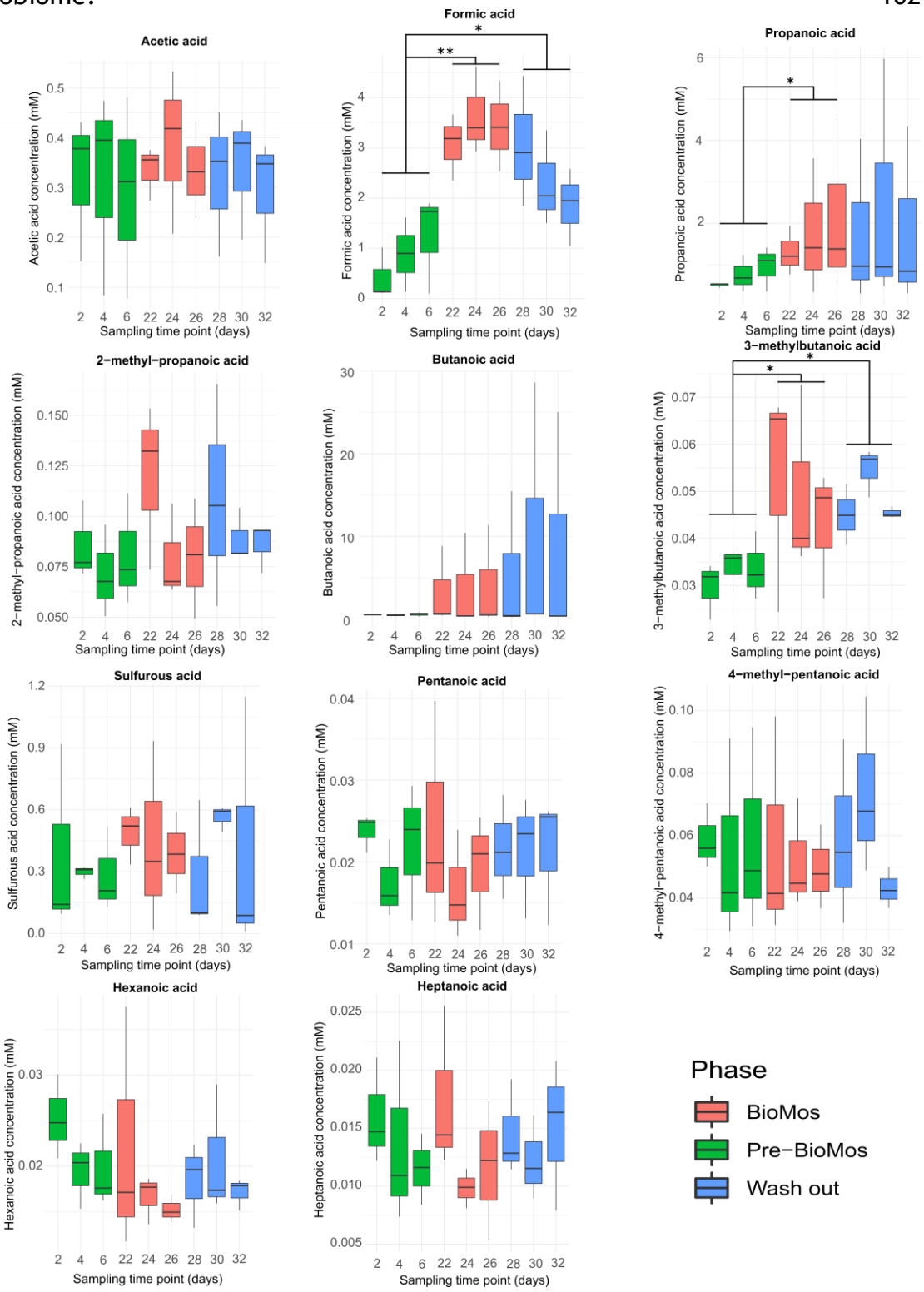


Figure 4-7 VFA responses in SalmoSim pyloric caecum compartment after Bio-Mos introduction and subsequent wash out period. *The figure above represents production of 11 volatile fatty acids in three different experimental phases: (i) SalmoSim fed on Fish meal alone without prebiotic addition (Pre-Bio-Mos: green), (ii) SalmoSim fed on Fish meal with addition of Bio-Mos (Bio-Mos: red), (iii) wash out period during which SalmoSim was fed on Fish meal without Bio-Mos (Wash out: blue). X axis represents the concentration of specific volatile fatty acid (mM) while the Y axis represents different sampling time points (days). The lines above bar plots represent statistically significant differences between different experimental phases. The asterisks show significance: (*: 0.01 ≤ p < 0.05; **: 0.05 ≤ p < 0.001; ***: p ≤ 0.001).*

Results shown in Figure 4-8 identify that in the Bio-Mos phase alone, a number of OTUs which showed a strong correlation with various VFAs, had already been picked up by differential abundance analysis (Figure 4-6), identifying statistically significant increases. OTUs belonging to *Agarivorans* and *Fusobacterium* genera were found to be positively correlated with propanoic and formic acid, but negatively correlated with 3-methyl butanoic acid. An OTU belonging to *Pseudoaltermonas* genus was found to be positively correlated with propanoic acid, but negatively correlated with 3-methyl butanoic acid, while other OTUs belonging to *Pseudoaltermonas* genus were found to be negatively correlated to propanoic acid. Finally, one OTU belonging to *Fusobacterium* was found to be negatively correlated to 3-methyl butanoic acid. While within Pre-Bio-Mos and Wash out phases, statistically significant Pearson correlations ($r>0.8$) were also identified between various OTUs and VFAs, however, these OTUs were not picked up in differential abundance analysis between those phases (Figure 4-6).

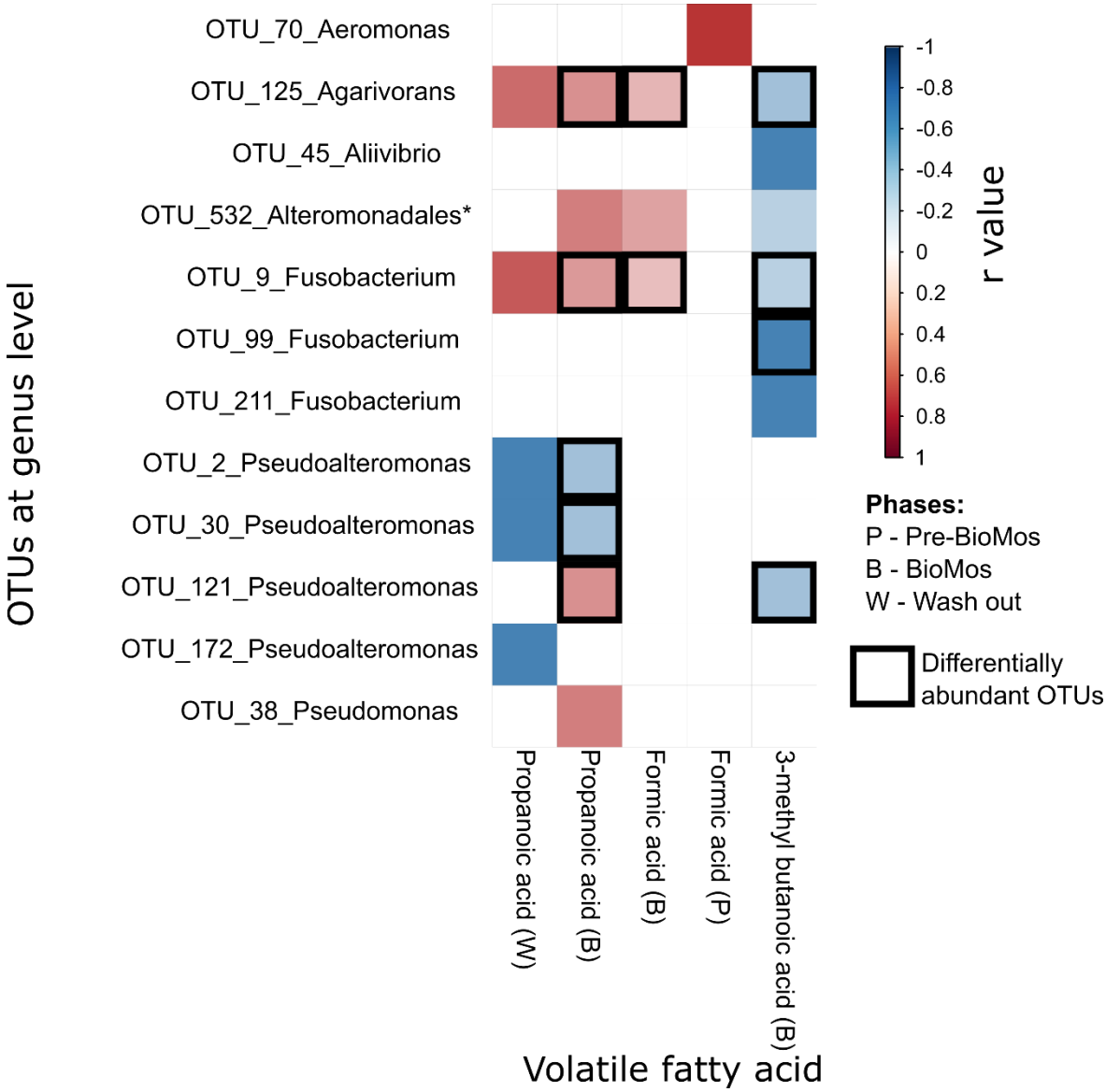


Figure 4-8 Pearson correlation coefficients across VFAs and taxonomic variables. *Statistically significant ($p<0.05$) and strongly correlated ($r>0.8$) Pearson correlation coefficients across a set of VFAs (that showed statistically significant change between feeds: propanoic, formic and 3-methyl butanoic acids) and taxonomic variables (OTUs summarised at genus level apart from * to order level) are shown in various experimental phases (Pre-Bio-Mos, Bio-Mos and Wash out). Blue colour represents negative correlations and red colour represents positive correlations, respectively. The boxes indicate that these OTUs in differential abundance analysis showed statistically significant increase from Pre-Bio-Mos to Bio-Mos phase*.

Figure 4-9 summarises measured ammonia (NH_3) concentration changes through the experiment. The data indicates a statistically significant increase in ammonia production between time points 2 and 4, and between time points 20 and 22, and statistically significant decrease in ammonia concentration between time points 30 and 32.

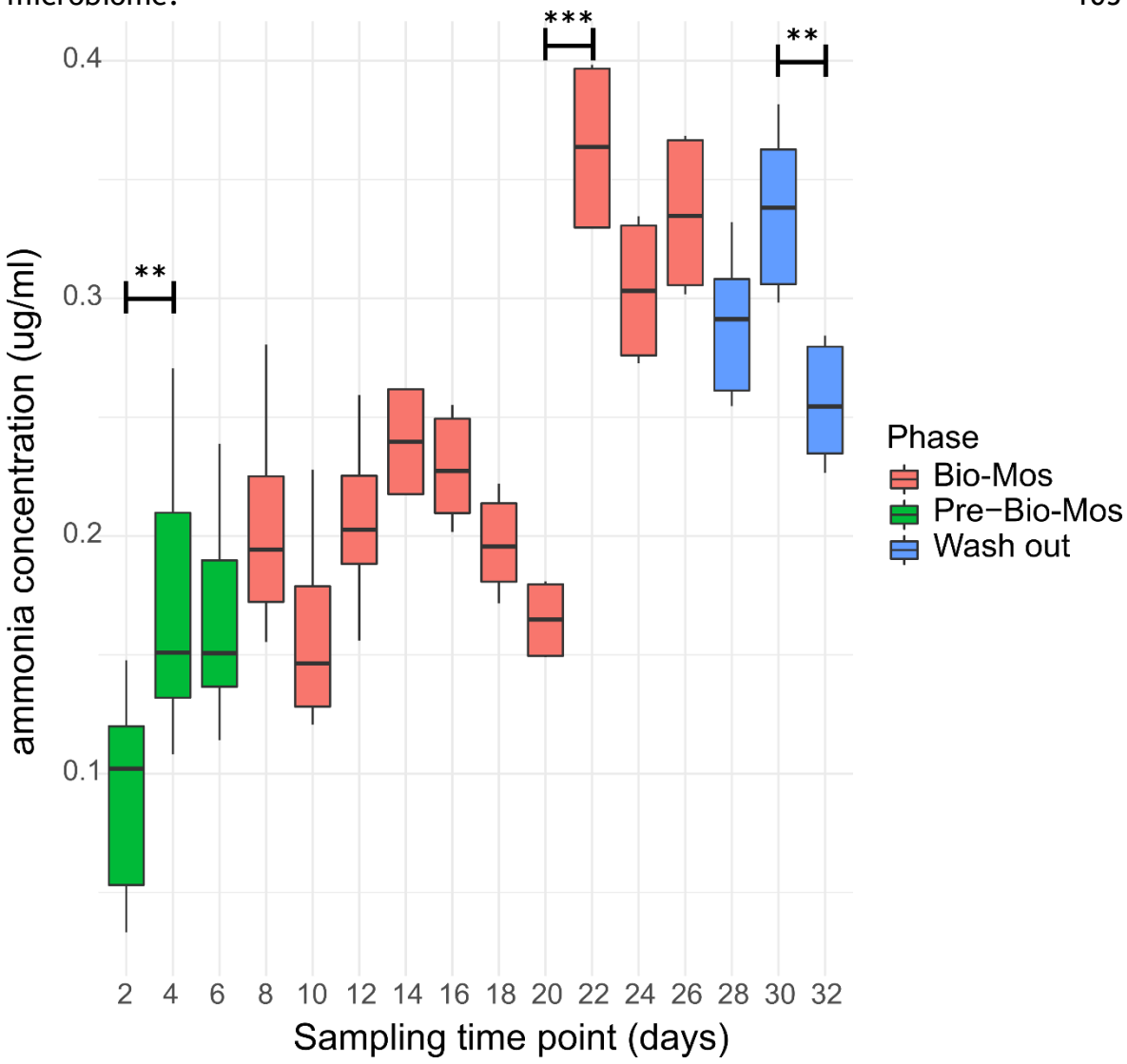


Figure 4-9 ammonia (NH_3) concentration in SalmoSim pyloric caecum compartment throughout experiment. *Ammonia (NH_3) production in three different experimental phases: (i) SalmoSim fed on Fish meal alone without prebiotic addition (Pre-Bio-Mos: green), (ii) SalmoSim fed on Fish meal with addition of Bio-Mos (Bio-Mos: red), (iii) wash out period during which SalmoSim was fed on Fish meal without Bio-Mos (Wash out: blue). X axis represents the concentration of ammonia (µg/ml) while the Y axis represents different sampling time points (days). The lines above bar plots represent statistically significant differences between sequential time points. The asterisks show significance: (*: 0.01 ≤ p < 0.05; **: 0.05 ≤ p < 0.001; ***: p ≤ 0.001)*

4.5 Discussion

Our study aimed at elucidating the effect of a commercially available MOS product (Bio-Mos) on the microbial communities within the gut content of Atlantic salmon using a newly developed artificial salmon gut simulator 'SalmoSim'. Inclusion of Bio-Mos within the tested feed did not affect microbial community diversity and richness in the SalmoSim system, nor did subsequent removal of the prebiotic during wash out. The biological replicate (the founding inoculum of each SalmoSim run) appears to be a major driver of variations in community composition and

structure throughout the experiment. This could be explained by the fact that feed used in the *in vitro* study was sterile, thus the bacterial communities retrieved within the SalmoSim system originated only from real salmon inoculums as in a previous experiment involving SalmoSim (Kazlauskaite et al. 2020). Our results indicate that bacterial community composition between Pre-Bio-Mos and Bio-Mos experimental phases was significantly different, but was statistically similar between Bio-Mos and Wash out periods. Similar trends were observed in the bacterial activity (VFA production) that showed statistically significant increases in formic, propanoic and 3-methylbutanoic acid concentrations during the shift from Pre-Bio-Mos to Bio-Mos phase, but no statistically significant change in bacterial activity between Bio-Mos and Wash out periods. The lack of change in bacterial composition and activity between Bio-Mos and Wash out period could be explained by the short time frame of the Wash out period, lasting only 6 days, compared to the 20-day Bio-Mos phase. This is potentially not long enough to see a reversal any of changes driven by Bio-Mos. Finally, a statistically significant increase in the ammonia production during Bio-Mos phase was observed at the later time points (between days 20 and 22), followed in the reduction in ammonia concentration during the Wash out period (between days 30 and 32), the potential drivers of which are discussed below.

Several studies have shown that in vertebrates (e.g. chicken, mouse, turkey) supplementing feed with MOS increases the production of propionate and butyrate by gut bacteria (Zdunczyk et al. 2005, Pan et al. 2009, Ao and Choct 2013), while other studies have not reported any effect of MOS on the VFA production (Gürbüz et al. 2010). In our study we report a statistically significant increase in the production of formic, propanoic and 3-methylbutanoic acids in the SalmoSim system associated with feed supplemented with Bio-Mos. In humans propionate is commonly absorbed and metabolised by the liver, where it impacts host physiology via regulation of energy metabolism (El Hage et al. 2020). It has also been associated with healthy gut histological development and enhanced growth in fish and shellfish (da Silva et al. 2016, Wassef et al. 2020). Formic acid, although frequently deployed as an acidifier in monogastrics to limit the growth of enteric pathogens (Luise et al. 2020), is not known to directly impact host phenotype. Similarly, except as the rare genetic disorder that occurs in humans, isovaleric acidemia, where the compound accumulates at high levels in the absence of

isovaleric acid-CoA dehydrogenase activity in host tissues (Vockley and Ensenauer 2006), isovaleric (3-methylbutanoic) acid is not expected to directly impact host phenotype either.

Further analysis identified that an increase in formic acid during the Bio-Mos phase positively correlated with OTUs belonging to *Agarivorans* (facultative anaerobic) and *Fusobacterium* (anaerobic) genera. While an increase in propanoic acid during the Bio-Mos phase also positively correlated with OTUs belonging to the *Agarivorans* (facultative anaerobic) and the *Fusobacterium* (anaerobic) genera as well as the *Pseudoaltermonas* (facultative anaerobic) genus, a negative correlation was found with two OTUs belonging to the same *Pseudoaltermonas* (facultative anaerobic) genus. Finally, only negative correlations were identified between the increased amount of 3-methyl butanoic acid in the Bio-Mos phase and OTUs belonging to *Pseudoaltermonas* (facultative anaerobic), *Fusobacterium* (anaerobic) and *Agarivorans* (facultative anaerobic) genera. All of these OTUs were found to not only be correlated with increased VFAs, but also to be differentially abundant between Pre-Bio-Mos and Bio-Mos phases, providing circumstantial evidence for a link between these microbes and the measured metabolites. The causal directionality between these genera and the respective VFAs is hard to establish. A strong positive correlation has been found previously in humans between the *Fusobacterium* genus and propanoic acid concentration (Riordan 2007). Propionate is a key substrate that can metabolised by several classes of methanogenic anaerobes (Mah et al. 1990) and may be driving the growth of the genera noted here. Equally, propionate is a major product of microbial metabolism of amino acids (Louis and Flint 2017), and it is likely here that more efficient protein metabolism in the system by certain genera is driving its abundance. An increase in ammoniacal nitrogen (ammonia) production was noted after the addition of Bio-Mos in all three replicates, albeit with a noticeable lag. Furthermore, although formate, propionate, isovalerate and ammonia show a downward trend after the removal of Bio-Mos, seemingly a longer wash-out period is required to allow VFA and ammonia levels to recover to their pre-Bio-Mos levels.

Previously published research has suggested that feed supplementation with MOS modulates immune response in animals by stimulation of the production of mannose-binding proteins which are involved in phagocytosis and activation of the

complement system (Franklin et al. 2005, Taschuk and Griebel 2012). Such relationships with host immunity are difficult to predict with a simplified *in vitro* system. It is thought the feed supplementation with MOS elevates the immune response within the host by increasing the lactic acid bacteria (LAB) levels in common carp (Momeni-Moghaddam et al. 2015). In the present study, an increase in differential abundance of *Carnobacterium* (LAB bacteria) from Pre-Bio-Mos to Bio-Mos phases was observed. This bacterial genus has been proposed as a potential probiotic when present within Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Robertson et al. 2000). The use of Carnobacteria as probiotics were shown to be correlated with increased survival of the larvae of cod fry and Atlantic salmon fry (Gildberg et al. 1995), rainbow trout (Irianto and Austin 2002), and salmon (Robertson et al. 2000). A fishmeal-based diet with limited carbohydrate content was used to perform this experiment, and has been previously linked to lower abundances of lactic acid producing bacteria when compared to microbial gut composition of Atlantic salmon fed on plant-based feed (Reveco et al. 2014). To enhance LAB growth even further alongside MOS in protein rich diets, some carbohydrate supplementation may be necessary.

Network analysis suggested a change in the distribution of connectivity of the microbial network during the Bio-Mos phase as compared to the Pre-Bio-Mos phase. The microbial network during the Bio-Mos phase shows higher modularity (nodes in the network tend to form denser modules), that is also reflected by a higher average of betweenness centralities within the Bio-Mos phase, a measure which represents the degree of interactive connectivity between nodes. Thus, feed supplementation with Bio-Mos may be correlated with more frequent species-species interactions, and a greater stability of network structure within the network. Stable microbial communities are also though to contribute to pathogen colonisation resistance via nutrient niche occupancy (Stecher et al. 2013, Romero et al. 2014, Xiong et al. 2019). However, a challenge experiment would be required to test this assertion.

4.6 Conclusions

Our study indicates a positive correlation between Bio-Mos supplementation and production of propanoic and formic acids, both of which are known to benefit

animal microbiome and health (Haque et al. 1970, EFSA 2014). Although, our *in vitro* model lacks a host component, previous studies involving the use of gut simulators to analyse the effectiveness of various pre-biotics were shown to produce similar results to *in vivo* trials (Sivieri et al. 2014, Duysburgh et al. 2020). Our data highlights the potential usefulness of various *in vitro* gut systems in fin fish aquaculture to study the effectiveness of feed additives.

Chapter 5 Contributions, Discussion and Conclusion

5.1 Research highlights

The use of *in vitro* systems to study and model the microbial communities of monogastric vertebrates is becoming increasingly widespread, with systems simulating: *Sus scrofa* (pig) (Tanner et al. 2014), *Gallus gallus* (chicken) (Card et al. 2017), *Canis lupus* (dog) (Duysburgh et al. 2020) and other vertebrate guts. Using *in vitro* gut simulators is also a widely accepted approach to study the human gut microbiome (Déat et al. 2009, Van Den Abbeele et al. 2010, Kim et al. 2016). An aim of this thesis was to design, build, validate, and use in practice an artificial Atlantic salmon gut simulator, SalmoSim.

The work conducted in Chapter 2 offered a foundation to both the design of the SalmoSim system and methods used in Chapters 3 and 4. The physiochemical conditions, required to design an Atlantic salmon gut simulator (such as pH, dissolved oxygen, temperature, flow rates, and more), were identified via experimentation, literature review, and data from industry partners. These conditions were then put into practice via a preliminary twenty-day SalmoSim replicate run study in which two identical SalmoSim runs were performed and the variability (experimental error) between two runs of the system via next generation sequence profiling of the 16S rRNA gene were assayed. Unfortunately, statistical analysis could not be performed on the current experiment due to insufficient replication as a result of a failure during one of the triplicate runs. Our results suggest visual differences between the SalmoSim system replicate runs using alpha diversity metrics. However, shared OTU analysis indicated that these differences were driven by rare OTUs, which comprise less than 1% of the total reads in both runs. Visual analysis of the alpha diversity metrics over time indicated that effective richness (number of OTUs) and effective Shannon diversity remained largely static throughout each replicate run regardless of gut compartment, with no observed visual pattern. Furthermore, this study identified a visual difference between the initial inoculum and SalmoSim system, in particular, the loss of the *Mycoplasma* genus in pyloric caecum and mid gut compartments. Finally, our results suggest that the microbial composition within

the stomach compartment is statistically different from the other two compartments.

The preliminary run performed in Chapter 2 acted as a pilot to identify future pitfalls and improvements allowing the enhancement of SalmoSim runs for Chapters 3 and 4. In order to improve the replicability between different replicate runs, the SalmoSim protocols were adapted for Chapter 3 and 4 by producing a pool of bile and mucous, and sampling at the same time of the day, as well as, considering run as a random effect during statistical analysis. Furthermore, to improve reproducibility, the SalmoSim system was further adapted in Chapter 4 to run triplicate experiments in parallel. In order to prevent the loss of taxa from founding inoculum to SalmoSim, in Chapters 3 and 4 the initial inoculum samples from real salmon were collected and processed under anaerobic conditions and a snap freezing method in liquid nitrogen was utilised instead of addition of glycerol before long term storage in -80^{o}C.

In Chapter 3 I focused on validating the system as a potential means of testing the impact of feeds on salmon gut microbial communities by comparing the performance and response of the *in vitro* simulator during a feed trial with parallel *in vivo* modulation of the gut microbial community in a cohort of marine phase Atlantic salmon. As in the trial SalmoSim replicate experiment described in Chapter 2, the SalmoSim microbiomes became increasingly stable over the first 20 days of the validation experiment. Furthermore, while in Chapter 2 the data identified the loss of taxa during the transfer of inoculum to the SalmoSim system, in the validation study SalmoSim microbiomes were indistinguishable ($p=0.230$) from their founding inocula at 20 days and the most abundant genera (e.g. *Psycrobacter*, *Staphylococcus*, *Pseudomonas*) proliferated within SalmoSim (OTUs accounting for 98% of all reads shared with founding communities), which indicates the success of the adapted inoculum collection protocol from Chapter 2. Regarding the validation, real salmon and SalmoSim responded similarly to the introduction of novel feed, with the majority of the taxa (96% Salmon, 97% SalmoSim) unaffected, while a subset of taxa (e.g. a small fraction of *Psychrobacter*) were differentially affected across both systems. Consistent with a low impact of the novel feed on microbial fermentative activity, volatile fatty acids profiles were not significantly different in SalmoSim pre- and post-feed

switch. By establishing stable and representative salmon gut communities, this study represents an important step in the development of an *in vitro* gut system as a tool for the improvement of salmon nutrition and welfare.

Overall, our results from the validation study in Chapter 3 indicate that SalmoSim can not only stably maintain the most abundant microbial communities from real salmon, but also demonstrates similar responses to experimental feed treatments as those seen in real salmon. These results are encouraging, however, given this study utilised a switch between two similar feeds that had little effect on the gut microbiota *in vivo,* further experimentation with SalmoSim would be beneficial. For example, the survival and influence of probiotics within the system or the influence of known prebiotics could also be assessed, as has been previously studied in other *in vitro* gut systems (Duysburgh et al. 2020). Thus, in Chapter 4 we assess Bio-Mos (commercially available pre-biotic currently used in the agri-feed industry) for potential use as a prebiotic growth promotor in salmonid aquaculture. Taking results from Chapter 2 we modified SalmoSim (enabling performance of triplicate experiment in parallel), to evaluate the impact of Bio-Mos on the host microbial communities. Inoculated from biological triplicates of adult farmed salmon pyloric caeca compartments, the microbial communities were stabilised in SalmoSim for 20-days as determined in Chapters 2 and 3, followed by a twenty-day exposure to the prebiotic and in turn followed by an six day 'wash out' period. Dietary inclusion of MOS resulted in a significant increase in formate ($p=0.001$), propionate ($p=0.037$) and isovalerate ($p=0.024$) levels, correlated with increased abundances of several, principally, anaerobic microbial genera (*Fusobacterium, Agarivorans, Pseudoalteromonas*). DNA metabarcoding with the 16S rDNA marker confirmed a significant shift in microbial community composition in response to MOS supplementation, with an observed increase in lactic acid producing *Carnobacterium*. In conjunction with previous *in vivo* studies linking enhanced volatile fatty acid production alongside MOS supplementation to host growth and performance, the data in Chapter 4 suggests that Bio-Mos may be of value in salmonid production. Finally, our data highlights the potential role of *in vitro* gut models to augment *in vivo* trials of microbiome modulators.

5.2 Future Research

5.2.1 Adapting the SalmoSim system for other fish species

Whereas studies have been performed using a simple *in vitro* batch method to study fermentation of various feeds using inocula of Nile tilapia (*Oreochromis niloticus*) and European sea bass (*Dicentrarchus labrax*), these systems lack complexity (Leenhouwers et al. 2008). In this thesis it was identified that in order to design and build an artificial gut simulator with similar complexity to SalmoSim the key parameters must be identified either via experimentation, literature review, or data from industry partners. These parameters include physiochemical conditions within fish's gut (pH, temperature, dissolved oxygen), feed media composition (for fish species raised in aquaculture setting the feed composition would need to be sourced from an industry partners), and flow rate between different gut compartments. If all these parameters can be determined, as well as access to fresh gut samples (for inoculation and collection of supplements, such as bile and mucous) of the fish species of interest, then there is no evidence to suggest that the adaptation of SalmoSim to other fish species (e.g. trout, carp) should not be possible. However, prior to application of these adaptations, re-validation of the system against an *in vivo* trial for the specific fish species would need to be carried out.

5.2.2 Introducing digestibility to SalmoSim system

In the aquaculture industry determination of the digestibility of nutrients in various feeds provides an indication of their nutritional value and is often considered as the first step in feed quality evaluation (Allan et al. 2000, Glencross et al. 2007, Liu et al. 2009, Luo et al. 2009). In order for the SalmoSim system to be used as a proxy for digestibility trials, and thus enhance its usefulness to the industry, the system and protocols would need to be adapted. While some *in vitro* systems used to assess digestibility in Atlantic salmon use enzymes sourced from pigs, and bovines, such as porcine pepsin/porcine trypsin, bovine chymotrypsin, and porcine peptidase (Stewart Anderson et al. 1993), the enzyme extracts from different parts of the digestive system from real salmon should be chosen to ensure accurate simulation of the *in vivo* digestion process. Thus, adaptations of SalmoSim should include the addition of enzyme extracts from the stomach and

pyloric caecum compartments of real salmon in order to hydrolyse ingredients within the feed being tested (Moyano et al. 2015). Furthermore, as the concentration of the digestive enzymes within the fish's gut fluctuates depending on circadian rhythms (Montoya et al. 2010, Yúfera et al. 2014) and different food intakes (feeding protocols) (Martínez-Llorens et al. 2021), the enzyme:subrate ratio for *in vitro* studies needs to be calculated. These calculations should include measurement of the range of values of total protease amylase, and lipolytic enzymes, released during gastric and intestinal digestion in fish of a specific size, and measuring the amount of protein in a feed, calculated by considering the daily ration for a specific fish size and the mean protein content of a commercial feed (Gilannejad et al. 2018). Furthermore, a dialysis loop could be introduced with a molecular weight cut-off, in order to filter out the low molecular weight products of digestion as they are formed as well as to strip out the nutrients, thus improving the microbiome model by making it more similar to real fish (Moyano et al. 2015). Finally, new methods need to be developed to assess the digestibility of various feeds and ingredients within SalmoSim system, such the Dumas method, to determine the total nitrogen content (Simonne et al. 1997), phthalaldehyde assay to determine the total amino acid absorption (Hamdan et al. 2009, Perera et al. 2010), and HPLC-MS (high performance liquid chromatograph – mass spectrometer) to determine the amino acid profile of the undigested sample and absorbed nutrients (Martinez-Montantildo et al. 2011, Márquez et al. 2013). The outputs of these analyses for various feeds and ingredients could then be compared to generic aquafeed protein ingredients, such as fish meal, feather meal, or soya meal.

5.2.3 Applying SalmoSim system to understand antimicrobial resistance transfer

In the past decade, antimicrobial compounds were increasingly used in agriculture and aquaculture to prevent and treat diseases as well as promoting animal growth (Van Boeckel et al. 2015). While several symbiotic commensals of the gut microbiome of higher organisms play a vital role in preventing the colonization by pathogens, the gastrointestinal tract can constitute a favourable environment for the transfer of multidrug resistance (MDR) plasmids. The gastrointestinal microbiome can act as a reservoir of antibiotic resistance genes that can be transferred to and from the resident microflora to pathogens and transient

colonizers, undermining the beneficial protective role of the commensal gut microbiota for the host as well as being cause for public concern (Haug et al. 2011, Card et al. 2017). *In vitro* systems such as bioreactors, fermenters and chemostats emulating the gastrointestinal tracts of different organisms offer ethical and safe *in vitro* platforms to model the selection, emergence, and dissemination of antibiotic resistance within the gut as well as the resulting repercussions on bacterial interactions. For example, an *in vitro* colonic fermentation model developed by Haug *et al.* to monitor horizontal antibiotic resistance gene transfer, demonstrated the transfer of a multiresistance plasmid to commensal bacteria in the presence of competing faecal microbiota, suggesting that commensal bacteria contribute to the increasing prevalence of antibiotic-resistant bacteria (Haug et al. 2011). Another *in vitro* system used to study the transmission of antimicrobial resistance used chemostats inoculated with human intestinal bacteria to study the effect of Tulathromycin (macrolide antibiotic used to treat bovine respiratory disease in cattle and swine) on colonization resistance, antimicrobial resistance, and virulence of the human gut microbiota (Hao et al. 2016). This study identified that the long-term exposure to high concentrations of Tulathromycin may damage the colonization resistance of the human gut microbiota and induce the development of antimicrobial resistance in *Enterococcus*. Finally, in a study using a recently developed *in vitro* chemostat system, which approximates the chicken caecal microbiota, colonization by an MDR Salmonella pathogen was simulated to examine the dynamics of transfer of its MDR plasmid harbouring the extended-spectrum beta-lactamase blaCTX-M1 (Card et al. 2017). The use of this system was successfully applied to analyse the impact of antibiotic administration on plasmid transfer and microbial diversity (Card et al. 2017). SalmoSim could be utilised to conduct amicrobial resistance transfer studies, using similar principles and experimental design outlined in these examples.

5.3 Concluding remarks

The work in this thesis has made important first steps in the designing and validating the artificial Atlantic salmon gut system SalmoSim. Future research can now use this validated system as is to address clear hypotheses focused on the Atlantic salmon microbiome interaction and response to various feeds, feed additives and drugs. Furthermore, the system could be improved further to start assessing digestibility. Finally, the steps of the system development described in

this thesis can be used as guidelines to develop various other systems representing other fish species.

Appendices

Appendix 1: Chapter 3 appendices

Table A3-1 16S rRNA gene-targeted group-specific primers. *Table lists primer sets that already published and validated in the literature. All primers were used on mouse faeces samples apart from Alphaproteobacterial specific primers that were used on marine biofilm samples.*

Group	Primer sequence	Source
Bacteroidetes F	GTTTAATTCGATGATACGCGAG	(Yang et al., 2015)
Bacteroidetes R	TTAASCCGACACCTCACGG	
Firmicutes F	GGAGYATGTGGTTTAATTCGAAGCA	
Firmicutes R	AGCTGACGACAACCATGCAC	
Actinobacteria F	TGTAGCGGTGGAATGCGC	
Actinobacteria R	AATTAAGCCACATGCTCCGCT	
Gammaproteobacteria F	TCGTCAGCTCGTGTYGTGA	
Gammaproteobacteria R	CGTAAGGGCCATGATG	
Betaproteobacteria F	AACGCGAAAAACCTTACCTACC	
Betaproteobacteria R	TGCCCTTTCGTAGCAACTAGTG	
Alphaproteobacteria F	CIAGTGTAGAGGTGAAATT	(Bacchetti De Gregoris et al., 2011)
Alphaproteobacteria R	CCCCGTCAATTCCTTTGAGTT	
General Bacteria F	ACTCCTACGGGAGGCAGCAGT	(Clifford et al., 2012)
General Bacteria R	TATTACCGCGGCTGCTGGC	
Mycoplasma F	AGCAGCCGCGGTAATACATAG	Generated by DECIPHER software*
Mycoplasma R	GAGCATACTACTCAGGCGGAT	
Lactobacillus F	CAGCAGTAGGGAATCTTCCACAA	
Lactobacillus R	CATGGAGTTCCACTCTCCTCTT	

Table A3-2 First round PCR primers used for the first round of NGS library preparation.

Primer	Name	Illumina 5′ sequencing primer (CS1/CS2)	Internal index	Heterogeneity spacer	16S rRNA gene v1 primer
Forward	27F	ACACTCTTTCCCTACACG ACGCTCTTCCGATCT	Index (8bp)	heterogeneity spacer (5/3 bp)	AGAGTTTGAT CMTGGCTCAG
Reverse	338R	GTGACTGGAGTTCAGAC GTGTGCTCTTCCGATCT	Index (8bp)	None	GCTGCCTCCC GTAGGAGT

Table A3-3 Second round PCR primers used for the NGS library preparation.

Primer name	Illumina MiSeq 3′ flow cell linker (i5/i7)	External index	Illumina 5′ sequencing primer (CS1/CS2)
Forward	AATGATACGGCGACCACCGAGATCTACAC	Index (8bp)	CACTCTTTCCCTACACGACGCT
Reverse	CAAGCAGAAGACGGCATACGAGAT	Index (8bp)	GTGACTGGAGTTCAGACGTGTGCTC

Table A3-4 OTUs prevalence analysis by sub-setting full dataset into multiple core OTUs. *The table summarises the number OTUs within each subset dataset (subset by the % of samples that share OTUs) and percentage of the total number of OTUs within the full dataset (100%). This table also shows the number of reads and the percentage of total reads (100%) within each of the subset datasets. Note: in the 60% subset three samples were lost as they did not retain any OTUs under that criteria: Id-val1-PC1, Id-Val2-MG4 and Id-Val2-PC1.*

% of samples that share OTUs	Number of OTUs (% of total OTUs)	Number of reads	% of total reads
80%	1 (0.10%)	77,528	2.18%
75%	1 (0.10%)	77,528	2.18%
60%	6 (0.61%)	186,285	5.23%
50%	13 (1.33%)	1,179,477	33.10%
40%	34 (3.48%)	2,796,009	78.47%
30%	65 (6.65%)	3,204,411	89.93%
All OTUs	978 (100%)	3,563,318	100%

Table A3-5 Bacterial group responses to feed change within different gut compartments in real salmon and SalmoSim based on qPCR data. *The table summarises the Estimated Marginal Means output for each mixed-effect linear model run with different qPCR measured relative abundance values identifying the difference between real salmon and SalmoSim response to feed change (Fish meal to Fish meal free diet) within different gut compartments (S - stomach, PC - pyloric caeca, and MG - mid gut). P>0.05 values identify no change in the bacterial group, p<0.05 identifies decrease (Est is negative), and p<0.05 identifies increase (Est is positive) in the relative abundance of target group after the feed change. Bold values identify similarities between SalmoSim and real salmon samples. The SalmoSim values used for this test involves stable SalmoSim time points: days 16, 18 and 20 (Fish meal diet - once bacterial communities adapted to the SalmoSim environment), and days 36, 38 and 40 (Fish meal free diet - once bacterial communities adapted to feed change).*

	real salmon			**SalmoSim**		
	S	**PC**	**MG**	**S**	**PC**	**MG**
Actinobacteria	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**
Alphaproteobacteria	p>0.05	p>0.05	p>0.05	p<0.001	p<0.001	p<0.001
Bacteroidetes	**p=0.005**	p=0.002	p=0.015	**p<0.001**	p>0.05	p>0.05
Betaproteobacteria	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**
Firmicutes	p>0.05	p>0.05	p>0.05	p=0.029	p=0.001	p=0.015
Gammaproteobacteria	p>0.05	**p>0.05**	**p>0.05**	p<0.001	**p>0.05**	**p>0.05**
Lactobacillus	p>0.05	**p>0.05**	p>0.05	p=0.017	**p>0.05**	p=0.002
Mycoplasma	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**	**p>0.05**

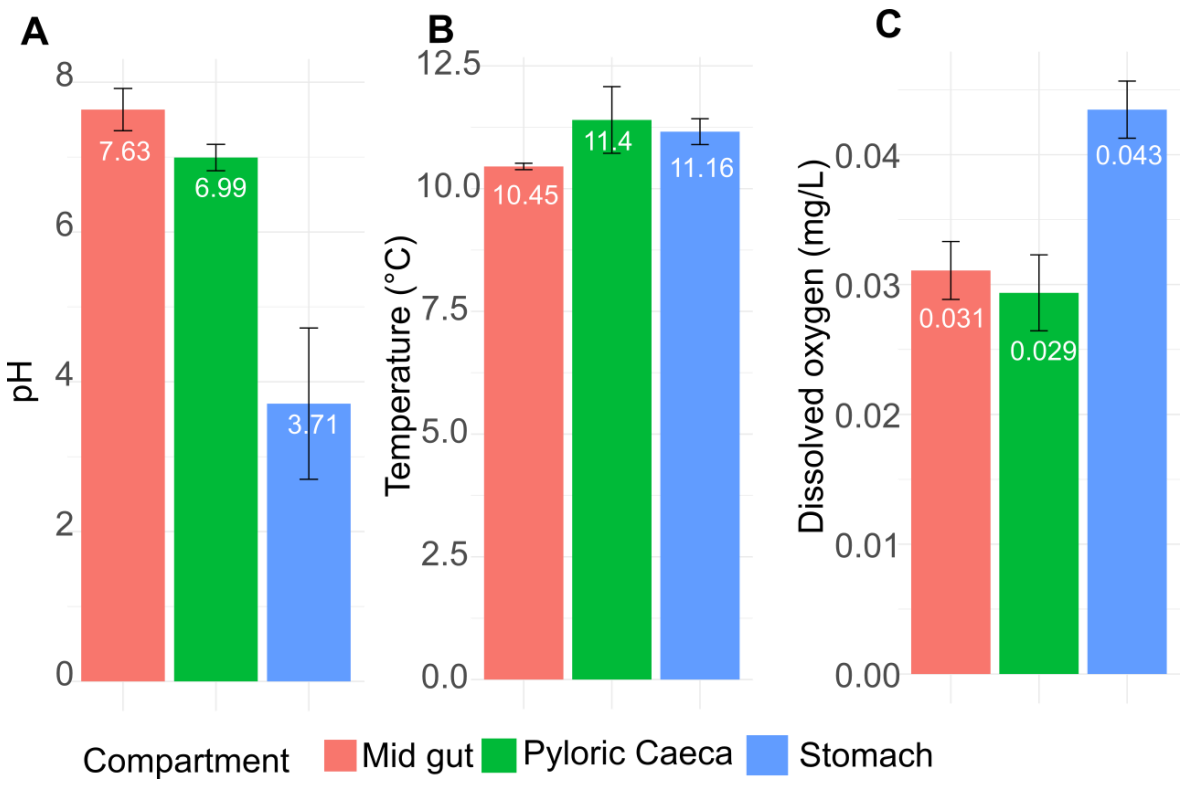


Figure A3-1 Physiochemical conditions measured within different real Atlantic salmon gut compartments. *1A-1C measured physicochemical conditions within real salmon (n=3) gut compartments: pH (**1A**), temperature (°C, **1B**), dissolved oxygen (mg/L, **1C**).*

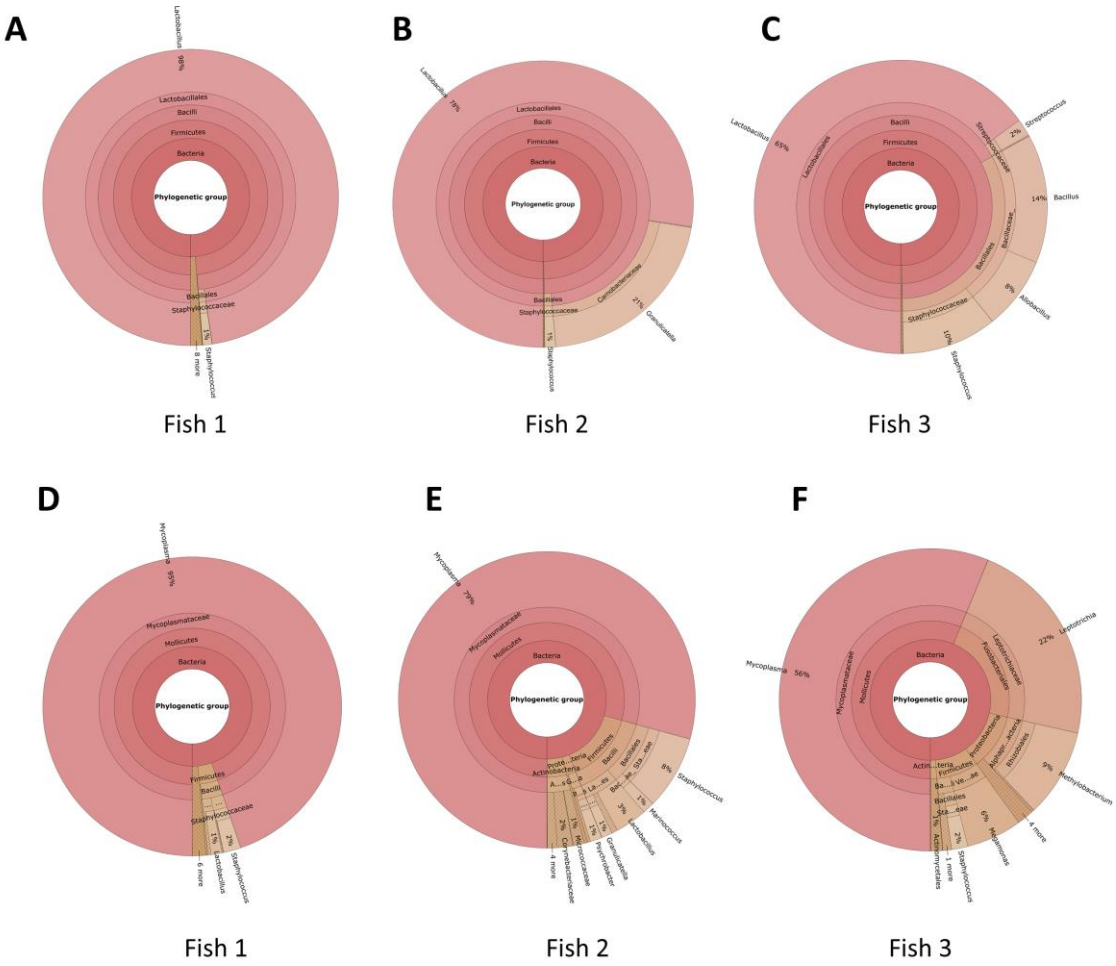


Figure A3-2 Specificity of the primers that target *Lactobacillus* and *Mycoplasma* genus. *The results in figure summarise bacterial genus targeted by Lactobacillus (Figure 1 A, B, C) and Mycoplasma (Figure 1 D, E, F) specific primer set. It shows that of all genus captured by Lactobacillus primer pair 98% were Lactobacillus in fish 1, 78% in fish 2, and 65% in fish 3. While of all genus captured by Mycoplasma primer pair 95% were Mycoplasma in fish 1, 79% in fish 2, and 56% in fish 3.*

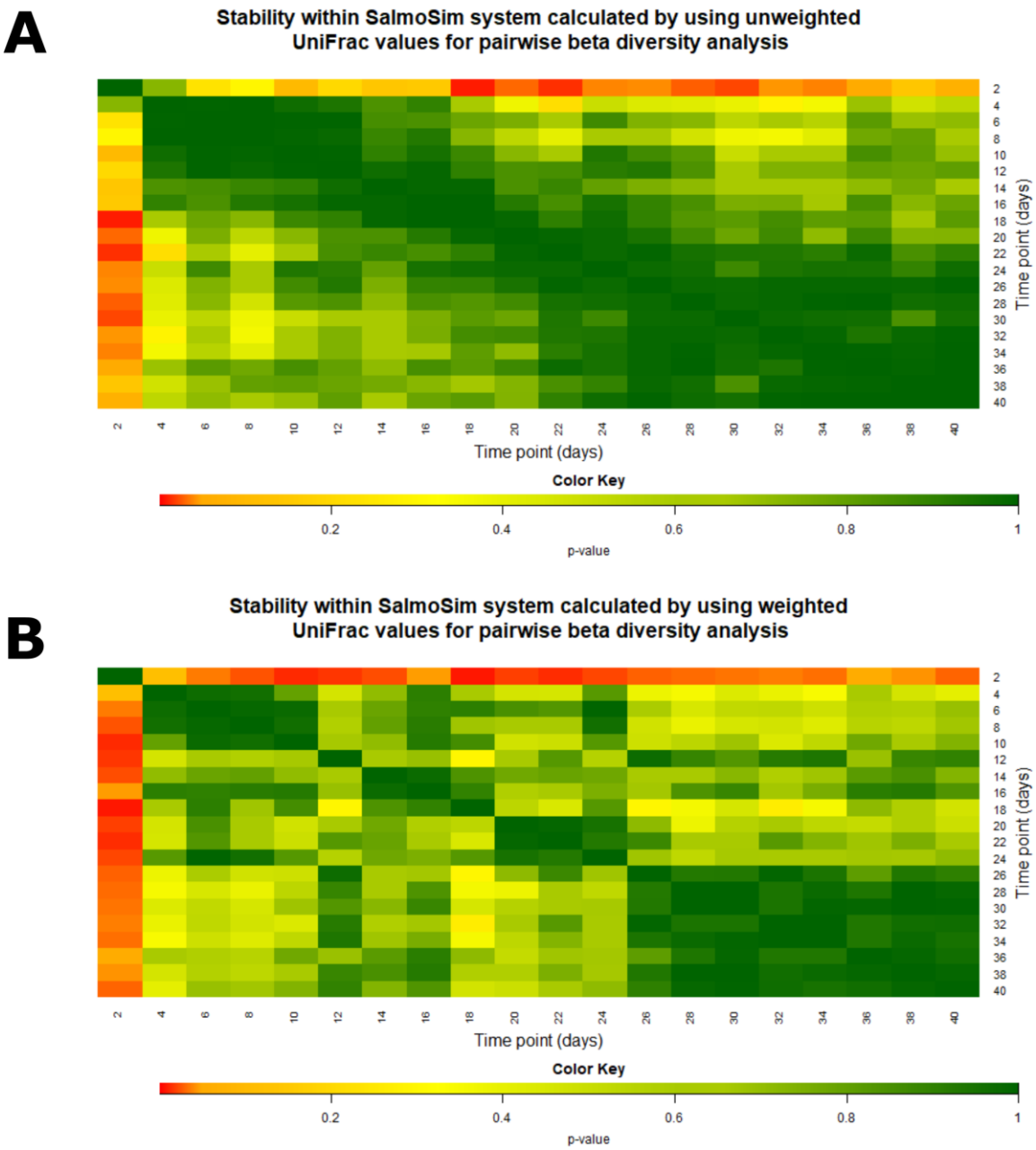


Figure A3-3 Stability within SalmoSim system calculated by using unweighted and weighted UniFrac values for pairwise beta diversity analysis. *The figure represents microbial stability within the SalmoSim system (data from all gut compartments combined) as the pairwise beta diversity comparison between different sampling time points (days), calculated by using* ***A*** *unweighted (0%) and* ***B*** *weighted (100%) UniFrac as a distance measure. A small p-value indicates that the two time points are statistically different, and p>0.05 indicates that two time points are not statistically different. The colour key illustrates the p-value: red end of spectrum denoting low p values (distinct compositions between time points) and dark green indicating high p values (similar compositions between timepoints).*

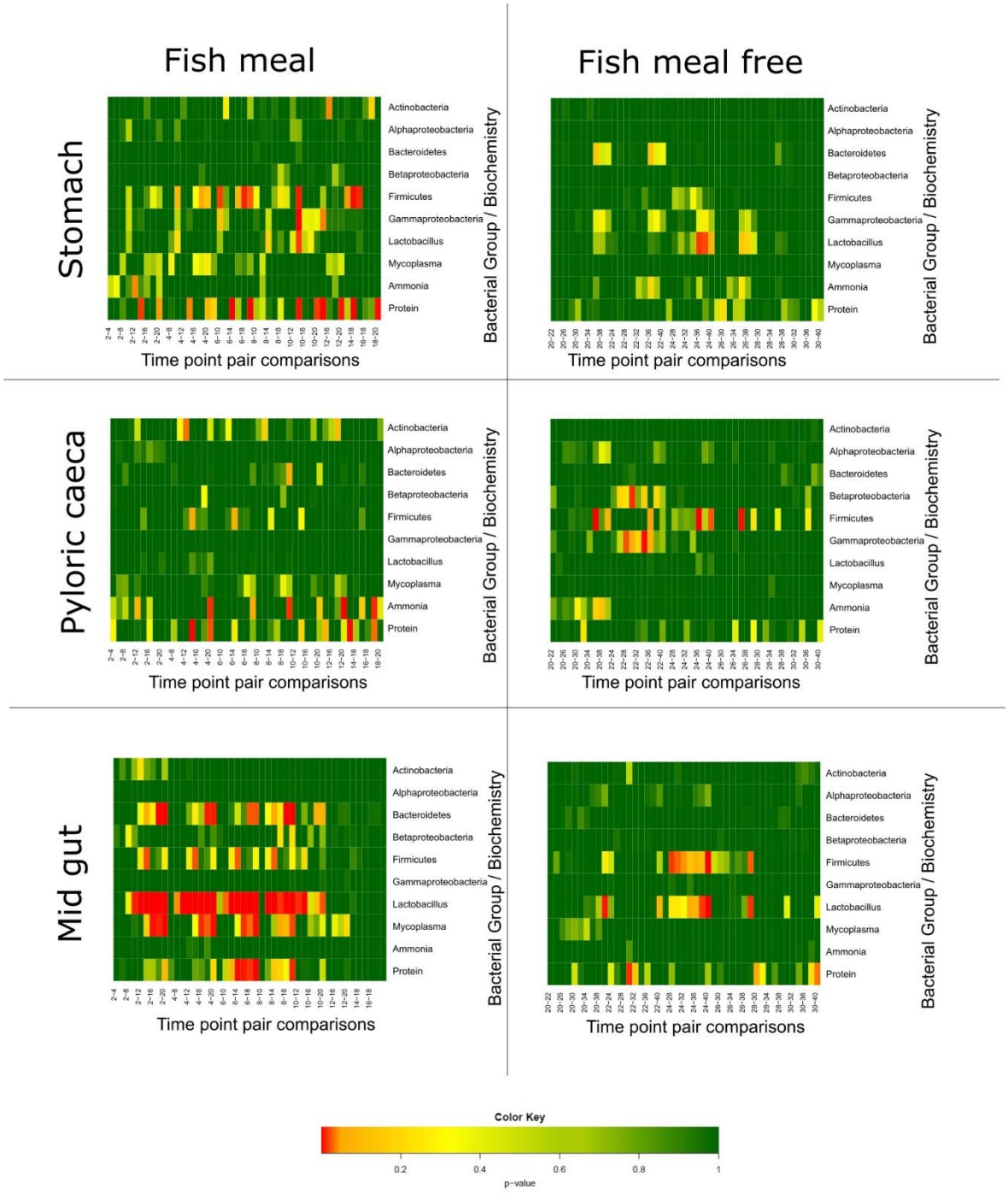


Figure A3-4 Measured value (qPCR, ammonia, and protein concentrations) stability within different SalmoSim compartments fed on Fish meal and Fish meal free diets. *The figure summarises the Estimated Marginal Means output for each mixed-effect linear model (Model 1) run with different values measured in different SalmoSim compartments (qPCR measurements, ammonia and protein concentrations) identifying the difference between different time points during the first (system fed on Fish meal diet) and last 20 days (system fed on Fish meal free diet) of validation experiment. A small p-value indicates that the two time points are statistically different, and p>0.05 indicates that two time points are not statistically different. The colour key illustrates the p-value: red end of spectrum denoting low p values (low correlation between time points) and dark green indicating high p values (no differences between timepoints).*

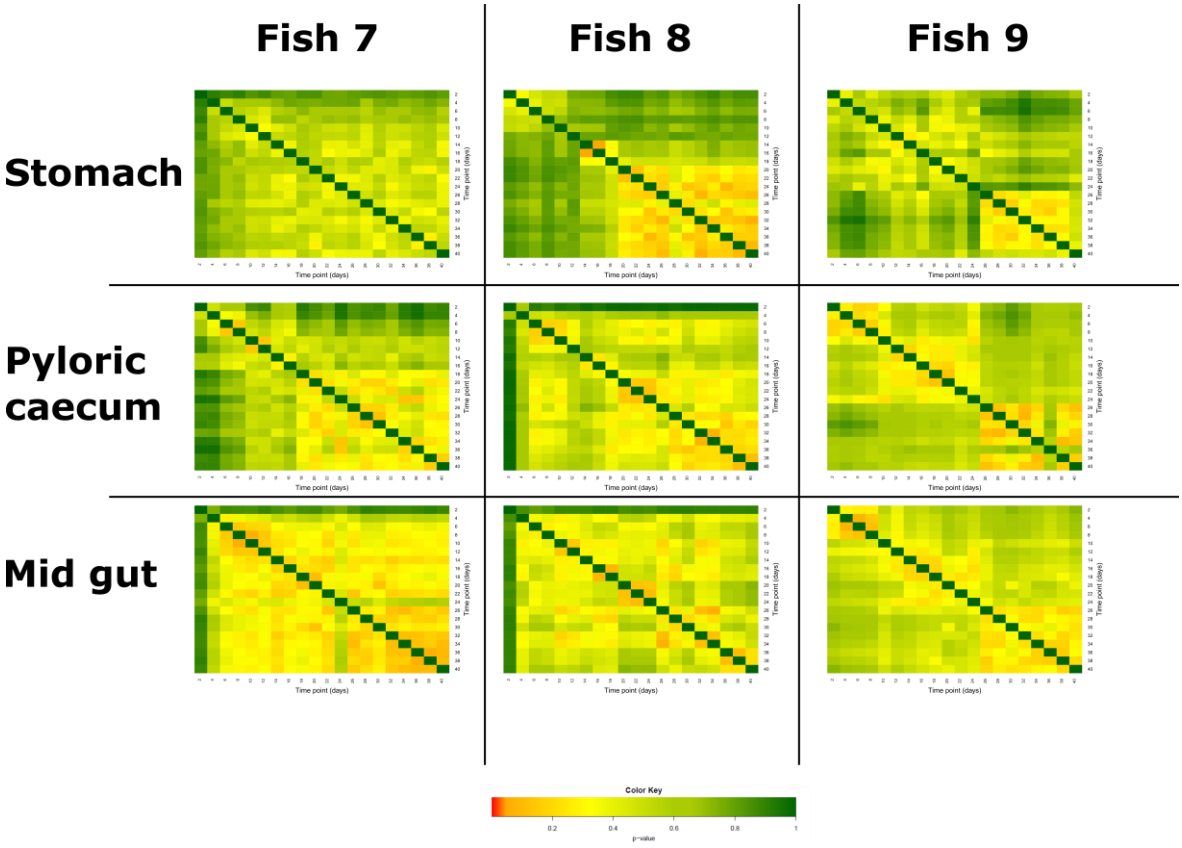


Figure A3-5 Stability within SalmoSim system, within different biological replicates and different gut compartments, calculated by using generalised UniFrac values for pairwise beta diversity analysis. *The figure represents microbial stability within the SalmoSim system (data separated by different biological replicates and gut compartments) as the pairwise beta diversity comparison between different sampling time points (days), calculated by using generalised (50%) UniFrac as a distance measure. A small p-value indicates that the two time points are statistically different, and p>0.05 indicates that two time points are not statistically different. The colour key illustrates the p-value: red end of spectrum denoting low p values (distinct compositions between time points) and dark green indicating high p values (similar compositions between timepoints).*

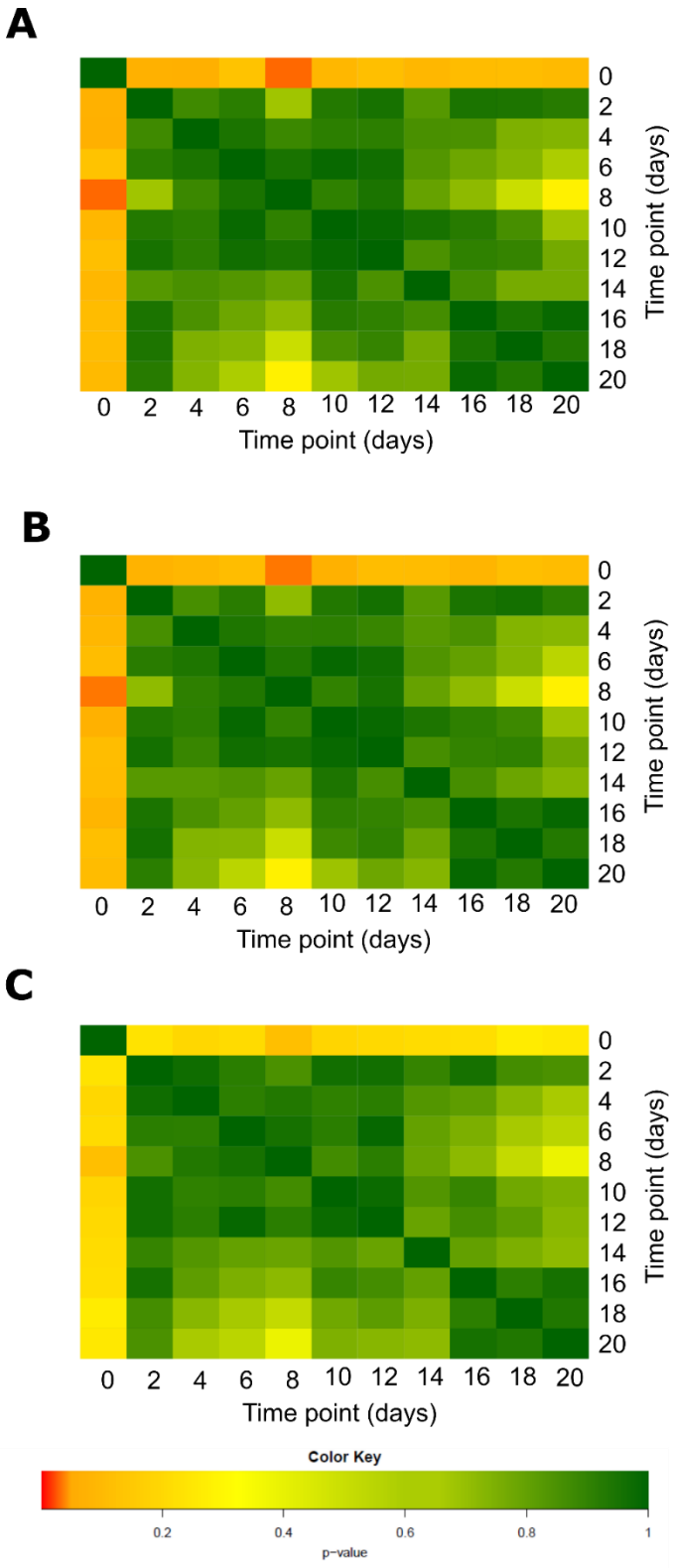


Figure A3-6 Stability within SalmoSim system calculated by using different UniFrac values for pairwise beta diversity analysis. *The figure represents microbial stability within the SalmoSim system (data from all gut compartments and two different technical replicate runs combined) as the pairwise beta diversity comparison between different sampling time points (days), calculated by using unweighted (**A**: 0%), generalised (**B**: 50%) and weighted (**C**: 100%) UniFrac as a distance measure. A small p-value indicates that the two time points are statistically different, and p>0.05 indicates that two time points are not statistically different. The colour key illustrates the p-value: red end of spectrum denoting low p values (distinct compositions between time points) and dark green indicating high p values (similar compositions between timepoints).*

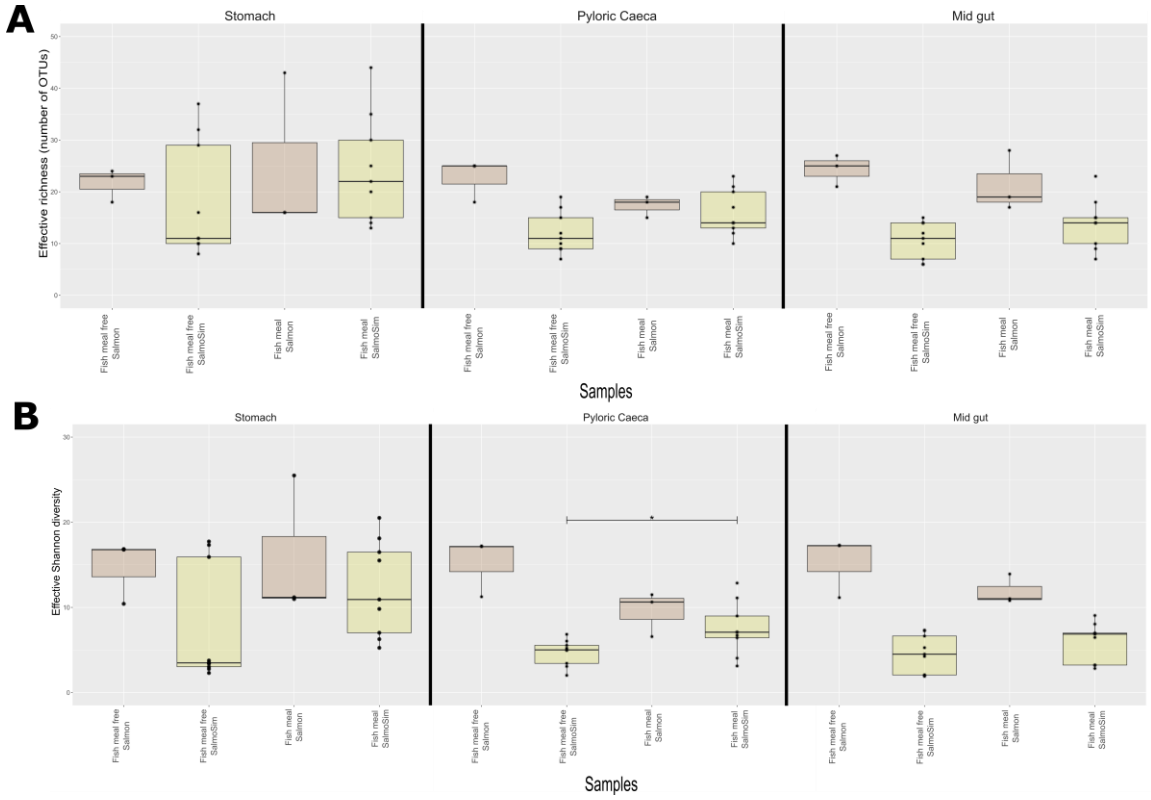


Figure A3-7 Calculated alpha-diversity metrics within different gut compartments of real salmon and SalmoSim fed on Fish meal and Fish meal free diets. *Figure represents different alpha diversity outputs within different gut compartments of real salmon in red and SalmoSim in yellow (stable time points: 16, 18 and 20 fed on Fish meal, and 36, 38 and 40 fed on Fish meal free diet) fed on Fish meal and Fish meal free diets.* ***A*** *represents effective richness (number of OTUs),* ***B*** *represents effective Shannon diversity. The lines above bar plots represent statistically significant differences after feed change. The stars flag the levels of significance: one star (*) for p-values between 0.05 and 0.01, two stars (**) for p-values between 0.01 and 0.001, and three stars (***) for p-values below 0.001.*

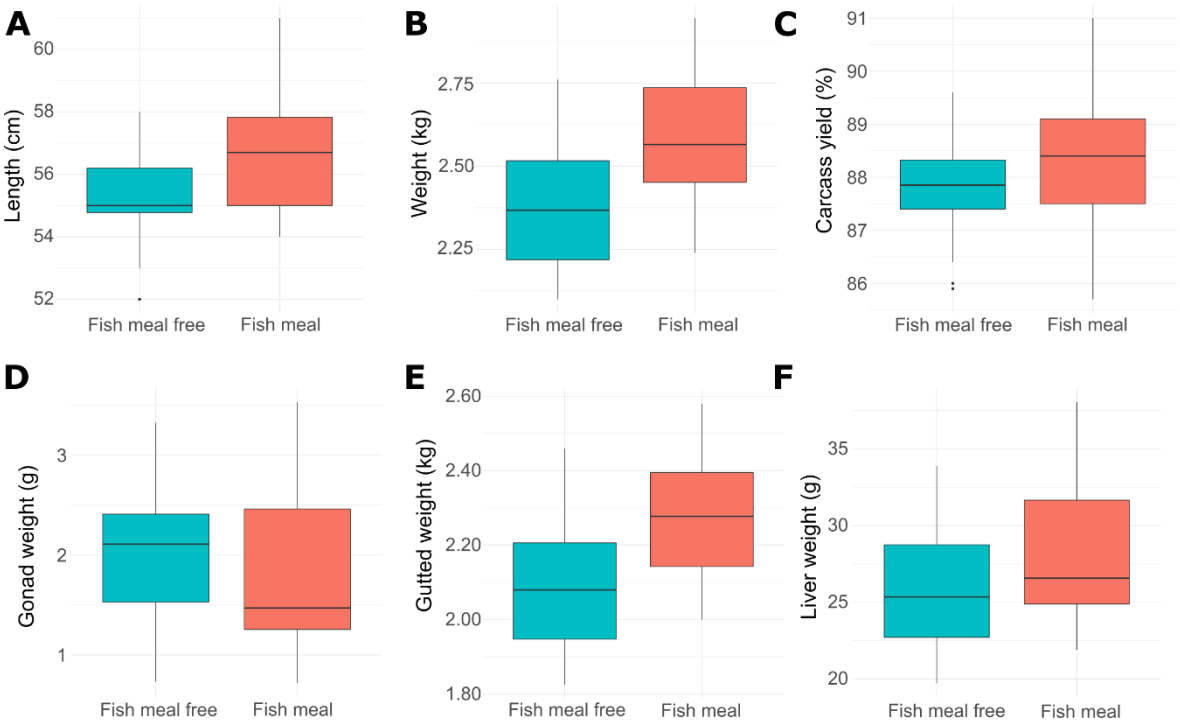


Figure A3-8 In vivo phenotypic fish performance fed on two different feeds. *Figure represents different phenotypic performance data of fish (n=32 per feed) fed on two different feed.* ***A*** *Atlantic salmon length in centimetres;* ***B*** *Atlantic salmon length in weight in kilograms;* ***C*** *Atlantic salmon percentage carcass yield;* ***D*** *Atlantic salmon gonad weight in grams;* ***E*** *Atlantic salmon gutted weight in kilograms;* ***F*** *Atlantic salmon liver weight in grams. Blue box plots represent data from salmon (n=32) fed on Fish meal free diet, and red represents Atlantic salmon fed on Fish meal diet (n=32).*

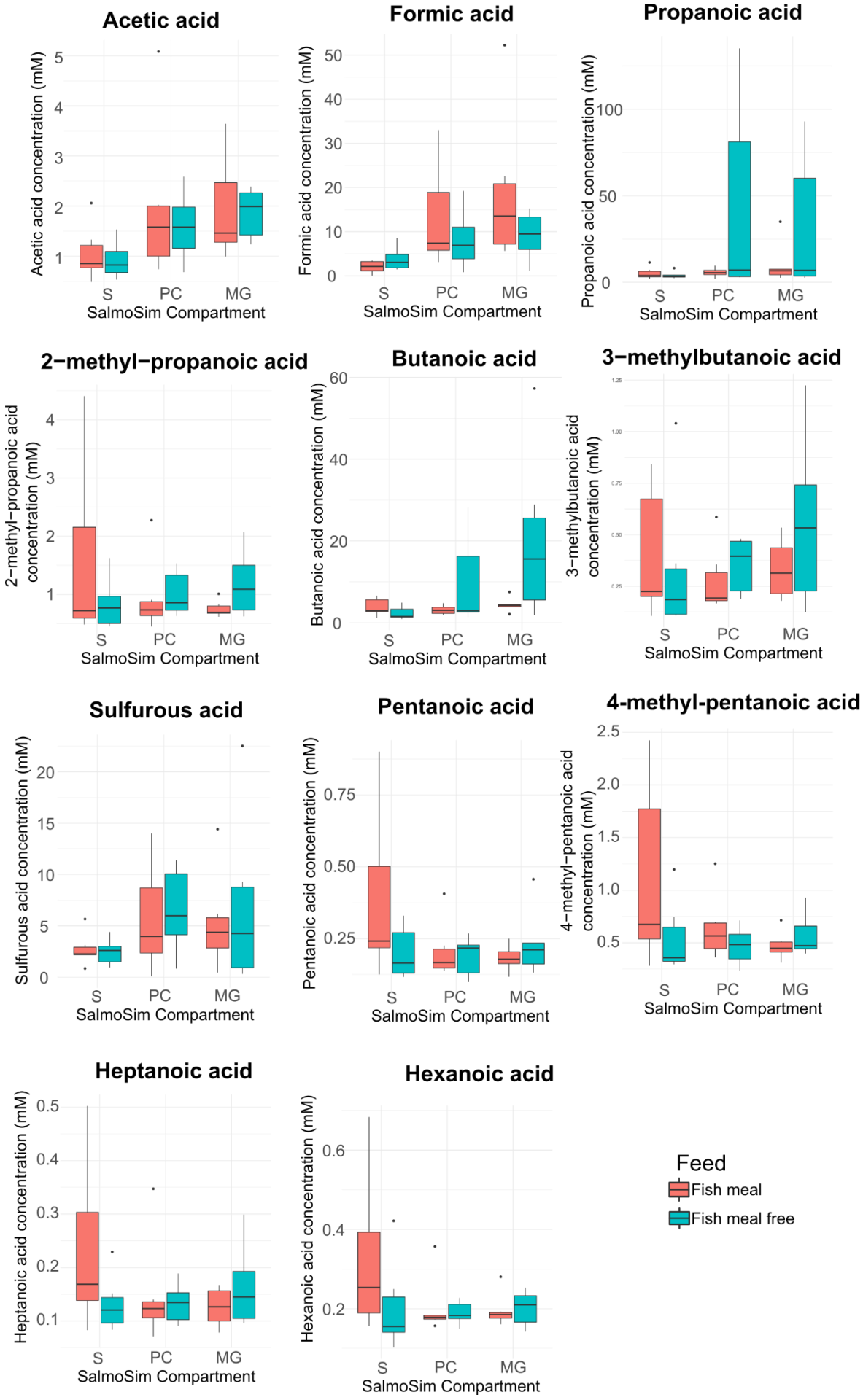

Acetic acid
Acetic acid concentration (mM)
Formic acid
Formic acid concentration (mM)
Propanoic acid
Propanoic acid concentration (mM)
2−methyl−propanoic acid
2−methyl−propanoic acid concentration (mM)
Butanoic acid
Butanoic acid concentration (mM)
3−methylbutanoic acid
3−methylbutanoic acid concentration (mM)
Sulfurous acid
Sulfurous acid concentration (mM)
Pentanoic acid
Pentanoic acid concentration (mM)
4-methyl-pentanoic acid
4−methyl−pentanoic acid concentration (mM)
Heptanoic acid
Heptanoic acid concentration (mM)
Hexanoic acid
Hexanoic acid concentration (mM)
S
PC
MG
SalmoSim Compartment
Feed
Fish meal
Fish meal free

Figure A3-9 VFA production within different SalmoSim compartments fed on different feeds. *Figure represents the production of 11 volatile fatty acids within SalmoSim system fed on Fish meal and Fish meal free diets within different gut compartments. Y axis represents the concentration of specific volatile fatty acid (mM) while the X axis represents each gut compartment (stomach, pyloric caeca, midgut). Red colour denoted Fish meal and blue – Fish meal free diets. The lines above bar plots represent statistically significant differences between different feeds and gut compartments. The stars flag the levels of significance: one star (*) for p-values between 0.05 and 0.01, two stars (**) for p-values between 0.01 and 0.001, and three stars (***) for p-values below 0.001.*

Supplementary methods for Chapter 3

1. qPCR data analysis

1.1. Performing qPCR analysis

The concentration of each DNA sample was measured using a Qubit® fluorometer (Thermo Fisher Scientific, USA), and dilutions were performed using Microbial DNA-Free Water (Qiagen, Valencia, CA, USA). Inoculums from all three real salmon gut compartments were diluted to 0.25 ng/µl. SalmoSim stomach samples were also diluted to 0.25 ng/µl, and pyloric caecum and midgut SalmoSim samples were diluted to 1 ng/µl. After, the qPCR analysis was performed on each DNA sample in duplicates by using SensiFAST™ SYBR® No-ROX Kit (Bioline, UK) and primer sets summarised in Supplementary Table 1 at a final concentration of 1 pM of each primer. Reaction conditions for all PCR reactions were 95°C for three minutes, followed by 40 cycles at 95°C for 5 seconds, 60°C for 10 seconds and 72°C for 20 seconds, followed by a final elongation step of 95°C for 10 minutes.

In order to measure the relative abundance of the target group (target determined by the specificity of the qPCR primer pairs); several steps were undertaken by adapting ΔΔCq method (Rao et al., 2013). First, the average quantitation cycle (Cq) value of each primer set negative control was found. This was followed by subtraction Cq value generated by using one of the primer pairs in Supplementary Table 1, from corresponding average Cq value of the corresponding negative control (generated with the same primer pair) in order to generate value X. After, the Cq value generated by using the general primer set was subtracted from the average Cq value of the corresponding negative control (generated using general primer set) in order to generate the value Y. Finally, the value X was divided by the value Y in order to find out the relative abundance of the target group with respect to the total number of bacterial 16S copies in the sample. The equations used for all these calculations are summarised below:

$$\mathrm{x} = average\ negative\ control\ Cq - Cq\ of\ target\ group$$
$$y = average\ of\ negative\ control\ Cq - Cq\ of\ general\ bacteria$$
$$relative\ abundance\ of\ target\ group = x \div y$$

This method was carried out for each sample, quantified using different primer sets targeting various bacterial taxon groups. Several published and validated primer sets in the literature were used (Supplementary Table 1). Primer sets targeting Mycoplasma, and Lactobacillus genus were designed by using DECIPHER software based on the data collected by Heys et al., 2020. These primers target specificity was analysed via amplicon sequencing of the products (See Supplementary Figure 2).

1.2. Investigating bacterial dynamics within SalmoSim system over time

In order to investigate the time taken for the measured values (qPCR of different bacterial groups, protein and ammonia concentrations) to stabilise within different gut compartments of the SalmoSim system, the data for all three SalmoSim runs (three biological replicates) was combined and then split-up by each separate SalmoSim compartment (stomach, pyloric caecum and midgut). These three resulting datasets were then further split-up in half: pre- and post-feed changes. The subdivided datasets by different SalmoSim gut compartment and different feed were used to run Model 1 with different values measured during a validation experiment considering run (biological replicates) as a random effect. The normality and heterogeneity of the residuals for of each model were checked by using Shapiro and Bartlett's tests. If these tests showed that residues were not normally distributed or not heterogeneous, the data were subsequently transformed by using Code 1 and then used to re-run Model 1. Finally, the post-hoc Estimated Marginal Means test (also known as Least-Squares Means) was used with Model 1 and ran with different values in order to investigate in-between individual time point comparison.

Model 1 = lme(value~ Time, random =c(~1|Run), data=**groupX**)

Model 1 Mixed effect linear model formula to investigate the effect of time taking run as a random effect. In Model 1 groupX denotes subdivided data sets by different SalmoSim compartments (stomach, pyloric caecum or midgut), and value denotes the qPCR results for one of the different targeted bacterial groups (Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Firmicutes, Gammaproteobacteria, Lactobacillus, Mycoplasma) or protein or ammonia concentrations. This model takes different SalmoSim runs as a random effect (biological replicates).

```
if(lambda!=0){y=((value)^lambda-1)/lambda}

if(lambda==0){y=log(value)}
```

Code 1 Code used to transform the data. Value in this code denotes the qPCR results for one of the different targeted bacterial groups (Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Firmicutes, Gammaproteobacteria, Lactobacillus, Mycoplasma) or protein or ammonia concentrations. Lambda values in 0.1 increments were tested.

1.3. Comparing *in vivo* and *in vitro* trials

In order to investigate whether a change in the feed from FMD to FMF results in similar trends measured between SalmoSim and real salmon samples, a combined data set was produced containing qPCR values measured in real salmon gut compartments (stomach, pyloric caecum and midgut of three fish fed on FMD and three fish fed on FMF) and SalmoSim compartments at the last three time points for both feeds (once bacterial communities were stabilised while feeding SalmoSim both FMF and FMD: days 16, 18, and 20 for FMD and days 36, 38, and 40 for FMF feed). This combined dataset was then separated by different SalmoSim gut compartments (stomach, pyloric caecum, and midgut). The subdivided datasets for each gut compartment for both real salmon (all samples) and SalmoSim (samples from only stable time points) were input into Model 2. In order to investigate how bacterial groups within different types of sample (SalmoSim or real salmon) react to the change in feed the Post-hoc Estimated Marginal Means test (also known as Least-Squares Means) was used in order to have a more detailed look at the effect of the interaction between feed and sample on the abundance of each target taxon.

Model 2 = lm(value~ Feed*samples, data=GroupX)

Model 2 Linear model formula to investigate the effect of interaction between feed (Fish meal and Fish meal free diets) and sample (real salmon and SalmoSim samples) on the qPCR values measured. In model 2, value denotes the qPCR results for one of the different targeted bacterial groups (Actinobacteria, Alphaproteobacteria, Bacteroidetes, Betaproteobacteria, Firmicutes, Gammaproteobacteria, Lactobacillus, Mycoplasma). Group X is a subset of dataset separated by different gut compartments (stomach, pyloric caecum and midgut). Feed identifies Fish meal and Fish meal 0 diets, and sample identifies real salmon and stable time point SalmoSim samples (days 16, 18 and 20 SalmoSim fed on Fish meal diet, and days 36, 38, and 40 SalmoSim fed on Fish meal free diet).

2. Volatile Fatty Acid (VFA) analysis

The measured VFA values were input to Model 4 including time point (sampling time point) and run (biological replicate of SalmoSim system) as random effects. This was followed by the Post-hoc test Estimated Marginal Means (also known as Least-Squares Means) in order to have a more detailed look at the effect of the interaction between feed and SalmoSim compartment affect the concentration of VFAs.

Model 3 = lmer(VFA~ Feed*Compartment+(1|Time point)+(1|Run))

Model 3 Mixed effect linear model to investigate the significance of different VFA concentrations between SalmoSim fed on Fish meal and Fish meal free diets within different SalmoSim compartments. In Model 3 VFA denotes the VFA values measured. This model takes run and time as random effects.

3. Beta-diversity analysis workflow

#' **Script**: Beta-Diversity analysis for SalmoSim

#' **Author**: Raminta Kazlauskaite (adapted from Rhea script produced by Ilias Lagkouvardos)

#' Calculate beta-diversity for microbial communities

#' based on permutational mulitvariate analysis of variances (PERMANOVA) using multiple distance matrices

#' computed from phylogenetic distances between observed organisms

#'

#' **Input**:

#' 1. Set the path to the directory where the file is stored

#' 2. Write the name of the normalized OTU table without taxonomy information

#' 3. Write the name of the mapping file that includes the samples groups

#' 4. Write the name of the OTU tree

#' 5. Write the name of the variable (sample group) used for comparison

#'

#'

#' **Output**:

#' The script generates three graphical outputs (pdf), one text file and a newick tree

#' 1. A phylogram with colour-coded group clustering

#' 2. MDS and NMDS plots showing information about beta-diversity across all sample groups

#' 3. MDS and NMDS plots of all pairwise comparisons

#' 4. The distance matrix

#' 5. Plot showing the optimal number of clusters

#' 6. Dendogram for all samples in a newick tree file

#'

#' **Concept**:

#' A distance matrix is calculated based on the generalized UniFrac approach

#' (Chen J, et al. Associating microbiome composition with environmental covariates using generalized UniFrac distances. 2012)

#' Samples are clustered based on the distance matrix using the Ward's hierarchical clustering method

#' To determine similarities between samples, a multivariate analysis is applied

```
#' and sample distribution is illustrated by means of MDS and NMDS (non-metric) plots

#' The Calinski-Harabasz (CH) Index is used to assess the optimal number of clusters the dataset was most robustly partitioned into


################################################################################
##
######          Set parameters in this section manually          ######
################################################################################
##


#' Please set the directory of the script as the working folder (e.g D:/studyname/NGS-Data/Rhea/beta-diversity/)

#' Note: the path is denoted by forward slash "/"

setwd("")     #<--- CHANGE ACCORDINGLY


#' Please give the file name of the normalized OTU-table without taxonomic classification

input_otu = ""          #<--- CHANGE ACCORDINGLY !!!


#' Please give the name of the meta-file that contains individual sample information

input_meta = ""          #<--- CHANGE ACCORDINGLY !!!


#' Please give the name of the phylogenetic tree constructed from the OTU sequences

input_tree = ""          #<--- CHANGE ACCORDINGLY !!!


#' Please give the column name (in the mapping file) of the categorical variable to be used for comparison (e.g. Genotype)

group_name = ""                #<--- CHANGE ACCORDINGLY !!!


################################################################################
##
######          Additional parameters                ######
################################################################################
##


#' Turn on sample labeling
```

```
#' 0 = Samples are not labeled in the MDS/NMDS plots

#' 1 = All Samples are labed in the MDS/NMDS plots

label_samples = 0


#' Determine which sample lable should appear

#' Write the name of samples (in quotation marks), which should appear in the MDS/NMDS plots, in the vector (c) below

#' If more than one sample should be plotted, please separate their IDs by comma (e.g. c("sample1","sample2"))

label_id =c("")


#' De-Novo Clustering will be perfomed for the number of samples or maximal for the set limit

#' Default Limit is 100

kmers_limit=20


######              NO CHANGES ARE NEEDED BELOW THIS LINE            ######


################################################################################
##

######                    Main Script                    ######

################################################################################
##


####################      Load all required libraries     ########################


# Check if required packages are already installed, and install if missing

packages <-c("ade4","GUniFrac","phangorn","cluster","fpc")


# Function to check whether the package is installed

InsPack <- function(pack)

{

  if ((pack %in% installed.packages()) == FALSE) {

    install.packages(pack,repos ="http://cloud.r-project.org/")

  }
```

```
}

# Applying the installation on the list of packages
lapply(packages, InsPack)

# Make the libraries
lib <- lapply(packages, require, character.only = TRUE)

# Check if it was possible to install all required libraries
flag <- all(as.logical(lib))
```

#################### **Read all required input files** ####################

```
# Load the tab-delimited file containing the values to be analyzed (samples names in the first column)
otu_file <- read.table (file = input_otu, check.names = FALSE, header = TRUE, dec = ".", sep = "\t", row.names = 1, comment.char = "")

# Clean table from empty lines
otu_file <- otu_file[!apply(is.na(otu_file) | otu_file =="",1,all),]

# Load the mapping file containing individual sample information (sample names in the first column)
meta_file <- read.table (file = input_meta, check.names = FALSE, header = TRUE, dec = ".", sep = "\t", row.names = 1, comment.char = "")

# Clean table from empty lines
meta_file <- data.frame(meta_file[!apply(is.na(meta_file) | meta_file=="",1,all),])

# Load the phylogenetic tree calculated from the OTU sequences
tree_file <- read.tree(input_tree)
```

```
# Create the directory where all output files are saved (is named after the target group name set above for comparisons)

dir.create(group_name)

#####################  Calculate beta-diversity  ###################

# OTU-table and mapping file should have the same order and number of sample names

# Order the OTU-table by sample names (ascending)

otu_file <- otu_file[,order(names(otu_file))]

# Transpose OTU-table and convert format to a data frame

otu_file <- data.frame(t(otu_file))

# Order the mapping file by sample names (ascending)

meta_file <- data.frame(meta_file[order(row.names(meta_file)),])

# Save the position of the target group name in the mapping file

meta_file_pos <- which(colnames(meta_file) == group_name)

# Select metadata group based on the pre-set group name

all_groups <- as.factor(meta_file[,meta_file_pos])

# Root the OTU tree at midpoint

rooted_tree <- midpoint(tree_file)

# Calculate the UniFrac distance matrix for comparing microbial communities: 0.0 – Unweighted UniFrac, 0.5 Balanced UniFrac and 1.0 Weighted UniFrac.

unifracs <- GUniFrac(otu_file, rooted_tree, alpha = c(0.0,0.5,1.0))$unifracs

# Weight on abundant lineages so the distance is not dominated by highly abundant lineages with 0.5 having the best power

unifract_dist <- unifracs[, , "d_0.5"]

################# Generate tree ########################
```

```
# Save the UniFrac output as distance object
all_dist_matrix <- as.dist(unifrac_dist)

# Apply a hierarchical cluster analysis on the distance matrix based on the Ward's method
all_fit <- hclust(all_dist_matrix, method = "ward.D2")

# Generates a tree from the hierarchically generated object
tree <- as.phylo(all_fit)
my_tree_file_name <- paste(group_name,"/phylogram.pdf",sep="")
plot_color<-rainbow(length(levels(all_groups)))[all_groups]

# Save the generated phylogram in a pdf file
pdf(my_tree_file_name)

# The tree is visualized as a Phylogram color-coded by the selected group name
plot(tree, type = "phylogram",use.edge.length = TRUE, tip.color = (plot_color), label.offset = 0.01)
print.phylo(tree)
axisPhylo()
tiplabels(pch = 16, col = plot_color)
dev.off()

################## Build NMDS plot ########################

# Generated figures are saved in a pdf file
file_name <- paste(group_name,"beta-diversity.pdf",sep="_")
pdf(paste(group_name,"/",file_name,sep=""))

# Calculate the significance of variance to compare multivariate sample means (including two or
more dependent variables)
# Omit cases where there isn't data for the sample (NA)
all_groups_comp <- all_groups[!is.na(all_groups)]
unifract_dist_comp <- unifract_dist[!is.na(all_groups), !is.na(all_groups)]
```

```
adonis<-adonis(as.dist(unifract_dist_comp) ~ all_groups_comp)

all_groups_comp<-factor(all_groups_comp,levels(all_groups_comp)[unique(all_groups_comp)])


# Calculate and display the MDS plot (Multidimensional Scaling plot)

s.class(

 cmdscale(unifract_dist_comp, k = 2), col = unique(plot_color), cpoint =

  2, fac = all_groups_comp, sub = paste("MDS plot of Microbial Profiles\n(p-value ",adonis[[1]][6][[1]][1],")",sep="")

)

if (label_samples==1) {

 lab_samples <- row.names(cmdscale(unifract_dist_comp, k = 2))

 ifelse (label_id != "",lab_samples <- replace(lab_samples, !(lab_samples %in% label_id), ""), lab_samples)

 text(cmdscale(unifract_dist_comp, k = 2),labels=lab_samples,cex=0.7,adj=c(-.1,-.8))

}


# Calculate and display the NMDS plot (Non-metric Multidimensional Scaling plot)

meta <- metaMDS(unifract_dist_comp,k = 2)

s.class(

 meta$points, col = unique(plot_color), cpoint = 2, fac = all_groups_comp,

 sub = paste("metaNMDS plot of Microbial Profiles\n(p-value ",adonis[[1]][6][[1]][1],")",sep="")

)

if (label_samples==1){

 lab_samples <- row.names(meta$points)

 ifelse (label_id != "",lab_samples <- replace(lab_samples, !(lab_samples %in% label_id), ""), lab_samples)

 text(meta$points,labels=lab_samples,cex=0.7,adj=c(-.1,-.8))

}


#close the pdf file

dev.off()
```

################ **NMDS for pairwise analysis** ###################

```
# This plot is only generated if there are more than two groups included in the comparison
# Calculate the pairwise significance of variance for group pairs
# Get all groups contained in the mapping file
unique_groups <- levels(all_groups_comp)
if (dim(table(unique_groups)) > 2) {

# Initialise vector and lists
pVal = NULL
pairedMatrixList <- list(NULL)
pair_1_list <- NULL
pair_2_list <- NULL

for (i in 1:length(combn(unique_groups,2)[1,])) {

 # Combine all possible pairs of groups
 pair_1 <- combn(unique_groups,2)[1,i]
 pair_2 <- combn(unique_groups,2)[2,i]

 # Save pairs information in a vector
 pair_1_list[i] <- pair_1
 pair_2_list[i] <- pair_2

 # Generate a subset of all samples within the mapping file related to one of the two groups
 inc_groups <-
  rownames(subset(meta_file, meta_file[,meta_file_pos] == pair_1
                |
                 meta_file[,meta_file_pos] == pair_2))

 # Convert UniFrac distance matrix to data frame
 paired_dist <- as.data.frame(unifract_dist_comp)

 # Save all row names of the mapping file
 row_names <- rownames(paired_dist)
```

```
# Add row names to the distance matrix
paired_dist <- cbind(row_names,paired_dist)

# Generate distance matrix with samples of the compared groups (column-wise)
paired_dist <- paired_dist[sapply(paired_dist[,1], function(x) all(x %in% inc_groups)),]

# Remove first column with unnecessary group information
paired_dist[,1] <- NULL
paired_dist <- rbind(row_names,paired_dist)

# Generate distance matrix with samples of the compared group (row-wise)
paired_dist <- paired_dist[,sapply(paired_dist[1,], function(x) all(x %in% inc_groups))]

# Remove first row with unnecessary group information
paired_dist <- paired_dist[-1,]

# Convert generated distance matrix to data type matrix (needed by multivariate analysis)
paired_matrix <- as.matrix(paired_dist)
class(paired_matrix) <- "numeric"

# Save paired matrix in list
pairedMatrixList[[i]] <- paired_matrix

# Applies multivariate analysis to a pair out of the selected groups
adonis <- adonis(paired_matrix ~ all_groups_comp[all_groups_comp == pair_1 |
                                 all_groups_comp == pair_2])

# List p-values
pVal[i] <- adonis[[1]][6][[1]][1]

}
```

```
# Adjust p-values for multiple testing according to Benjamini-Hochberg method
pVal_BH <- p.adjust(pVal,method="BH", n=length(pVal))

# Generated NMDS plots are stored in one pdf file called "pairwise-beta-diversity-nMDS.pdf"
file_name <- paste(group_name,"pairwise-beta-diversity-NMDS.pdf",sep="_")
pdf(paste(group_name,"/",file_name,sep=""))

for(i in 1:length(combn(unique_groups,2)[1,])){
   meta <- metaMDS(pairedMatrixList[[i]], k = 2)
   s.class(
    meta$points,
    col = rainbow(length(levels(all_groups_comp))), cpoint = 2,
    fac = as.factor(all_groups_comp[all_groups_comp == pair_1_list[i] |
                        all_groups_comp == pair_2_list[i]]),
    sub = paste("NMDS plot of Microbial Profiles\n ",pair_1_list[i]," - ",pair_2_list[i], "\n(p-value
",pVal[i],",","," corr. p-value ", pVal_BH[i],")",sep="")
   )
}
dev.off()

# Generated MDS plots are stored in one pdf file called "pairwise-beta-diversity-MDS.pdf"
file_name <- paste(group_name,"pairwise-beta-diversity-MDS.pdf",sep="_")
pdf(paste(group_name,"/",file_name,sep=""))

for(i in 1:length(combn(unique_groups,2)[1,])){
 # Calculate and display the MDS plot (Multidimensional Scaling plot)
 s.class(
  cmdscale(pairedMatrixList[[i]], k = 2), col = rainbow(length(levels(all_groups_comp))), cpoint =
   2, fac = as.factor(all_groups_comp[all_groups_comp == pair_1_list[i] |
                        all_groups_comp == pair_2_list[i]]), sub = paste("MDS plot of Microbial
Profiles\n ",pair_1_list[i]," - ",pair_2_list[i], "\n(p-value ",pVal[i],","," corr. p-value ",
pVal_BH[i],")",sep="")
 )
}
```

```
dev.off()


}


################################################################################
#
######                Write Output Files                ######
################################################################################
#


# Write the distance matrix table in a file

file_name <- paste(group_name,"distance-matrix-gunif.tab",sep="_")

write.table( unifract_dist_comp, paste(group_name,"/",file_name,sep=""), sep = "\t", col.names =
NA, quote = FALSE)

write.table( unifract_dist_comp, "distance-matrix-gunif.tab", sep = "\t", col.names = NA, quote =
FALSE)

write.tree(tree,"samples-Tree.nwk",tree.names = FALSE)


# Graphical output files are generated in the main part of the script

if(!flag) { stop("

   It was not possible to install all required R libraries properly.

         Please check the installation of all required libraries manually.\n

         Required libaries:ade4, GUniFrac, phangorn")

}


################################################################################
#
######                End of Script                ######
################################################################################
#
```

Appendix 2: Chapter 4 appendices

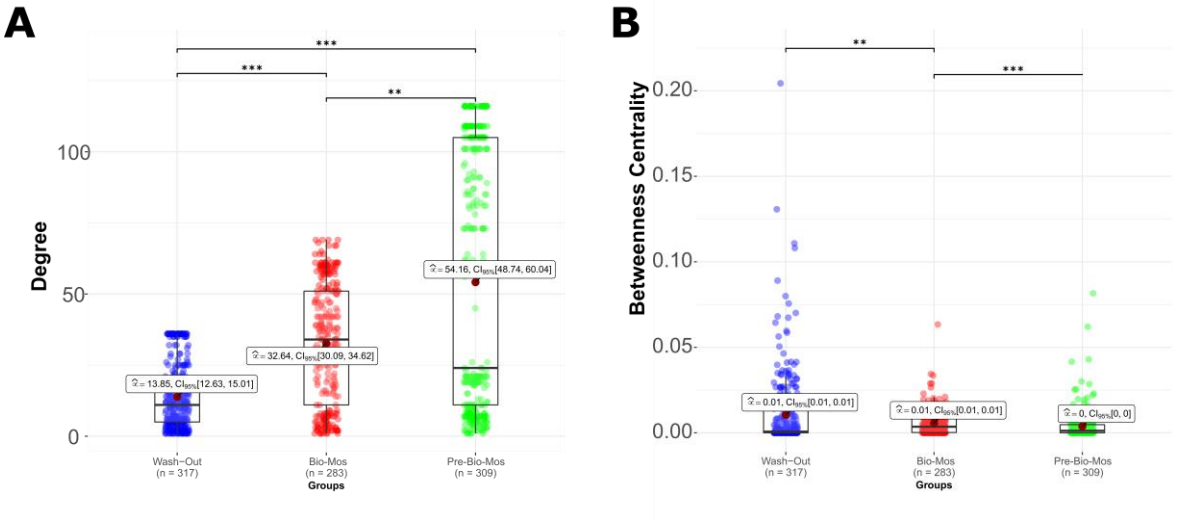


Figure A4-1 Comparison of key network analysis indicators between different experimental phases (Pre-Bio-Mos, Bio-Mos and Wash out). *Figure compares key characteristics of networks produced for three experimental phases: Pre-Bio-Mos (green), Bio-Mos (red), and Wash out (blue). **A** compares degree of each network; **B** betweenness centrality. The asterisk show significance: (*: 0.01 ≤ p < 0.05; **: 0.05 ≤ p < 0.001; ***: p ≤ 0.001).*

Table A4-1 Beta diversity and differential abundance values from the comparison of microbial composition between different phases (Pre-Bio-Mos, Bio-Mos and Wash out). *The table summarises different beta-diversity analysis outputs calculated by using different distances: phylogenetic (unweighted, balanced, and weighted UniFrac) and ecological (Bray-Curtis and Jaccard's), between different experimental phases: Pre-Bio-Mos, Bio-Mos and Wash out. Numbers represent p-values, with p-values <0.05 identifying statistically significant differences between compared groups. The comparisons are shown for 3 different datasets: (i) All (completed data set containing all the samples sequenced), (ii) a Subset (containing all samples for Pre-Bio-Mos and (iii) the Wash out period, but only stable samplings from Bio-Mos period (time points 22, 24 and 26)). The last row indicates the number of differentially abundant OTUs between Phases of interest.*

Test		Data	Pre-Bio-Mos vs Bio-Mos	Bio-Mos vs Wash out	Pre-Bio-Mos vs Wash out
UniFrac	Unweighted (0%)	All	0.042	0.029	0.001
		Subset	0.002	0.184	0.001
	Balanced (50%)	All	0.023	0.207	0.001
		Subset	0.007	0.648	0.001
	Weighted (100%)	All	0.022	0.37	0.002
		Subset	0.002	0.717	0.001
Bray-Curtis		All	0.034	0.04	0.001
		Subset	0.003	0.727	0.001
Jaccards		All	0.018	0.05	0.001
		Subset	0.001	0.8	0.001
Differential abundance		Subset	149	5	138

Appendix 3: The Role of the Gut Microbiome in Sustainable Teleost Aquaculture.

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Review

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THE ROYAL SOCIETY PUBLISHING

The role of the gut microbiome in sustainable teleost aquaculture

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As the most diverse vertebrate group and a major component of a growing global aquaculture industry, teleosts continue to attract significant scientific attention. The growth in global aquaculture, driven by declines in wild stocks, has provided additional empirical demand, and thus opportunities, to explore teleost diversity. Among key developments is the recent growth in microbiome exploration, facilitated by advances in high-throughput sequencing technologies. Here, we consider studies on teleost gut microbiomes in the context of sustainable aquaculture, which we have discussed in four themes: diet, immunity, artificial selection and closed-loop systems. We demonstrate the influence aquaculture has had on gut microbiome research, while also providing a road map for the main deterministic forces that influence the gut microbiome, with topical applications to aquaculture. Functional significance is considered within an aquaculture context with reference to impacts on nutrition and immunity. Finally, we identify key knowledge gaps, both methodological and conceptual, and propose promising applications of gut microbiome manipulation to aquaculture, and future priorities in microbiome research. These include insect-based feeds, vaccination, mechanism of pro- and prebiotics, artificial selection on the holobiome, in-water bacteriophages in recirculating aquaculture systems (RAS), physiochemical properties of water and dysbiosis as a biomarker.

1. Introduction

Since its conception in the 1980s describing soil ecology [1], the term microbiome has evolved into an intensely studied area of research. In recent decades, this area has begun expanding from an anthropocentric and medically dominated field, into a taxonomically broad field, examining research questions in non-model species, from trees [2] to frogs [3], and increasingly, fish. The diversification in microbiome studies has been driven by increased access to next generation sequencing (NGS), a tool that is not reliant upon culture-based techniques, which often require previous knowledge of target microbes.

Currently, gut bacterial communities have been assessed in over 145 species of teleosts from 111 genera, representing a diverse range of physiology and ecology (figure 1*a*), often with similarities in bacterial phyla composition between fish species, dominated by Bacteroidetes and Firmicutes [5,6]. Non-model taxa from an array of aquatic ecosystems have had their gut microbiomes sequenced using NGS, with studies extending beyond species identification, into hypothesis testing which was once only feasible in model systems. Examples of studies on non-model teleost gut microbiomes range from those demonstrating rapid gut microbiome restructuring after feeding in clownfish (*Premnas biaculeatus*) [7] to the effect of differing environmental conditions, such as dissolved oxygen content, on the gut microbial diversity of blind cave fish (*Astyanax mexicanus*) [8].

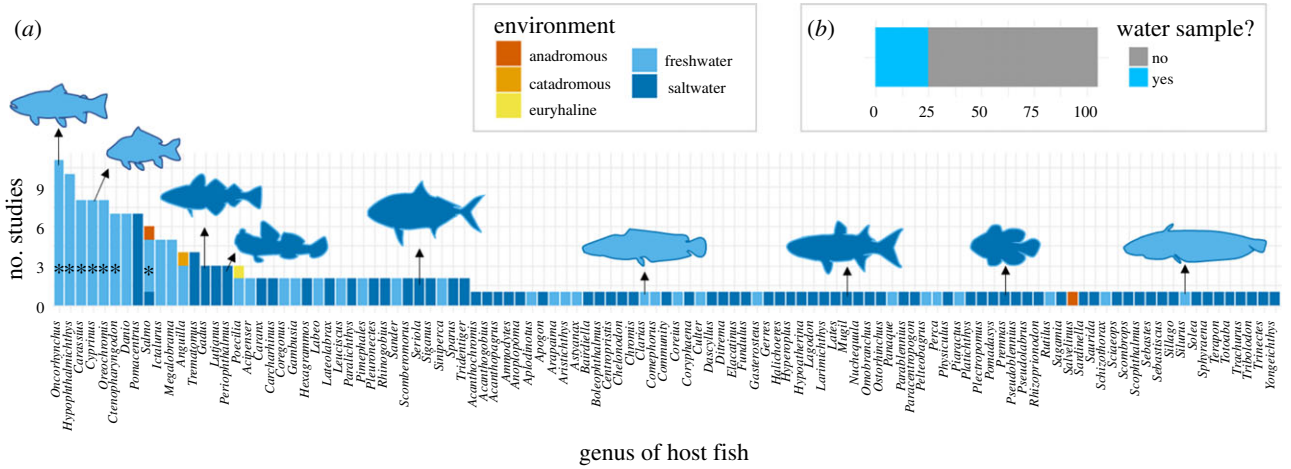

Figure 1. (*a*) Number of studies on the gut microbiome using NGS broken down by the genus of fish that the study was conducted on, as well as the environment those fish same from. Asterisk represents salmonid, carp and talapia. (*b*) The number of studies that assessed the water microbial communities. Gut microbiome studies were compiled using Web of Science [4] and only include studies that implemented NGS. It is acknowledged that total microbiome research extends further than this. Further information on search terms and filtering can be found in the electronic supplementary material. (Online version in colour.)

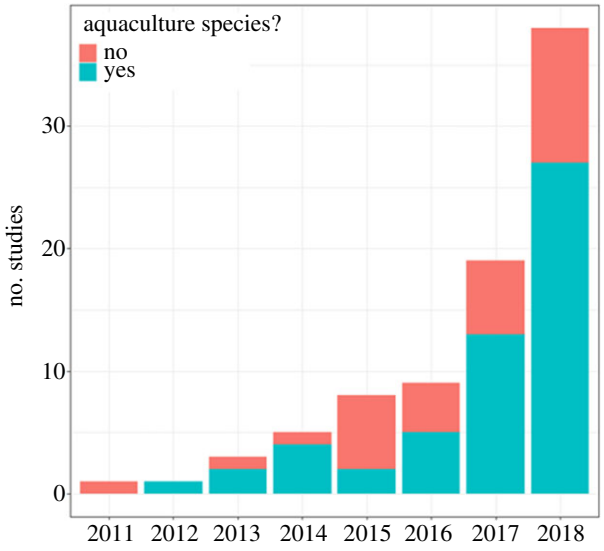

Figure 2. Growth in the studies using NGS on fish gut microbiomes, including food aquaculture species (aquaculture status taken from FishBase [12]). Further information on search terms and filtering can be found in the electronic supplementary material. (Online version in colour.)

Interest in the gut microbiome of fish has accelerated for many reasons, as not only do teleosts represent the most diverse vertebrate group [9], they are also of significant economic importance, including in aquaculture [10]. Aquaculture now provides over 45% of fish-based food products globally [11], and influence of the aquaculture industry on teleost gut microbiome research is demonstrated by the research questions tackled, with a clear bias towards salmonids (genera: *Oncorhynchus* and *Salmo*), carp (genera: *Hypophthalmichthys*, *Carassius*, *Cyprinus* and *Ctenopharyngodon*) and tilapia (genus: *Oreochromis*) (figure 2).

Rapid growth of the aquaculture industry has led to mounting pressure to make it more sustainable [13], and here we discuss four key components relevant to its sustainability in the context of the teleost gut microbiome: diet, immunity, artificial selection and closed-loop systems. We highlight some key deterministic factors important to aquaculture, although as shown in figure 3, there are numerous interacting ecological processes. More in-depth reviews focusing on these specific interactions are available, for example, interactions between the gut microbiome and the immune system [14], energy homeostasis [15] and physiology [16]. Understanding and manipulating microbial–host–environmental interactions (figure 3*a*) and associated functional capacity in these areas could contribute substantially towards achieving a more sustainable aquaculture industry. We identify potential for future research, both methodological and conceptual. Other microbiomes are known to impact host function, in particular, the skin microbiome and its relationship to immunity [17], however, due to their differing ecology [18] and aquaculture applications [19], the gut microbiome will remain our focus here.

2. Diet

The gut microbiome has long been linked with diet, yielding insights into the commensal relationship between certain microbes and host. It has been shown that the teleost gut microbiome produces a range of enzymes (carbohydrases, cellulases, phosphatases, esterases, lipases and proteases) which contribute to digestion [10,20]. More intimate relationships also exist, for example, anaerobic bacteria in the teleost gut have a role in supplying the host with volatile fatty acids [21], an end product of anaerobic fermentation that provides energy for intestinal epithelial cells [22]. Gut microbes also synthesize vitamins and amino acids in the gut of aquatic vertebrates [23,24]. For example, the amount of vitamin B_{12} positively correlated with the abundance of anaerobic bacteria belonging to the genera *Bacteroides* and *Clostridium*, in Nile tilapia (*Oreochromis niloticus*) [25]. Here, we discuss this host–microbe relationship in the context of contemporary aquaculture, with a focus on two timely issues: fishmeal and starvation.

(a) Fishmeal

Fishmeal is an efficient energy source containing high-quality protein, as well as highly digestible essential amino and fatty acids [26], which is included in feed for a range of teleost species. Fish used in fishmeal production is, however,

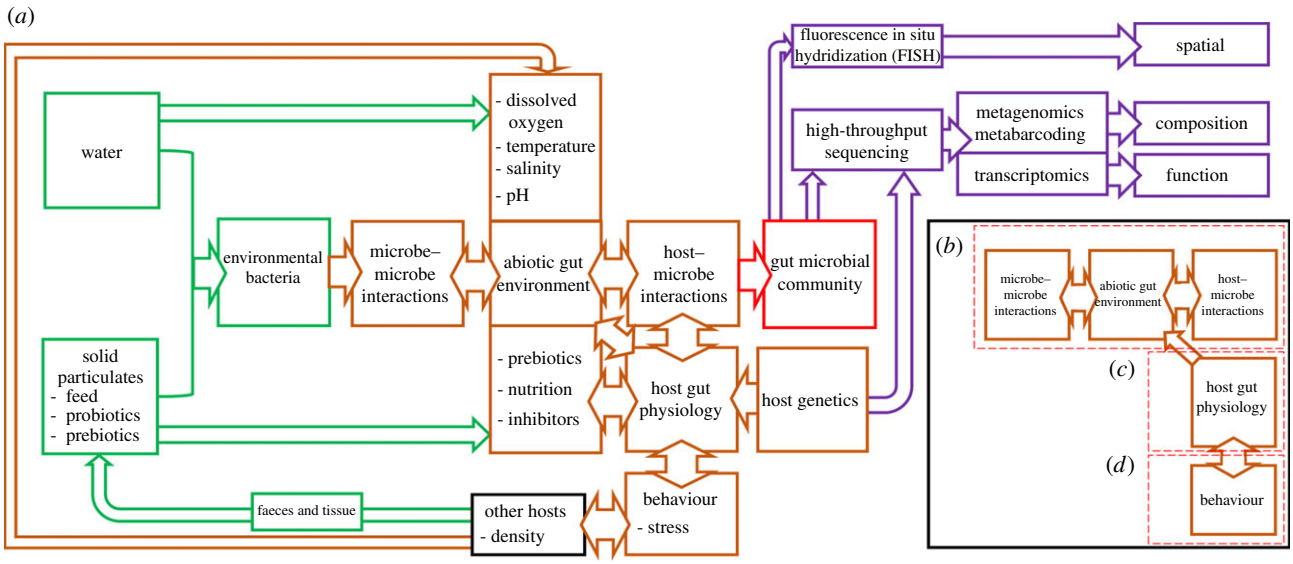

Figure 3. (*a*) Schematic view of the deterministic processes that influence gut microbial communities in fish. Community assemblage of bacteria in the gut starts with inputs from the environment (green), such as the bacteria within the water column, or in solid particulates of biofilm, sediment and feed. Once ingested, these bacteria are influenced by interacting deterministic processes (brown) such as the host's abiotic gut environment, interaction with the hosts' physiology through the gut lining and its secretions, as well as interactions between other microbiomes. The outcome (red) is final community assembly, which can be characterized using an array of cutting-edge molecular techniques (purple). A subset of the boarder interactions is provided, with focus on (*b*) microbe–environment–host interactions, (*c*) host gut physiology and (*d*) behaviour. (Online version in colour.)

predominantly sourced from capture fisheries, putting pressure on already overfished stocks [13]. Despite a global decrease in fishmeal production, from an average of 6.0 million tonnes between 2001 and 2005 to 4.9 million tonnes between 2006 and 2010 [27], and growth in plant-based substitutes (e.g. wheat gluten, soya bean protein and pea protein), some aquaculture species still require a proportion of fish-sourced amino acids and proteins [28].

As dietary changes can alter the fish gut microbiome [29], there has been a considerable rise in the number of studies investigating the influence of alternative plant-protein sources on host–microbe interactions. Plant-protein sources have been shown to disturb the gut microbiota of some fish, with the production of antinutritional factors (factors that reduce the availability of nutrients) and antigens, impeding host resilience to stress [30], metabolism [31] and immune functioning [32]. Fish fed plant-protein-based diets can exhibit alterations in their intestinal morphology including disruption to the lamina propria and mucosal folds [33], which may modify attachment sites for commensal bacteria [34], and can therefore impact microbial composition [32,35].

Insect meal is increasingly used in aquafeed as a protein source with a high nutritional value [36], and several studies have demonstrated its potential use in manipulating the gut microbiome in fish [37,38]. As insects are chitin rich, these diets have been associated with prebiotic effects, through increased representation of beneficial commensal bacteria such as *Pseudomonas* sp. and *Lactobacillus* sp., which in turn improves performance and health in some fish [37]. Despite this, however, the beneficial effects of chitin are species specific, with Atlantic cod (*Gadus morhua*) and several cyprinid species demonstrating increased growth rates on diets with varying levels of chitin, whereas tilapia hybrids (*O. niloticus* × *O. aureus*) and rainbow trout (*Oncorhynchus mykiss*) both display decreased growth rates [39]. Chitin can therefore not be described as a probiotic for all species. The influence of insect meal on microbial-mediated functions also remains underexplored, with little known about the extent to which species-specific responses to a chitin-rich diet are microbially mediated [40], offering scope for future research.

(b) Starvation

Starvation is common in the production of valuable species such as salmon [41], sea bream [42], halibut [43] and cod [44], prior to handling, transportation and harvest, but is also used as a method to improve fillet quality. However, starvation is likely to have a substantial impact on host–microbe interactions (figure 3*b*). Gut microbial communities of the Asian seabass (*Lates calcarifer*), for example, shifted markedly in response to an 8-day starvation period, causing enrichment of the phylum Bacteroidetes, but a reduction of Betaproteobacteria, resulting in transcriptional changes in both host and microbial genes [45]. Perturbation to the gut microbiome could lead to the opening of niches for other commensal or even pathogenic bacteria [46], especially if this is combined with the compromised immune system of a stressed host [47] (figure 3*d*). Even if all fish are terminated shortly after starvation, gut microbial community changes before termination could cause long-term impacts to the microbial composition of water and biofilters in closed recirculating aquaculture systems (RAS). RAS systems will be discussed in greater detail later in this review.

3. Immunity

Gut microbial communities have strong links to immunity [48], which is pertinent in fish as they are in constant contact with water, a source of pathogenic and opportunistic commensal microbes [49]. In addition to this, fish cultured intensively are often stocked at high densities, allowing for easier transmission of microbes. Therefore, a microbially diverse gut microbiome in aquaculture is important to prevent unfavourable microbial colonization [50], and although the mechanisms are not fully

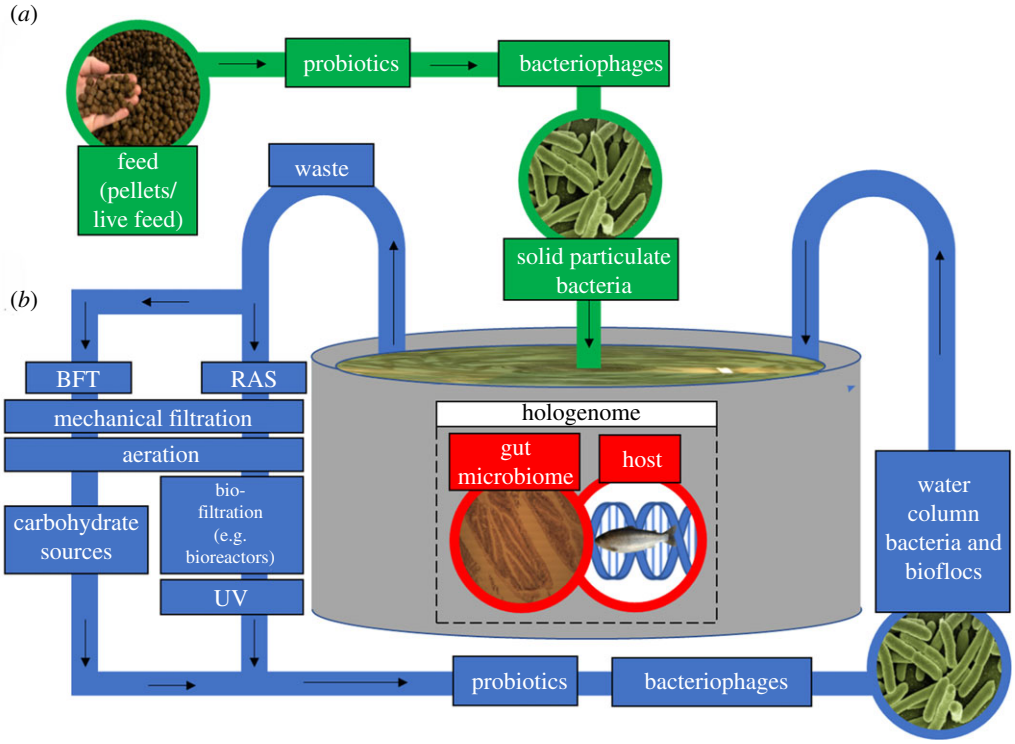

Figure 4. Schematic diagram of (*a*) feed inputs (green), (*b*) water processing (both RAS and BFT) (blue) and the (*c*) species being cultivated, along with its gut microbiome (red). (Online version in colour.)

understood, some key processes have been identified. For example, *Bacillus* and *Lactobacillus*, two common probiotic genera of bacteria used in aquaculture, are able to stimulate expression of inflammatory cytokines in the fish gut [51], increase the number of mucus layer producing goblet cells [52] and increase phagocytic activity [53]. Furthermore, comparison in gene expression between gnotobiotic zebrafish (*Danio rerio*) and conventionally reared zebrafish has shown bacteria induced expression of myeloperoxidase, an enzyme that allows neutrophil granulocytes to carry out antimicrobial activity [54]. Colonizing microbes can also modulate host gene expression to create favourable gut environments, thereby constraining invasion by pathogens [23], while also promoting expression of proinflammatory and antiviral mediators genes, leading to higher viral resistance [55]. Reducing viral and bacterial pathogens, such as *Vibrio* sp. and *Aeromonas* sp., is important for fish health in aquaculture, and will be discussed further in the context of closed-loop systems later in the review.

The interaction between the gut microbiome and the immune system is bilateral, for example, secretory immunoglobulins in fish recognize and coat intestinal bacteria to prevent them from invading the gut epithelium [56]. Similarly, in wild three-spined stickleback (*Gasterosteus aculeatus*), a causal chain (diet → immunity → microbiome) was discovered, demonstrating the impact of diet on fish immunity and thus the microbial composition of the gut [57]. Understanding microbial–host–environmental interactions like this are crucial for aquaculture, where, as previously discussed, diet is often manipulated.

(a) Antibiotics

As most antibiotics used in aquaculture display broad-spectrum activity, they can affect both pathogens and non-target commensal microbes [58]. Oxytetracycline is one of the most widely used veterinary antibiotics, with 1500 metric tonnes applied between 2000 and 2008 to salmon aquaculture in Chile [59]. However, oxytetracycline was seen to reduce gut microbial diversity in Atlantic salmon (*Salmo salar*), while enriching possible opportunistic pathogens belonging to the genus *Aeromonas*, and leading to a high prevalence of multiple tetracycline resistance-encoding bacterial genes [60]. Long-term exposure to oxytetracycline has also been reported to negatively affect growth, immunity and nutrient digestion/metabolism in Nile tilapia (*O. niloticus*) through antibiotic-induced disruption to the microbiota [61], causing considerable changes in the representation of Bacteroidetes and Firmicutes.

Vaccination has become a widespread prophylactic measure applied in aquaculture to improve immune functioning and disease resilience in farmed fish [62]. One study attempted to identify potential alterations in the microbiota structure and localized immune responses caused by a novel recombinant vaccine against *Aeromonas hydrophila* in grass carp (*Ctenopharyngodon idella*) [63]. Results from their study suggest that oral vaccines can target *Aeromonas* sp. through activation of innate and adaptive immune defences within the intestine without causing large disturbances in non-target microbiota populations. Given the importance of the immune response in regulating the gut microbiome [64], only a small number of studies have investigated the influence of vaccines on the resident microbiota composition and function in fish, providing grounds for future study.

(b) Pro- and prebiotic supplementation

In view of the challenges associated with antibiotics, studies have examined the impact of alternative, prophylactic measures such as pro- and prebiotics (figure 4*a*). As literature on the types of pro- and prebiotics used in aquaculture have been reviewed elsewhere [65,66], as well as their effectiveness [67,68], we focus here on the ability of these compounds to induce changes in host physiology and function through shifts in the gut

microbiome. As has already been discussed, *Bacillus* sp. and *Lactobacillus* sp. have a beneficial effect on immunity and are suggested to provide an alternative approach to controlling disease in aquaculture. Targeted microbiota manipulation using these same bacteria have also been reported to exert beneficial effects on fish growth through (i) alterations in gut morphology [69], leading to improved digestion and metabolism [70] and (ii) microbial-mediated regulation of the genetic components involved in growth and appetite control [71,72]. Recently, the establishment of *Lactobacillus* probiotic bacteria within the gut microbiota was also associated with improved learning/memory capacity and changes in shoaling of zebrafish [73,74], indicating a potential gut–brain interaction pathway similar to what is described in higher vertebrates [75].

Research into the modulation of gut microbial communities using prebiotic compounds has expanded also. Certain dietary components have been reported to induce changes in gut morphology within the fish host, including vacuolation of enterocytes [76] and enhancing mucosal barrier integrity [77]. Improved mucosal protection and disease resilience are thought to be driven by microbes and associated microbial metabolites. Several prebiotics have been reported to manipulate the resident microbiota community of a host in favour of Firmicutes and short-chain fatty acid producing communities [78]. Mechanistic pathways remain elusive, however, with additional research required.

4. Artificial selection

Within aquaculture, selection has been applied routinely to increase production by enhancing desirable traits such as growth and disease resilience [79,80]. Recent evidence suggests, however, that host genetics plays a fundamental role in determining the gut microbiota in fish [81]. The 'hologenome' concept proposes that the host organism, along with their commensal microbial community, form one unit of selection [82]. Host physiology, for example, is determined in part by the host's genome and has the ability to shift gut microbiome composition, as demonstrated in zebrafish, whereby host neural activity and subsequent gut motility is able to destabilize microbial communities [46] (figure 3*c*). Although not described in teleosts, the reverse has also been seen, whereby microbial communities are able to regulate the host's gut through: (i) serotonin signalling [83,84], (ii) macrophages and enteric neurons interactions [85], (iii) metabolism of bile salts [86] and possibly, (iv) metabolism of short-chain fatty acids such as butyrate [87]. The host–microbe relationship means that traits selected during breeding programmes may be traits from the hologenome. Pyrosequencing studies have also shown significant changes in the microbial community composition of genetically improved fish compared with domesticated individuals [88,89]. Artificial selection has also been demonstrated on single species of bacteria, with *Aeromonas veronii* selected to exhibit greater colonization success in gnotobiotic zebrafish [90]. Environmental filtering of the reservoir of bacteria surrounding the fish generates the potential for improving colonization success of commensal bacteria. Currently, bacterial communities selected by breeding programmes could be neutral, sympathetic or antagonistic to the goals of artificial selection, and understanding this relationship will be vital in manipulating the hologenome.

5. Closed aquaculture systems

Many environmental problems plague current aquaculture practices. In addition to those already discussed, there are also issues with parasite transmission to wild fish [91], interactions between wild and escaped farmed fish [92], and release of faeces and excess feed into the environment [93]. One way to better control these problems is to remove aquaculture from ecosystems and bring it into a land-based setting [94].

(a) Manipulating environmental microbiota

RAS and biofloc technology (BFT) are forms of aquaculture which use microbial communities to minimize excess nutrients and pathogens in rearing water (figure 4). In these systems, microbial reconditioning of the rearing water is vital as fish are stocked at high densities, resulting in elevated levels of organic material, which can promote microbial growth [95]. Selection of competitive, slow-growing K-strategist bacteria shifts the community from autotrophy to heterotrophy activity. Such shifts allow for a microbial community which maintains both water quality, through nutrient recycling, and inhibits the growth of fast-growing, opportunistic r-strategists, which include many bacterial pathogens such as *Aeromonas* sp. [96,97]. RAS and BFT could therefore be combined with vaccination against bacterial pathogens such as *Aeromonas* sp., as previously discussed, to reduce infections. The selection of K-strategist microbial communities differ between RAS and BFT. In RAS; K-selection is achieved by passing rearing water through heterotrophic biofilters [98], whereas in BFT, a high carbon to nitrogen ratio within rearing water is conditioned by the addition of carbohydrate sources, favouring heterotrophic K-strategist bacteria [99]. High-carbon conditions in BFT systems also promote nitrogen uptake into microbial biomass, which forms protein-rich bacterial 'flocs' that supplement feed [100].

Manipulation of microbes associated with live feed cultures is critical to the production of fish larvae as live feeds often contain opportunistic pathogens (figure 4*a*), resulting in stochastic mortality [64]. While traditional approaches involve non-selective, temporary methods (i.e. physical/chemical disinfection [101]), more recent efforts have shifted towards targeted manipulation through probiotics, for example, the successful use of *Phenylobacterium* sp., *Gluconobacter* sp. and *Paracoccus denitrificans* in rotifer (*Brachionus plicatilis*) production [102]. Lytic bacteriophages have also proven somewhat successful in reducing the prevalence of opportunistic pathogens, such as *Vibrio* sp. [103–105]. Live feed also appears to play a critical role in the delivery and establishment of colonizing gut microbiota in fish larvae upon first feeding [106]. Supplementation of live feed cultures with beneficial microbes, such as the previously mentioned *Lactobacillus* spp. and *Pediococcus* sp., has become common practice in hatcheries, with beneficial effects on growth, mucosal immunity and stress tolerance of larvae [17,107,108]. Bacteriophages and probiotics have also been applied directly to tank water (figure 4*b*); probiotics such as *Bacillus* spp. preventing fish mortality from *Vibrio* spp. infections [109] and *Flavobacterium columnare*-infecting phages have been shown to persist in RAS for up to 21 days [110]. Far less is known about the application of probiotics directly to tank water when compared with feed application [111]; however, and the use of bacteriophages is still in its infancy, providing potential for future research.

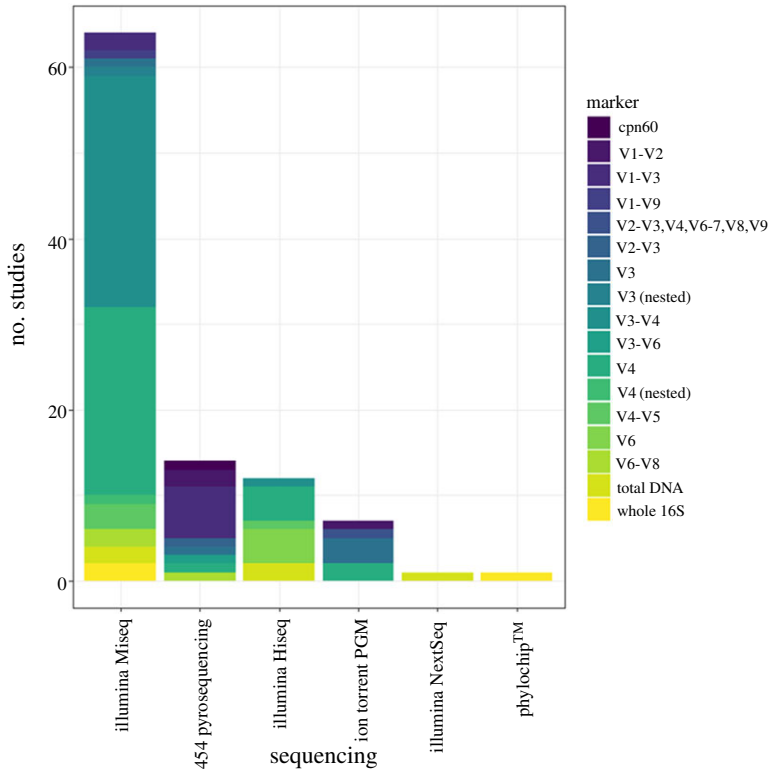

Figure 5. Methodological approaches used in high-throughput sequencing of fish gut microbiomes, broken down by the type of sequencing platform and genetic marker. Marker types are predominantly variable regions (V) within the 16S ribosomal RNA gene. Further information on search terms and filtering can be found in the electronic supplementary material. (Online version in colour.)

(b) Controlling environmental variables

Changes in abiotic conditions in the water column propagate into the gut, as seen with dissolved oxygen concentration [8]. Such parameters are hard to control within the natural environment, but closed-loop systems provide consistent abiotic conditions, and allow for other variables, such as hologenome (figure 4*c*), to be manipulated with greater ease. The effect of many important physiochemical water properties (e.g. nitrate, ammonia and phosphate) on the teleost gut microbiome has not been studied, however, let alone how these properties interact [112]. Salinity is another important physiochemical property for the gut microbiome in many aquaculture species. When Atlantic salmon transition from freshwater to saltwater, individuals can experience a 100-fold increase in gut bacteria, combined with a shift in dominant microbial taxa [113]. Increasing salinity in RAS systems can, however, negatively impact nitrate removal in bioreactors [114], highlighting the importance of understanding interacting physiochemical properties.

(c) Dysbiosis as a stress biomarker

The use of closed-loop systems is a progression to a more intensive method of aquaculture, mirroring the progression seen in animal agriculture, and a crucial element to sustainable intensification is welfare. It is possible to measure fish welfare through physiological and behavioural indicators, with a current focus on identifying stress. The microbiome has been identified as another potential biomarker [64] due to its interaction with the host immune system, and its responsive nature to stressors [115,116]. Therefore, identifying imbalances in the gut microbiome, or dysbiosis, could be a useful predictor of stress-related syndromes, which could ultimately lead to mortality. Using non-invasive faecal samples could complement other non-invasive stress biomarkers, such as water cortisol [117], allowing for the optimization of husbandry, alerting operators to chemical (e.g. poor water quality, diet composition imbalance, accumulation of wastes), biological (e.g. overcrowding, social dominance, pathogens), physical (e.g. temperature, light, sounds, dissolved gases) or procedural (e.g. handling, transportation, grading, disease treatment) stressors [118]. More research is needed, however, in assessing the reliability and accuracy of faecal microbiome sampling in identifying stress.

6. Conclusion and future applications

The teleost gut microbiome has a clear role in the future of aquaculture, and although research has come a long way in recent decades, there are still many areas of gut microbiome research that require further development. As highlighted in figure 1*b*, there are still key elements lacking from many studies, particularly those assessing metacommunity composition, with the lack of water samples being particularly glaring. The ability to sample the environmental metacommunity with ease is one of the strengths of using a teleost model. Another methodological problem that will hinder comparability, reproducibility and metanalysis of fish gut microbiome datasets is the varying degree of sequencing platforms and markers (figure 5). A solution to this problem would be to focus on one marker, and one sequencing platform, with many metabarcoding microbiome studies adopting the V3 and V4 regions, sequenced on Illumina platforms. It is noted, however, that different markers and sequencing platforms work better in some systems with no simple fit-all approach. Therefore, tools that incorporate differences in taxonomic

identification that arise through using different methodological approaches will be vital in comparing datasets.

Current findings, as summarized here, show that the teleost gut microbiome plays an important role in aquaculture, however, the literature is dominated with studies performed on mammals, leading to limited data on functional capacity of fish gut microbiomes [64]. Furthermore, a knowledge gap exists between ascertaining the composition of the microbiome and understanding its function, partly due to the complexity and variability in the ecology of teleost gastrointestinal tracts [119] and unknown bacterial taxa. More specifically, however, it has been caused by the lack of synthesis between multiple cutting-edge molecular techniques. Progression in teleost gut microbiome research will depend on combining function (RNA sequencing), composition (metabarcoding and metagenomics) and spatial distribution (fluorescence *in situ* hybridization). Understanding host genetic diversity (population genomics) and expression (RNA sequencing) of that diversity, all while incorporating environmental variation, will also be vital.

Finally, there are many areas in which synergies between gut microbiomes and aquaculture can be made. These have been highlighted through the review, but, in summary, include a better understanding of the gut microbiome with respect to insect-based feeds, vaccination, mechanism of pro- and prebiotics, artificial selection on the hologenome, in-water bacteriophages in RAS/BFT, physiochemical properties of water and dysbiosis as a biomarker.

Data accessibility. All data collected in the systematic review can be found in the electronic supplementary material.

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Appendix 4: Neutral Processes Dominate Microbial Community Assembly in Atlantic Salmon, *Salmo salar*

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Neutral Processes Dominate Microbial Community Assembly in Atlantic Salmon, *Salmo salar*

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ABSTRACT In recent years, a wealth of studies has examined the relationships between a host and its microbiome across diverse taxa. Many studies characterize the host microbiome without considering the ecological processes that underpin microbiome assembly. In this study, the intestinal microbiota of Atlantic salmon, *Salmo salar*, sampled from farmed and wild environments was first characterized using 16S rRNA gene MiSeq sequencing analysis. We used neutral community models to determine the balance of stochastic and deterministic processes that underpin microbial community assembly and transfer across life cycle stage and between gut compartments. Across gut compartments in farmed fish, neutral models suggest that most microbes are transient with no evidence of adaptation to their environment. In wild fish, we found declining taxonomic and functional microbial community richness as fish mature through different life cycle stages. Alongside neutral community models applied to wild fish, we suggest that declining richness demonstrates an increasing role for the host in filtering microbial communities that is correlated with age. We found a limited subset of gut microflora adapted to the farmed and wild host environment among which *Mycoplasma* spp. are prominent. Our study reveals the ecological drivers underpinning community assembly in both farmed and wild Atlantic salmon and underlines the importance of understanding the role of stochastic processes, such as random drift and small migration rates in microbial community assembly, before considering any functional role of the gut microbes encountered.

IMPORTANCE A growing number of studies have examined variation in the microbiome to determine the role in modulating host health, physiology, and ecology. However, the ecology of host microbial colonization is not fully understood and rarely tested. The continued increase in production of farmed Atlantic salmon, coupled with increased farmed-wild salmon interactions, has accentuated the need to unravel the potential adaptive function of the microbiome and to distinguish resident from transient gut microbes. Between gut compartments in a farmed system, we found a majority of operational taxonomic units (OTUs) that fit the neutral model, with *Mycoplasma* species among the key exceptions. In wild fish, deterministic processes account for more OTU differences across life stages than those observed across gut compartments. Unlike previous studies, our results make detailed comparisons between fish from wild and farmed environments, while also providing insight into the ecological processes underpinning microbial community assembly in this ecologically and economically important species.

KEYWORDS aquaculture, fish, host-microbe, microbial communities, microbial ecology, microbiome

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Worldwide, salmonid aquaculture accounted for over 9 billion euros in 2014 (1), with the industry rapidly expanding to feed a growing global population. As such, the need to further current knowledge of core host processes, such as energy allocation, physiology, and behavior, is at the forefront of salmonid research. Previous studies on mammals, fish, and invertebrates have implicated the gut microbiota in a number of these processes (2). To date, there are limited studies discussing the bacterial diversity and functional diversity of fish intestinal microbiota (e.g., references 3 and 4). In salmon, no studies have addressed the relative roles of neutral (stochastic) and selective (deterministic) processes in shaping gut communities, which are key to understanding the importance of the host environment in driving community assembly (5).

The life cycle of Atlantic salmon, *Salmo salar*, is complex, with individuals employing a number of different life history strategies (reviewed in reference 6). Most forms are anadromous, completing a juvenile stage in freshwater, a long migration to the ocean for maturity, and a return migration back to the original freshwater rearing grounds for spawning. To transition from the juvenile "parr" stage, individuals must smoltify to enter the marine environment. Smoltification encompasses all physiological, developmental, and behavioral changes that accompany this life stage transition (7). Changes include silvering of the skin and darkened fin margins alongside the reorganization of major osmoregulatory organs including the gills, gut, and kidney in order to develop seawater tolerance (7, 8). Following maturity in marine waters, individuals must then physiologically reacclimate to the freshwater environment to which they return to reproduce. Studies have shown that individuals respond differently to stress according to life stage, with smolts more responsive to stress than parr, measured by increased levels of plasma cortisol (9, 10). Each transition between life cycle stages to enable individuals to survive and thrive in a different environment will likely impact the resident host-associated microbiota.

The gut microbiota in salmonids is thought to be largely shaped by dietary and environmental factors, although initial bacterial colonization of the gastrointestinal tract begins shortly after hatching (11). Salmonids are gastric fishes. Their guts are characterized by a clearly defined stomach followed by a pylorus with attached blind vesicles called pyloric ceca as well as a relatively short and nonconvoluted posterior (mid and distal) intestine leading to the anus (12). Attempts have been made to map the microbial diversity of different gut compartments in onshore saltwater recirculation systems, but it is unclear where either pyloric ceca or stomach has been sampled (13). A number of studies have demonstrated the impact of diet on the resident gut microbiota (14, 15). It has been shown that certain diets, such as soybean protein concentrate, can cause dysbiosis of the gut microbiota by increasing the bacterial diversity to include those not typically associated (16). The core gut microbiota of wild Atlantic salmon is typically characterized by the key presence of *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* in freshwater life stages and *Tenericutes* (genus *Mycoplasma*) in marine-phase adults (4). Only a minority of core operational taxonomic units (OTUs) is thought to be conserved across both freshwater and saltwater phases in the wild (4). In contrast to wild salmon, the microbiota of farmed salmon seems to be more stable during the transition from freshwater to saltwater (17).

There is considerable debate in the literature around the role of gut microbes in host health and ontology across taxa (e.g., reference 18). One step toward understanding the relationship between microbes and their host is to establish whether the host environment has any impact on microbial community structure. For example, there is evidence in both vertebrate and invertebrate systems that some species can lack a resident microbiome altogether (e.g., reference 19). By combining next-generation sequencing and modeling approaches, one can assess the relative contribution of stochastic and deterministic processes in driving community assembly to indicate whether host-associated microbes are indeed any different from those in the immediate environment. One such approach is via the application of neutral community models (NCMs) (e.g., reference 20). Neutral theory assumes species are "neutral" in their

ecological niches, and community assembly is the result of stochastic dispersal and drift whereby organisms are randomly lost and replaced by migration from the source metacommunity (21). In contrast, "nonneutral" deterministic theory predicts that environmental (e.g., intrahost) conditions and interspecific interactions determine microbial species abundance (22). Due to their wide-ranging relevance, NCMs have successfully been applied to the understanding of microbial community assembly and have successfully predicted community structures (23–26). Arguably, the most robust NCM is by Sloan et al. (20), as it calibrates Hubbell's neutral theory and is able to reproduce patterns throughout variously sized samples (23). However, despite the clear benefits of NCMs, they are not without controversy, with some arguing that they only explain a very small percentage of variance in host organisms (e.g., reference 27).

In the current study, we use 16S rRNA gene MiSeq sequencing analysis and NCMs (20) to examine microbial community assembly and transfer between different life history stages and digestive compartments in Atlantic salmon, *S. salar*. In a wild salmon system, we compare the microbiota within the midgut of each freshwater life cycle stage, including parr, smolt, and returning adults, alongside the midgut of marine-phase adults. We also analyze adult salmon gut microbial communities, sampled from an aquaculture fishery, to assess microbial diversity and function in different sections of the digestive tract. Finally, we are also able to compare community composition and taxonomic and functional diversity as well as determine the role of neutral and nonneutral processes in community assembly and transfer in salmon from both farmed and wild environments.

RESULTS

Richness comparisons for farmed and wild salmon. We undertook surveys of both functional and taxonomic richness among our study groups, including direct comparisons between midgut richness in farmed and wild salmon. Among wild salmon, we observed a significant decline in the number of taxa present throughout the life cycle, although retuning adults held a greater diversity of microbes than that of marine-phase adults ($P < 0.001$) (Fig. 1). In farmed salmon, the lowest richness was observed in the pyloric cecum, significantly lower than richness levels in the stomach ($P = 0.008$), midgut ($P = 0.012$), or bile duct ($P = 0.003$) (Fig. 1). Interestingly, taxonomic richness in wild, adult marine-phase fish ($n = 47$) was significantly lower than that observed in the farmed adults ($P = 0.021$). Functional richness estimates generated by modeling whole microbial metagenomes from 16S data using Tax4Fun (28) indicated similar patterns of statistical significance in variation to taxonomic richness among wild samples (i.e., declining with maturation between life cycle stages) (Fig. 2). Functional richness estimates among different gut compartments in farmed salmon support a reduction in diversity in the pyloric cecum compared to that in all other compartments; however, the bile duct also appears different, with a richer functional repertoire than the midgut ($P = 0.003$) and stomach ($P = 0.029$) (Fig. 2). Interestingly, functional repertoire comparisons between the midgut of farmed and wild marine-phase salmon suggest no significant differences, despite large differences in taxonomic diversity ($P = 0.720$) (Fig. 2).

Taxonomic diversity and compositional differences between life history stages, gut compartments, and farmed and wild salmon. Pairwise comparisons of beta diversity among all pairs of samples are shown in Table 1. Significant divergence was observed among farmed adults and both freshwater and marine wild individuals (Fig. 3). Multiple instances of significant compositional divergence were also observed between gut compartments in farmed fish, especially in relation to comparisons with the pyloric cecum. Life cycle stage had a significant effect on microbial community composition, as we have observed previously (4). Microbial genera that showed significant differential abundance between gut compartments and life cycle stages are summarized in Fig. S1 and S2 in the supplemental material. Again, life cycle stage-specific differences are described extensively in Llewellyn et al. (4). In terms of differential taxonomic abundance between gut compartments, the stomach is most frequently an outlier, being

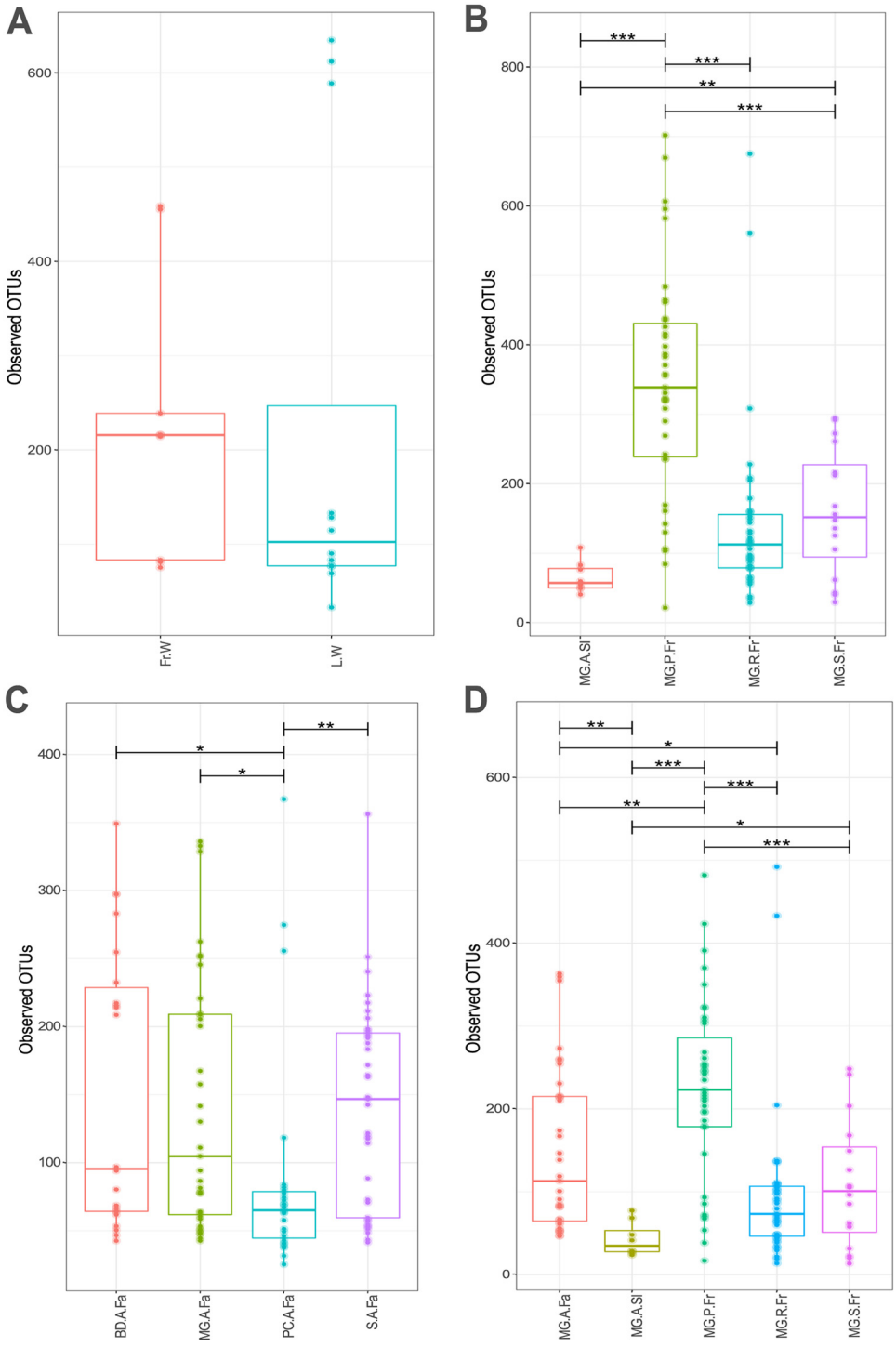
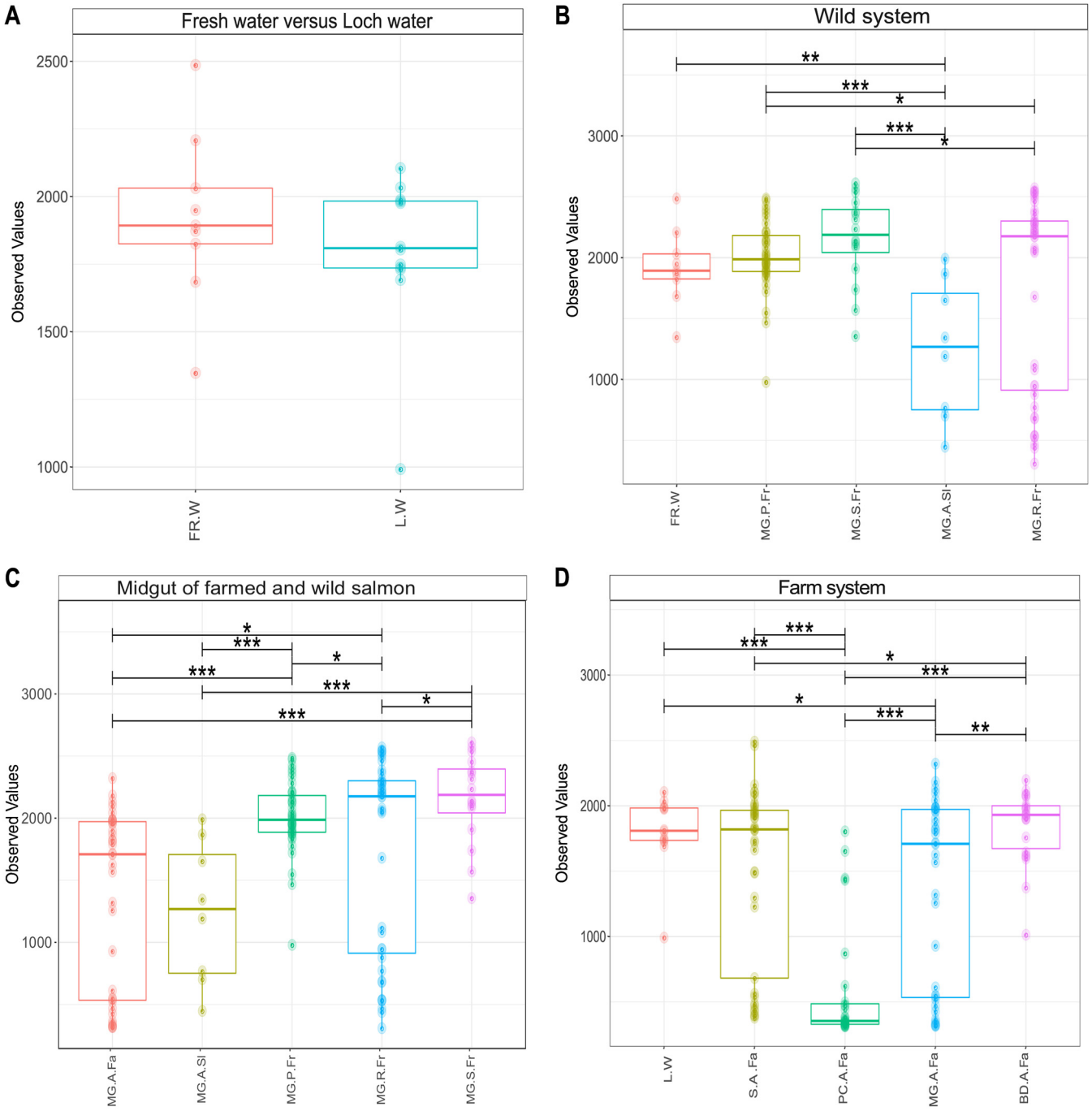


FIG 1 Alpha diversity measured in terms of richness of OTUs found across samples. (A) Comparison between freshwater (Fr.W) and loch water (L.W). (B) Comparison between the midgut of wild individuals sampled according to life cycle stage, including marine-phase adults (MG.A.Sl), parr (MG.P.Fr), smolt (MG.S.Fr), and returning adults (MG.R.Fr). (C) Different gut compartments of farmed subadults including midgut (MG.A.Fa), stomach (S.A.Fa), pyloric cecum (PC.A.Fa), and bile duct (BD.A.Fa). (D) Midgut of wild individuals sampled according to life cycle stage and midgut of farmed subadults (MG.A.Fa). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.



L.W : Loch Water ; **FR.W** : Fresh Water
MG.A.Sl : Midgut of Salt Water Adult ; **MG.P.Fr** : Midgut of Fresh Water Parr ;
MG.R.Fr : Midgut of Fresh Water Returning Adult ; **MG.S.Fr** : Midgut of Fresh Water Smolt
BD.A.FA : Bile duct of Adult Farm ; **S.A.FA** : Stomach of Adult Farm ;
PC.A.FA : Pyloric Caecum of Adult Farm ; **MG.A.FA** : Midgut of Adult Farm

FIG 2 Functional diversity found across samples. (A) Comparison between freshwater (Fr.W) and loch water (L.W). (B) Comparison between the midgut of wild individuals sampled according to life cycle stage, including marine-phase adults (MG.A.Sl), parr (MG.P.Fr), smolt (MG.S.Fr), and returning adults (MG.R.Fr). (C) Midgut of wild individuals sampled according to life cycle stage and midgut of farmed subadults (MG.A.Fa). (D) Different gut compartments of farmed subadults including midgut (MG.A.Fa), stomach (S.A.Fa), pyloric cecum (PC.A.Fa), and bile duct (BD.A.Fa). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TABLE 1 Mean pairwise beta diversity identifying significant differences in microbial profile across the environment, gut compartment, and life cycle stage of *S. salar*[a]

Sample	Beta-diversity of gut compartment and environmental samples in farm system (*P* value)					
	Bile duct	Feed	Loch Water	Midgut	Pyloric Coecum	Stomach
Bile duct	.	0.103	0.167	0.244	**0.020**	0.071
Feed		.	0.230	**0.020**	**0.042**	**0.020**
Loch Water			.	**0.021**	**0.020**	**0.020**
Midgut				.	**0.015**	0.071
Pyloric Coecum					.	**0.039**
Stomach						.
	Beta-diversity of gut compartment and environmental samples in both wild and farm systems (*P* value)					
	Adult Farm	Marine Adult	Parr	Returning Adult	Smolt	Freshwater
Loch Water	No comparison	No comparison	No comparison	No comparison	No comparison	**0.001**
Adult Farm	.	**0.001**	**0.001**	**0.001**	**0.001**	No comparison
Marine Adult		.	**0.001**	**0.001**	**0.016**	No comparison
Parr			.	**0.004**	**0.001**	No comparison
Returning Adult				.	**0.001**	No comparison
Smolt					.	No comparison
	Beta-diversity of midguts and environmental samples in wild system					
	Fresh water	Marine Adult	Parr	Returning Adult		Smolt
Fresh water	.	**0.016**	**0.002**	**0.002**		**0.002**
Marine Adult		.	**0.003**	**0.003**		**0.010**
Parr			.	**0.002**		**0.002**
Returning Adult				.		**0.002**
Smolt						.

[a]All relevant comparisons (GUniFrac with PERMANOVA test) are stated, and the corresponding significance value (adjusted *P* value, Benjamini-Hochberg test) included.

highly enriched for *Aliivibrio*, *Weissella*, *Lactobacillus*, *Photobacterium*, *Paracoccus*, and *Pantoea* species. The pyloric cecum is highly enriched for *Mycoplasma* species while *Paracoccus* and *Lactobacillus* species show lower abundance. High levels of enrichment for *Mycoplasma* species in the pyloric cecum likely account for the position of this gut compartment as an outlier on the basis of beta diversity estimates. The lowest abundance of *Mycoplasma* species was found in the bile, which also corresponds to the compartment from which no host cellular material was included in the DNA extraction.

Neutral model in shaping community assembly. Differences in beta diversity among microbial communities may result from neutral sampling effects (e.g., demographic bottlenecks) rather than adaptation of microbes to different environments. To explore the role of neutral processes in determining microbial community assembly, we first deployed the Sloan neutral model (20) in both the farmed and wild systems. In the farmed system, we examined the relative role of neutral and deterministic processes in a sequential, stepping-stone pattern (Fig. 4), moving from a combined food and water source through the gut system (Fig. 4, bar plot, neutral model hybrid). We noted a preponderance of OTUs that fitted the neutral model among all comparisons. Many of the OTUs that accounted for those that did not fit the neutral model were assigned to *Mycoplasma* species (indeed no *Mycoplasma* sp. OTUs fitted the neutral model), which can be observed in Fig. 4 as well as in Table S1 in the supplemental material. *Aliivibrio*, *Lactobacillus*, and *Paracoccus* species were also among those that showed nonneutral patterns of colonization in farmed fish (see Table S1). Figure 5 shows similar analyses describing OTU abundances among wild salmon. Again, neutral processes best account for the presence of the majority of OTUs among different life cycle stages compared to their freshwater source communities. Overall, however, deterministic processes account for more OTU differences between life cycle stages than between gut compartment communities (Fig. 4 and 5). The intestines of returning adults appear to contain the largest number of OTUs that show evidence of host adaptation compared to the abundance and diversity of their source microbes in the freshwater environment, as

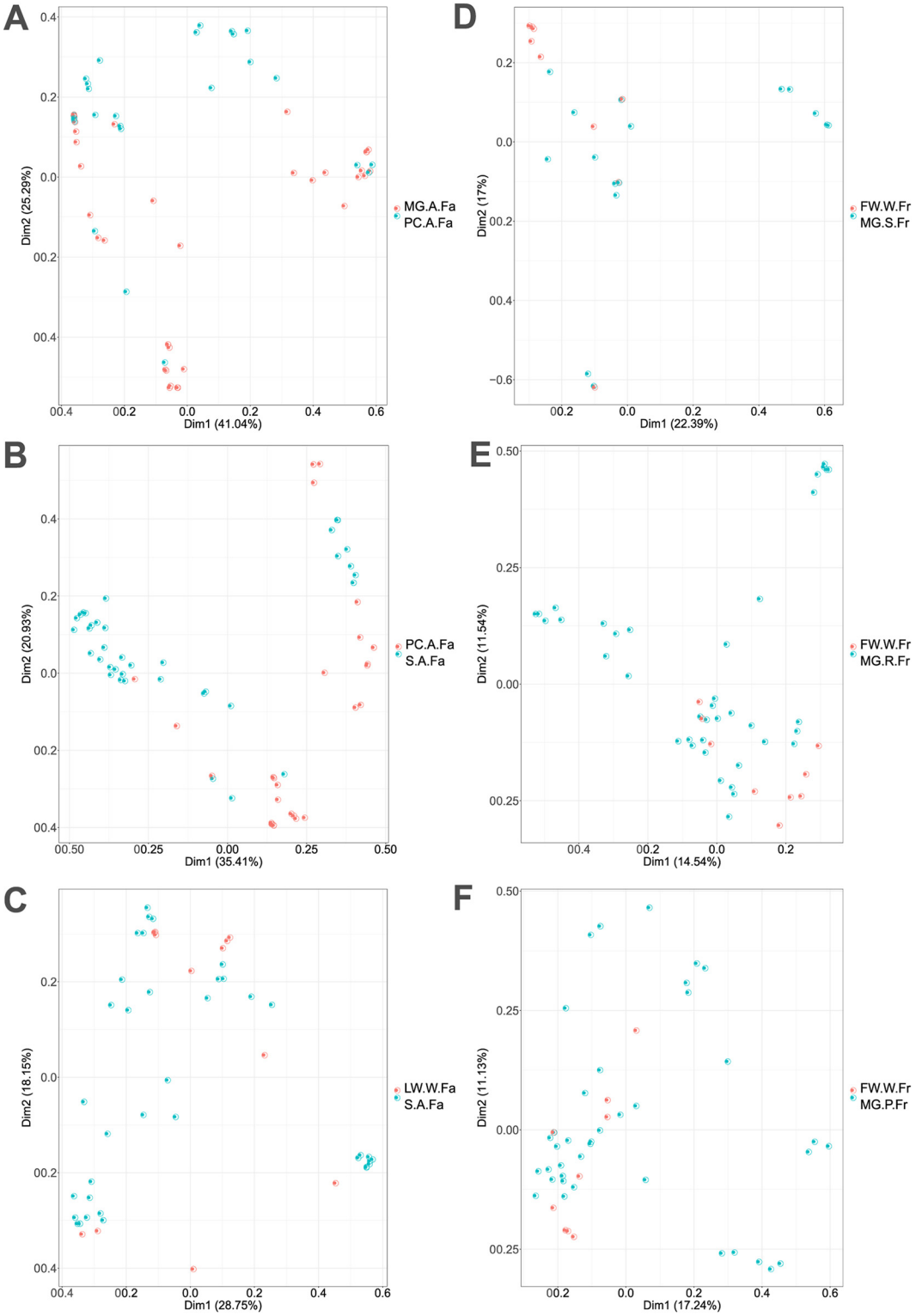


FIG 3 Principal coordinates analysis (PcoA) plot showing the mean pairwise beta diversity in microbial profile across the environment, gut compartment, and life cycle stage of *S. salar*. (A) Comparison between the midgut and pyloric cecum of farmed individuals. (B) Pyloric cecum and stomach of farmed adults. (C) Environmental loch water to stomach of farmed adults. (D) Fresh water and midgut of wild smolt. (E) Freshwater and midgut of returning wild adults. (F) Freshwater and midgut of wild parr.

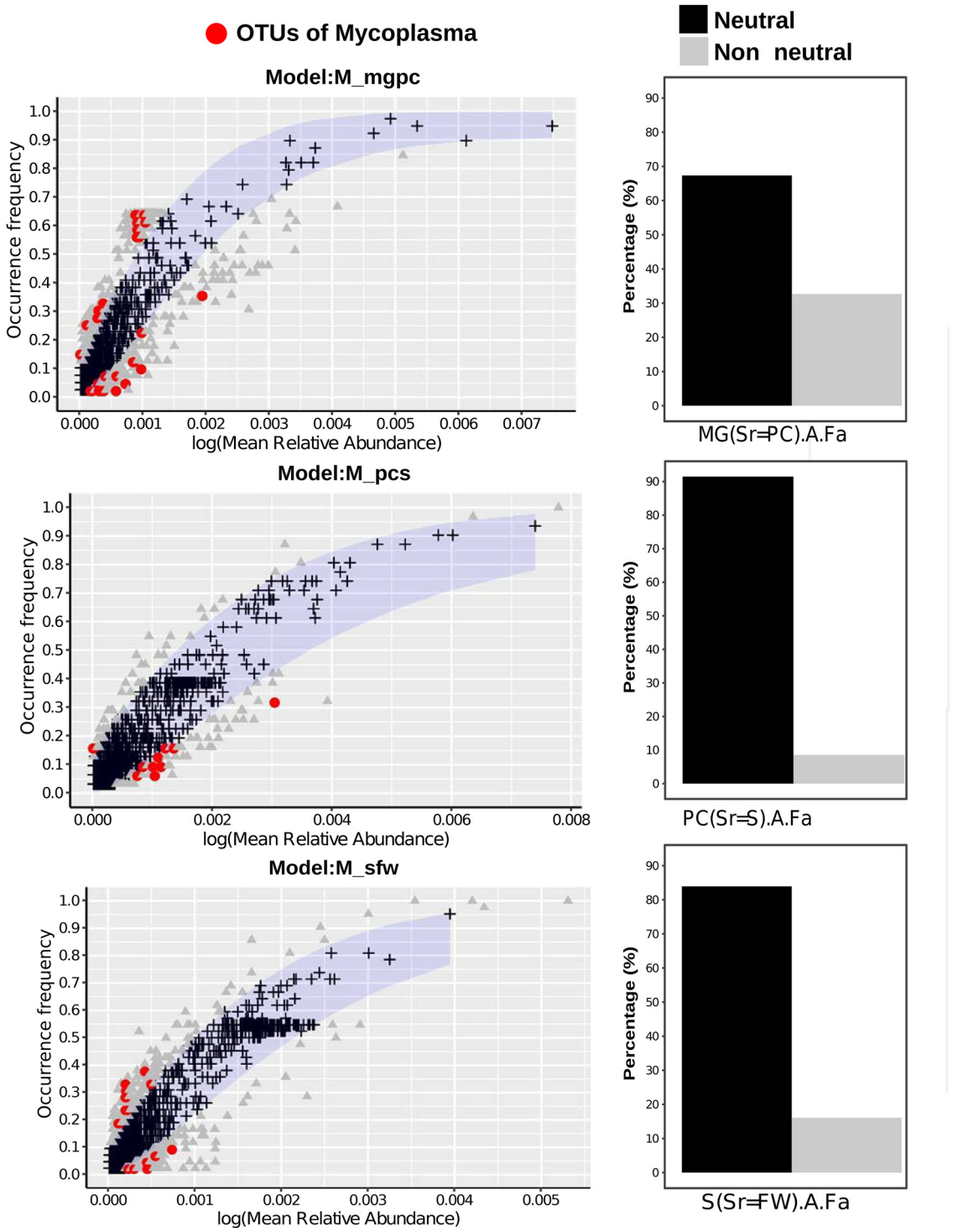


FIG 4 Demographic variation of community neutrality (measured as percentage) across differing samples of farmed *S. salar*. Neutrality is measured as the migration rate from the source community. Different gut compartments of subadult farmed individuals were compared to environmental feed and water samples (FW) as the source community, before being compared sequentially through the digestive tract as follows: stomach (S), pyloric cecum (PC), midgut (MG), bile duct (BD). Neutral processes are shown in black while nonneutral are depicted in gray. Selection of comparisons to show how well the OTUs fit the neutral model. Neutral OTUs are shown in black, nonneutral are depicted in gray, while the red is *Mycoplasma* sp. OTUs. We see no *Mycoplasma* sp. OTUs that fit the neutral model. The roles of OTUs from the pyloric cecum as the source community to the midgut (top), stomach compartment to the pyloric cecum (middle), and combined food and water to the stomach compartment (bottom) are shown.

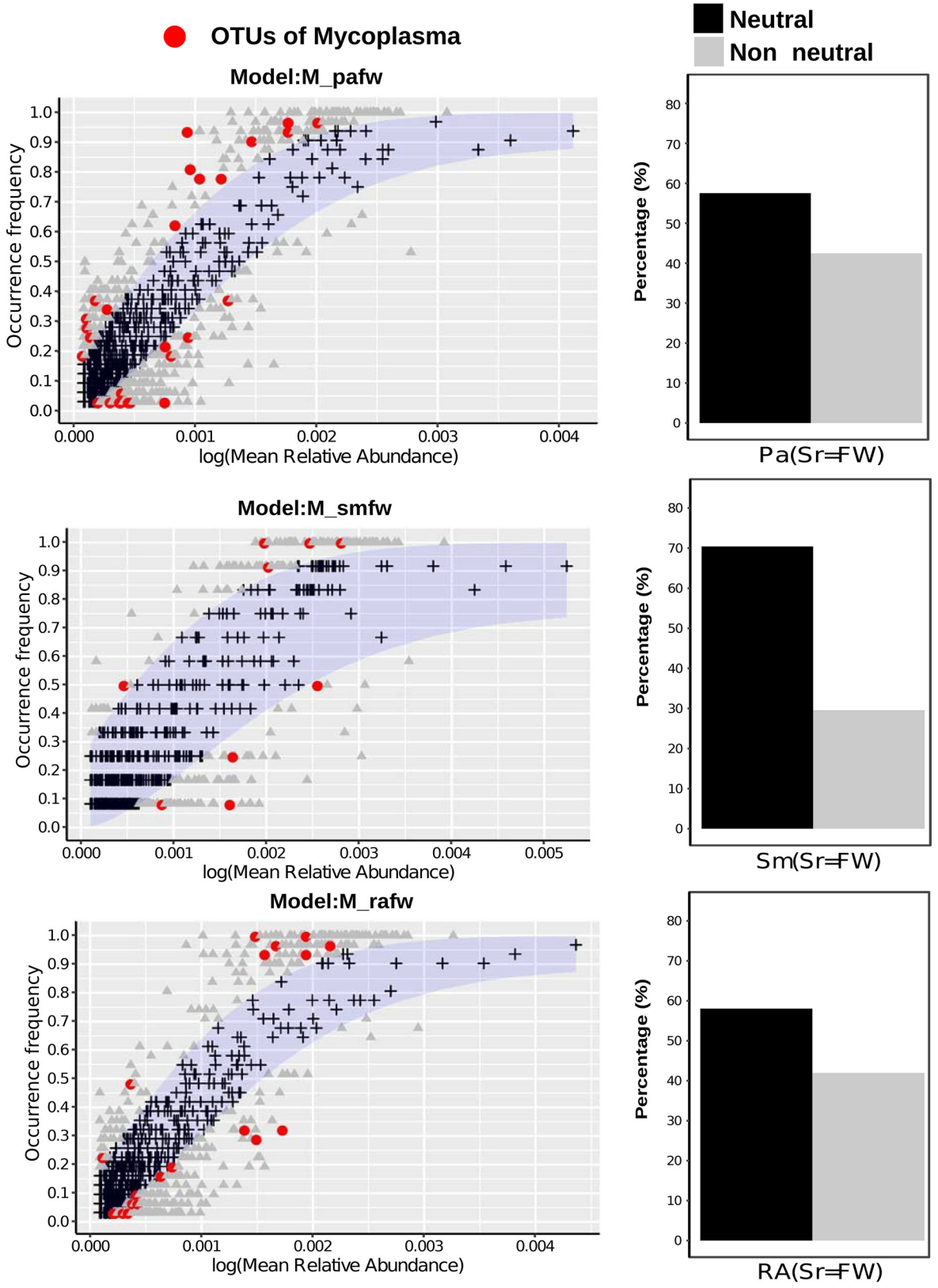


FIG 5 Demographic variation of community neutrality (measured as percentage) across differing samples of wild *S. salar*. Neutrality is measured as the migration rate from the source community. The midgut of different life cycle stages of wild individuals was sampled and compared to environmental water samples as the source community before being compared sequentially through life cycle stages as

(Continued on next page)

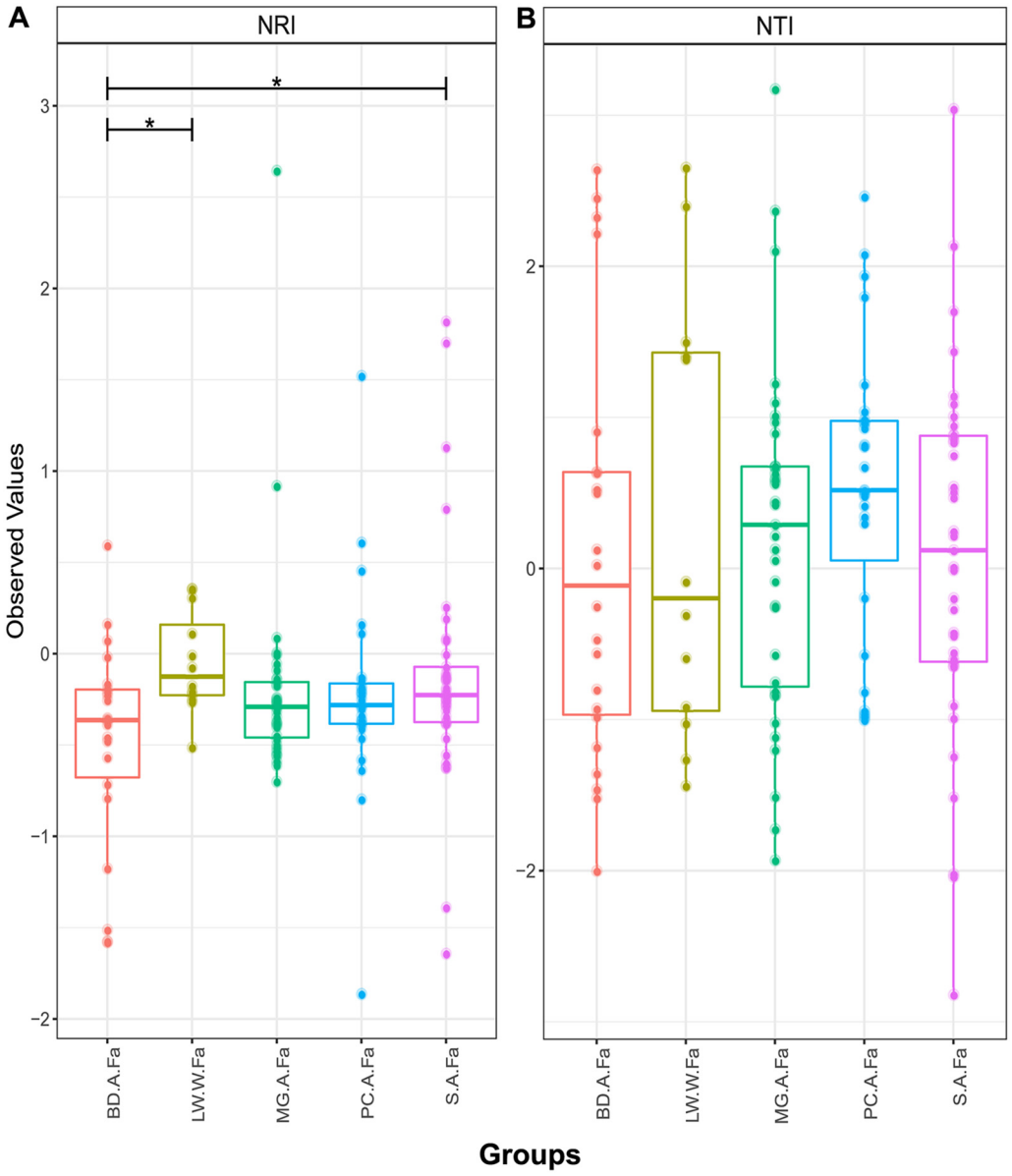


FIG 6 Indices of phylogenetic (NRI) (A) and taxonomic (NTI) (B) dispersion among the gut compartments and environmental communities associated with farmed fish. Samples include stomach (S.A.Fa), pyloric cecum (PC.A.Fa), midgut (MG.A.Fa), bile duct (BD.A.Fa), and loch water (LW.W.Fa). Significant differences are indicated with an asterisk (*, $P < 0.05$).

well that of their source microbes in marine adults (Fig. 5). We also explored the goodness of fit of mycoplasmas and found that *Mycoplasma* OTU abundance in wild fish, as with farmed fish (Fig. 5), was poorly explained by the neutral model (Fig. 5). Stegen's (29) indices of taxonomic (nearest taxonomic index [NTI]) and phylogenetic (net relatedness index [NRI]) dispersion among the gut compartments and environmental communities associated with farmed fish (Fig. 6) largely support the findings from Sloan's model (Fig. 4), with little deviation from neutral expectations overall with

FIG 5 Legend (Continued)
follows: parr (Pa), smolt (Sm), marine adult (MA), and returning adult (RA). Neutral processes are shown in black while nonneutral are depicted in gray. Selection of comparisons to show how well the OTUs fit the neutral model. Neutral OTUs are shown in black, nonneutral are depicted in gray, while the red is *Mycoplasma* sp. OTUs. We see no *Mycoplasma* sp. OTUs that fit the neutral model. The roles of OTUs from combined food and water as the source community to the parr life cycle stage (top), food and water to smolt (middle), and food and water to returning adult (bottom) are shown.

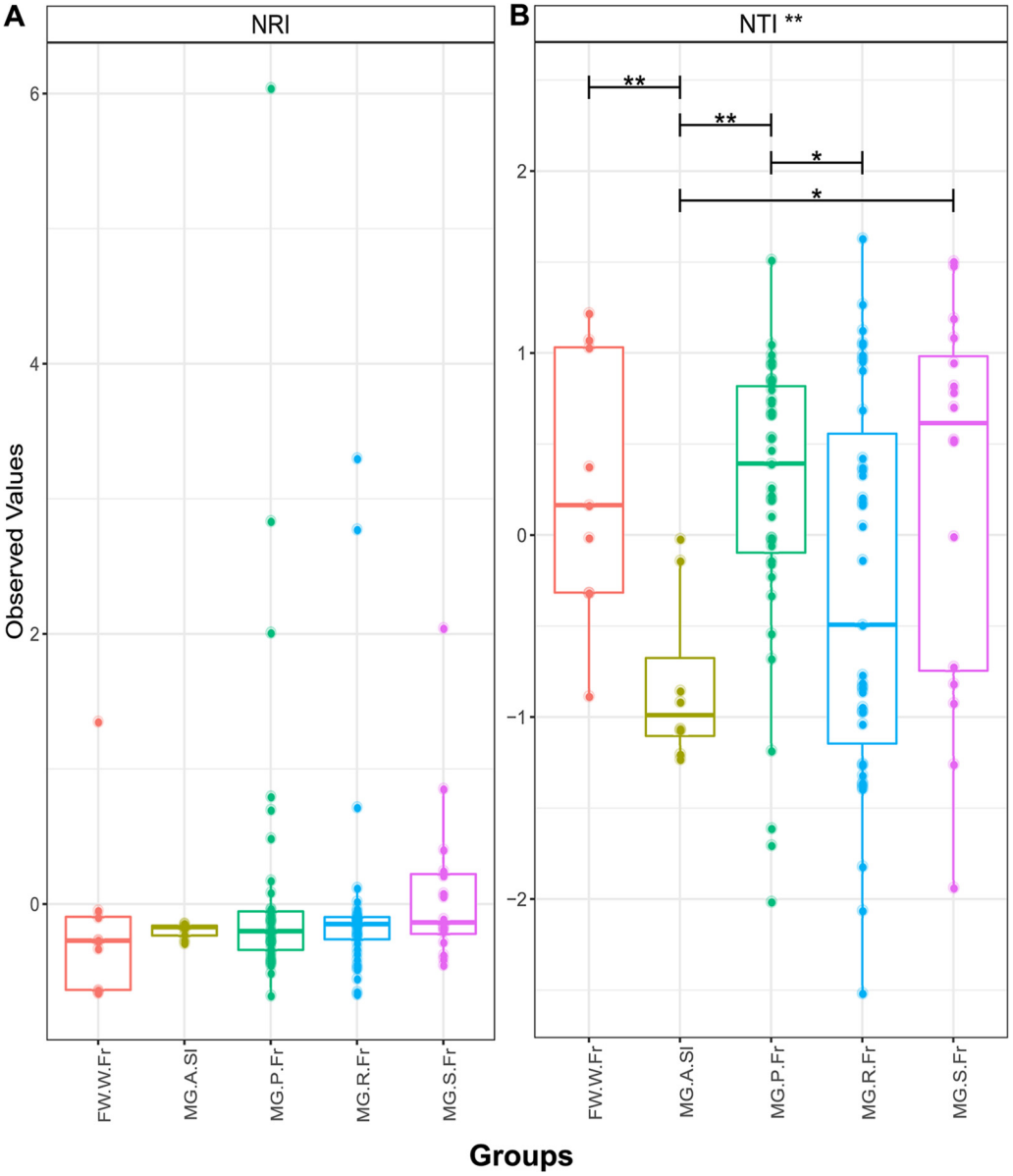


FIG 7 Indices of phylogenetic (NRI) (A) and taxonomic (NTI) (B) dispersion among the midguts of different life cycle stages of wild salmon. Samples include combined food and water (FW.W.Fr), smolt (MG.S.Fr), parr (MG.P.Fr), returning adult (MG.R.Fr), and marine adult (MG.A.Sl). Significant differences are indicated with asterisks (*, $P < 0.05$; **, $P < 0.01$).

the exception of some weakly significant differences in NRI between the bile duct, stomach, and environmental microbes (see Table S1 in the supplemental material). Among wild life cycle stages, NRI scores are generally negative, although values from fish do not deviate from their freshwater source community, suggesting no genuine effect (Fig. 4). NTI scores, conversely, are strongly negative in marine-phase salmon. A comparison to the freshwater sample is not relevant, and local sampling of microbes from Greenland's marine environment was not possible. Significant declines in NTI values between parr and adults (marine-phase and returning) support an increasingly important role of the host habitat in filtering community diversity (Fig. 7) and may link to the declining OTU richness also observed in alpha diversity analyses (see Fig. 1).

DISCUSSION

Our study explores the ecological processes underpinning community assembly in Atlantic salmon, makes detailed comparisons between farmed and wild fish, and makes

direct comparisons between the gut compartments of farmed Atlantic salmon from sea cages. In the wild fish, we see declining microbial community richness, both taxonomic and functional, as fish mature through different life cycle stages alongside an increasing role for the host in filtering microbial communities. In gut compartments of farmed fish, the neutral models suggest that the majority of microbes appear to be transient, with a limited subset of gut microflora apparently adapted to the host environment, among which *Mycoplasma* spp. are dominant. In our data, while taxonomic richness estimates between the guts of wild and farmed marine-phase salmon show significant differences, their predicted functional richness is stable.

Our findings that the environmental microbes are the source of salmon intestinal microbes in parr are consistent with findings that initial bacterial colonization of the gastrointestinal tract begins shortly after hatching (11). However, microbes from the environment apparently continue to actively colonize later life cycle stages (smolts and returning adults) directly from the water, and the majority of OTUs fit a neutral model assuming freshwater as the origin. Burns et al. (21) found that the proportion of OTUs fitting a neutral model, with respect to environmental sources, declined linearly with age during early zebrafish development. This is presumably due to increasingly selective filtering by the host environment (21). Similar patterns were not observed in our data, although all salmon studied were at a much later stage of development than the embryonic zebrafish investigated by Burns et al. (21). We did, however, note an increasingly important role for host filtering in comparisons between life cycle stages (parr and smolt, smolt and marine adult, marine adult and retuning adult). These data corresponded broadly with increasingly negative NTI values among later life cycle stages and suggest that a subset of host-adapted, taxonomically related OTUs come to dominate the *S. salar* microbiome as it matures. The declining trend in OTU richness observed across life cycle stages in our study is also consistent with observations that gut OTU richness declines with age in juvenile Atlantic salmon (30). As we noted in a previous study based on the wild salmon data set (4), *Mycoplasma* spp. are a dominant presence, especially among adults, with others observing the same phenomenon (31, 32). The inability of neutral models to explain the abundance of any *Mycoplasma* sp. OTUs in any comparisons in the current study suggests that these organisms may be highly adapted to the host environment.

A principal aim of our study was to establish the diversity of microbes among different sections of the Atlantic salmon gut. A previous attempt to map the diversity of microbes across different gut compartments did so in a recirculating aquaculture system (RAS) (13). Differences between this study and ours may, therefore, reflect variation in the environmental source communities, given their likely importance in defining microbial community structure. In our study, the great majority of microbial OTUs experienced no host filtering as they pass through the gut from the environment (feed and water). Comparisons with Gajardo et al. (13) are further frustrated by a lack of anatomical precision in the definition of different gut compartments. In this sense, the adoption of a standardized nomenclature and anatomical map, akin to that presented by Løkka et al. (12), would benefit the research community. Particularly abundant microbial OTUs from the intestines of farmed fish in our study included *Aliivibrio*, *Mycoplasma*, *Lactobacillus*, and *Paracoccus* species (see Fig. S1 in the supplemental material), many of which did not follow the neutral model. We find a number of similarities to others who have characterized the gut of farmed Atlantic salmon in open mariculture (31, 32). The abundance of *Paracoccus* species in our system, especially the stomach, may in part be explained by its abundance in the feed (data not shown). As with wild samples, the lack of compliance of any *Mycoplasma* sp. OTUs with neutral models supports some form of active adaptation to the host environment. The pyloric cecum, a region of densely packed epithelial folds and the site of most nutrient absorption in Atlantic salmon, was most enriched for *Mycoplasma* sp. OTUs. Many *Mycoplasma* species are intracellular commensals or pathogens (e.g., references 33 and 34). If *Mycoplasma* spp. recovered from the samples here share a similar lifestyle, abundant gut epithelial cells in constant contact with the digesta in this pyloric cecum

may represent a permissive microbial habitat. Further work, potentially involving *in situ* visualization of microbes in the gut (e.g., reference 35), could reveal more.

The dominance of *Mycoplasma* species in both farmed and wild fish may not be an example of evolutionary convergence. Marine salmon farms are very frequently placed along the costal migratory routes of their wild congeners. Pathogen and parasite transfer between farmed and wild fish is a major consideration of coastal economies (e.g., reference 36). It is entirely possible that commensals like *Mycoplasma* spp. can also pass between farmed and wild fish in a similar fashion. Other microbial species were shared between farmed and wild marine-phase salmon (e.g., *Aliivibrio* and *Photobacterium* species); however, microbial taxonomic diversity was notably lower in the wild. Estimates of functional diversity suggested that this decline in taxonomic diversity had little impact on functional diversity in the midguts of farmed or wild salmon. However, predictive algorithms for microbiome function based on 16S data must be approached with caution, as microbes from nonmodel organisms are usually underrepresented in KEGG databases (28).

In conclusion, our study updates the "map" of microbial communities that colonize the different gut compartments of salmon. However, the predominance of neutral processes dominating the stepwise colonization of the salmon gut indicates a powerful role for the environment, not the host, in defining the microbial communities therein. Nonetheless, many of the most abundant gut OTUs were nonneutral in their colonization dynamics, suggesting that the host might be exerting a powerful influence over a small subset of important taxa. Between life cycle stages in wild salmon, more evidence of host filtering is apparent—declining alpha diversity with age and a relatively larger number of OTUs that do not fit a neutral model. One explanation for this could be due to wild fish having a more varied diet, as diet is well known to be a determining factor on the host microbiome (e.g., reference 37). We hope one role of this work will be to focus attention on the microbes that consistently do show signs of adaptation to the gut environment, the mycoplasmas, for example. Further work is required to understand what specific adaptive role such microbes may play in salmon host digestion and physiology as well as to illuminate how these organisms interact with their host.

MATERIALS AND METHODS

Sample collection in aquaculture setting. Farmed *Salmo salar* subadults (3 to 5 kg) were sampled from marine cages at an aquaculture farm site at Corran Ferry, near Fort William, Scotland, in autumn 2017. Samples of environmental microbes were collected concurrently by filtering 500 ml of sea cage water ($n = 14$) through a 0.22-μm nitrocellulose membrane filter (Millipore, USA) (e.g., reference 4). Samples of pellet feed ($n = 13$) were also collected and stored at −80°C until DNA extraction. Individual fish were dissected using aseptic technique, and samples of several gut compartments excised and flash frozen in liquid nitrogen as follows: stomach ($n = 42$), pyloric cecum ($n = 31$), bile fluid ($n = 23$), and "midgut" (approximately 20 cm from the vent; $n = 39$). Gut samples were taken via the excision of a short section of gut wall alongside gut contents to minimize potential sampling bias between adherent/planktonic microbes. A full representation of the sampling method is presented (Fig. 8).

Sample collection in wild setting. Wild *S. salar* specimens were collected from sites in Ireland, Canada, and west Greenland. Several life cycle stages were targeted in freshwater (Burrishoole and Erriff rivers, West Ireland [$n = 9$]; St. Jean and Trinite rivers, QC, Canada—parr [1+ age class representing 1 year after hatching; $n = 32$], smolt [$n = 12$], and returning adults [$n = 31$]) and marine settings (Sisimut, Manitsoq, Greenland, feeding subadults [$n = 9$]). Contents of mid and distal intestines were collected and flash frozen. Environmental microbes were sampled via the same microfiltration protocol as before at all freshwater sites. Details of sample collection from this wild *S. salar* cohort have been described previously (4). A full representation of the sampling method is presented (Fig. 8).

DNA extraction from gut contents and 16S rRNA gene amplification. DNA purification from all aquaculture samples, including both gut and environmental samples, was achieved using a QIAamp stool kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol (e.g., reference 38) with an additional ceramic bead-beating step (60 s) to break down the tissue samples. DNA extraction from wild samples was achieved using an MO BIO PowerSoil kit as described previously (39). As such, we limit direct alpha and beta diversity comparisons between farmed and wild fish. Amplification of the 16S V4 hypervariable region of the universal rRNA 16S gene (40) was achieved using redundant, tagged primers 519_f, 5′-CAGCMGCCGCGGTAA-3′, and 785_r, 5′-TACNVGGGTATCTAATCC-3′, at a final concentration of 1 pM of each primer. V4 was chosen in light of its widespread use to profile vertebrate-associated microbiota as well as its suitability for Illumina paired end sequence read lengths at the time of sequencing (40). Each primer was 5′ tagged with a common 22-bp tag for Illumina barcode attachment

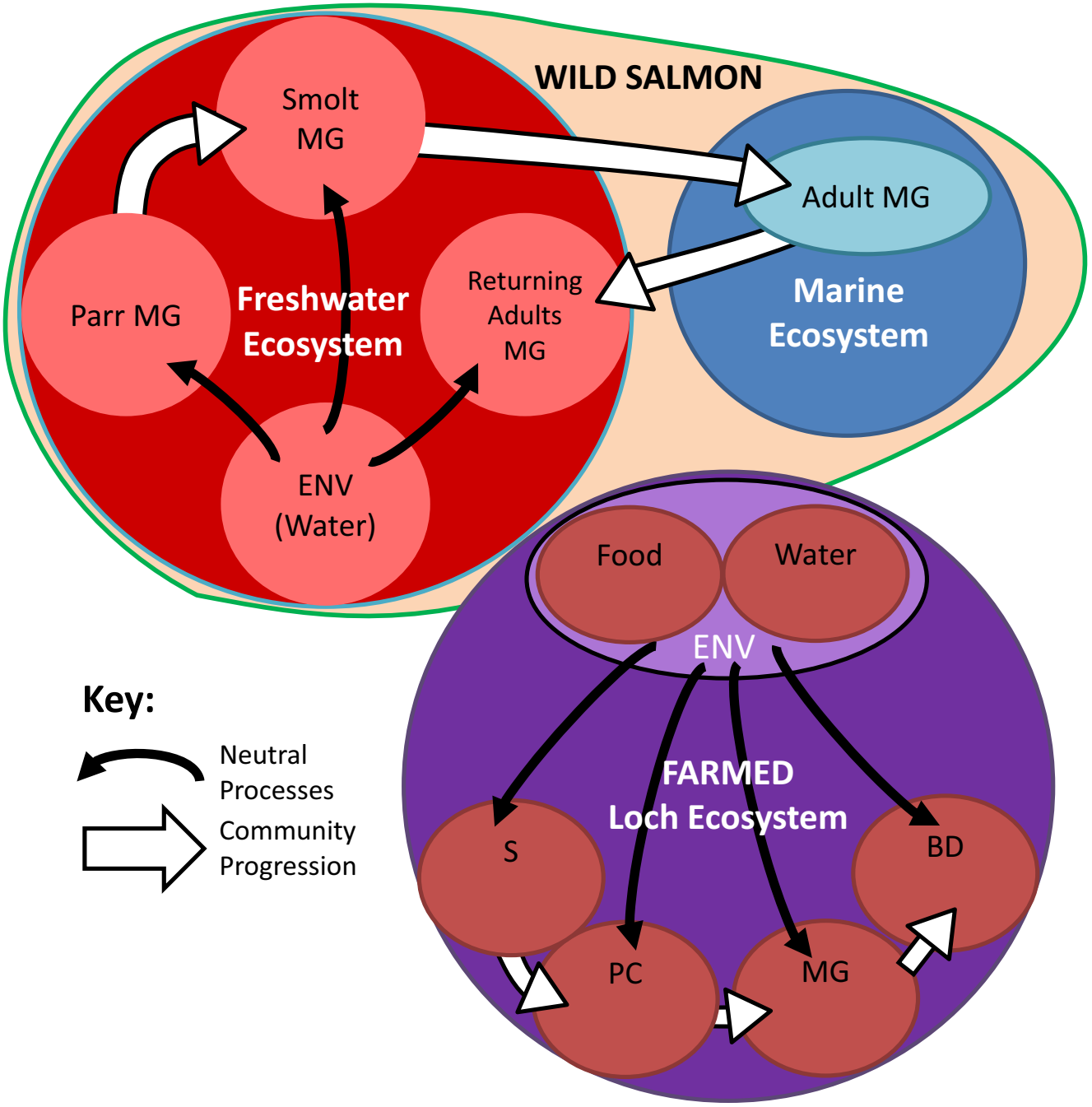


FIG 8 Representation of sampling methods within farmed and wild populations of Atlantic salmon (*Salmo salar*). The midgut (MG) of different life cycle stages of wild individuals was collected and analyzed for microbial diversity, abundance, and richness and compared to environmental water samples. Different life cycle stages included parr, smolt, marine-phase adult, and returning adult. In a farmed aquaculture system, samples were collected from different gut compartments of subadults, including stomach, pyloric cecum, midgut, and bile duct, and compared to environmental samples consisting of feed pellets and water.

(CS1-ACACTGACGACATGGTTCTACA; CS2-TACGGTAGCAGAGACTTGGTCT). Reaction conditions for the first round PCR were 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final elongation step of 72°C for 10 min. The second round PCR, which enabled the addition of the multiplex identifiers (barcodes), involved only six cycles and otherwise identical reaction conditions to the first. Frequent miss-priming was observed in primary PCRs, especially in samples including high volumes of salmon tissue, resulting in either a single ca. 200-bp amplicon or two amplicon sizes (one at 200 bp, a further at the expected ca. 300 bp). Poor amplification efficiency was a feature of all PCRs. Sequencing of the smaller amplicon and comparison with NCBI databases revealed high sequence similarity to the mitochondrially encoded *S. salar* 12S ribosomal gene (data not shown). Gel extraction

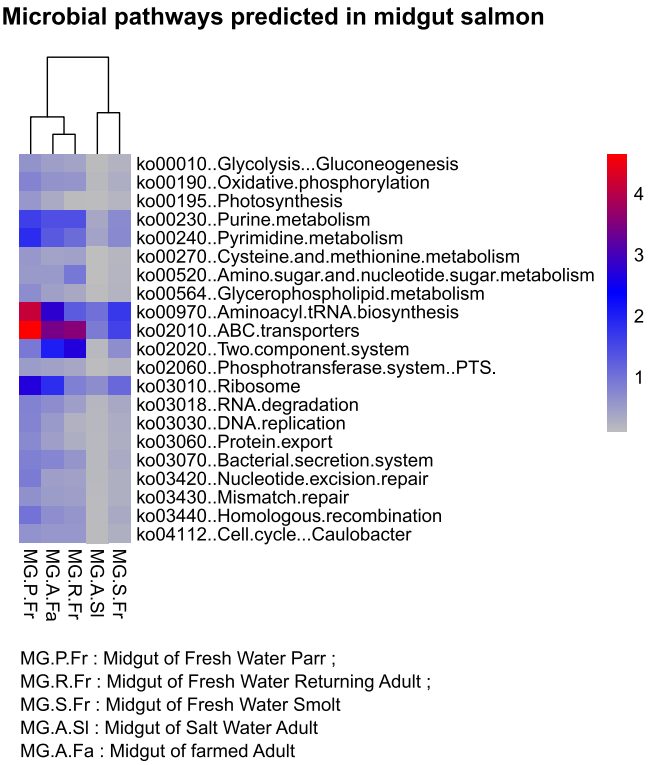


FIG 9 Heatmap showing the predicted pathways in the midgut of Atlantic salmon.

of 300-bp products was achieved using a PureLink gel extraction kit (Thermo) prior to a second round PCR (8 cycles) to incorporate Illumina barcodes for multiplex library preparation (see supplemental material for custom barcode sequences). The sequencing platform used was Illumina MiSeq with a read length of 300 bp.

Analysis of 16S rRNA gene amplicons. Sequence analysis was performed with our bioinformatic pipeline as described previously (4). Firstly, we used Sickle version 1.2 (41) to trim sequencing reads (>Q30 Phred quality score) and screen sequencing errors (>Q23) in forward reads (R1) of the 16S rRNA V4 hypervariable region. Due to poor read quality of R2, we discarded them from the analysis to avoid the significant loss of data size after R1 and R2 merging and synchronization. After read filtration processing, all samples that counted for lower than 8,000 reads were discarded from the analysis. Sample sizes are included above. Secondly, after screening for size (>100 bp) and homopolymer errors with mothur (42), the 12,759,456 filtered reads were clustered in operational taxonomic units (OTUs) using USEARCH version 9 at 97% identity. We used the algorithm UNOISE2 to filter out chimeric sequences produced during PCR amplification cycles. Subsequently, for the taxonomic assignment, the 7,109 clustered OTUs were annotated using the Silva database (version 123), and a tree of OTUs clusters was constructed using the algorithm SINTAX (43). The OTU table was converted to biological observation matrix (BIOM) format in order to predict the function categories and metabolic pathways using Tax4Fun software (28). Analysis of variance (ANOVA) and Wilcoxon tests were employed to compare functional categories.

Post-OTU statistical analysis. The alpha diversity distribution and differences within the microbiome of farmed and wild fish were plotted and analyzed for significance using the Rhea package (44). Briefly, the significance of alpha diversity indexes (richness and evenness) and beta diversity (phylogenetic distance) differences between groups were assessed using rank statistics tests (Kruskal-Wallis/Wilcoxon). The computed *P* values of pairwise comparisons in alpha and beta diversity were corrected for multiple testing using the Benjamini-Hochberg method (45). Beta diversity was measured using generalized UniFrac (46). Permutational multivariate analysis of variance (PERMANOVA) method (47) was applied on the GUniFrac distance matrices to determine the significant separation of experimental groups. Nonmetric multidimensional scaling (NMDS) was performed to visualize GUniFrac distances (46) in a reduced space of two dimensions (48). To detect significant differences in composition and abundance between groups, we used the nonparametric Kruskal-Wallis rank sum test (49) as the normality distribution of OTU data is rarely assumed.

Neutral and deterministic models of microbial community assembly. To investigate the role of neutral processes in microbiome assembly, we fitted the distribution of OTUs to a neutral model suggested by Sloan et al. (20), and recently implemented by others (e.g., reference 21), using nonlinear least-squares based on fitting beta distributions. The estimated migration rate (*m*) is the probability that a random loss (death or immigration) of an OTU in a local community is replaced by dispersal from the

metacommunity source. The comparisons of community assembly demographic and time fates between gut compartments and life cycle stages are highlighted (Fig. 8). In the gut compartment comparisons, the source communities were defined in a sequential fashion (water and feed as source for stomach, stomach as source for pyloric cecum, etc.) to assess the progression of microbes through the digestive tract. For life cycle comparisons among wild fish, source communities were defined either as the water sample or as the preceding life cycle stage. Predicted versus observed OTU frequencies from the neutral model were compared to highlight the percentage of OTUs that fit the model with a confidence interval of 95%. The goodness of fit to the neutral model was assessed using R^2 as the coefficient of determination. We also complemented Sloan's model by a second measure adapted from Stegen et al. (29) using the Picante package (50) to explore patterns of phylogenetic (net relatedness index [NRI]) and taxonomic (nearest taxonomic index [NTI]) relatedness within sample groups. These indices measure the extent of the overdispersion and underdispersion of relatedness at different timescales (NRI distant, NRI recent)—with an expectation that communities whose membership is primarily the result of neutral processes should approximate zero. Based on the broad assumption that taxonomically and/or phylogenetically similar groups might share a similar niche, underdispersion indicates habitat filtering and overdispersion intraspecific competition (29).

Tax4Fun (28) was used to predict the functional content of microbial communities based on 16S rRNA data sets (all prokaryotic KEGG organisms are available in Tax4Fun for Silva version 123 and KEGG database release 64.0). In Tax4Fun, the MoP-Pro approach (51) was employed to provide precomputed 274 KEGG pathway reference profiles. The ultrafast protein classification (UProC) tool (52) generated the metabolic profiles after normalizing the data for 16S rRNA gene copy numbers. The inferred nature of these functional predictions is highlighted in Fig. 9.

Data availability. All sequence data were deposited into the NCBI database under accession number PRJNA594310.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.3 MB.

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A.B., R.K., L.M., and J.K. collected the samples, performed experiments, and collected data. B.C. and U.Z.I. performed the data analysis. B.C., U.Z.I., C.H., and M.S.L. interpreted the data. C.H. wrote the original draft of the manuscript. W.T.S., P.M., and M.S.L. conceived and designed the study. All authors contributed to revisions of the manuscript.

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Appendix 5: Genome erosion and evidence for an intracellular niche – exploring the biology of mycoplasmas in Atlantic salmon

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Genome erosion and evidence for an intracellular niche – exploring the biology of mycoplasmas in Atlantic salmon

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A B S T R A C T

Mycoplasmas are the smallest autonomously self-replicating life form on the planet. Members of this bacterial genus are known to parasitise a wide array of metazoans including vertebrates. Whilst much research has been significant targeted at parasitic mammalian mycoplasmas, very little is known about their role in other vertebrates. In the current study, we aim to explore the biology of mycoplasmas in Atlantic Salmon, a species of major significance for aquaculture, including cellular niche, genome size structure and gene content. Using fluorescent *in-situ* hybridisation (FISH), mycoplasmas were targeted in epithelial tissues across the digestive tract (stomach, pyloric caecum and midgut) from different development stages (eggs, parr, subadult) of farmed Atlantic salmon (*Salmo salar*), and we present evidence for an intracellular niche for some of the microbes visualised. *Via* shotgun metagenomic sequencing, a nearly complete, albeit small, genome (~0.57 MB) as assembled from a farmed Atlantic salmon subadult. Phylogenetic analysis of the recovered genome revealed taxonomic proximity to other salmon derived mycoplasmas, as well as to the human pathogen *Mycoplasma penetrans* (~1.36 Mb). We annotated coding sequences and identified riboflavin pathway encoding genes and sugar transporters, the former potentially consistent with micronutrient provisioning in salmonid development. Our study provides insights into mucosal adherence, the cellular niche and gene catalog of *Mycoplasma* in the gut ecosystem of the Atlantic salmon, suggesting a high dependency of this minimalist bacterium on its host. Further study is required to explore and functional role of Mycoplasma in the nutrition and development of its salmonid host.

1. Introduction

Mycoplasmas are a diverse group of bacteria known to parasitise a wide array of metazoans, plants, invertebrates and vertebrates, including fisheries (Razin, 1992). *Mycoplasma* had been isolated from multiple fish species, (Carp: *Cyprinus carpio;* Tench *Tinca tinca*; Trout: *Salmo trutta*; Eel: *Anguilla anguilla;* Sheat fish: *Silurus glanis*; Mosaic threadfin: *Trichogaster leeri*; cichlid:*Tropheus sp.*; Plaice: *Pleuronectes platessa*; Salmon: *Salmo salar*; goldfish: *Carassius aurarus*; Brook lamprey: *Lampetra planeri*) by the early 1980s (Kirchhoff and Rosengarten, 1984). More recently, several studies have identified *Mycoplasma* from marine teleosts using culture-free approaches. Mudsucker (*Gillichthys mirabilis*) and pinfish (*Lagodon rhomboids),* for example, have been identified as having gut microbiomes rich in *Mycoplasma* (Egerton et al., 2018). However, salmonids in particular are frequently reported to be colonised by *Mycoplasma* (Holben et al., 2002; Llewellyn et al., 2016). This is especially true in Atlantic salmon (*Salmo salar*), both in wild and in farmed settings (Holben et al., 2002; Zarkasi et al., 2016). In some cases, *Mycoplasma* phylotypes can comprise >70% of the total microbial reads recovered from salmon intestines (Heys et al., 2020; Llewellyn et al., 2016). The distribution and biological role of *Mycoplasma* in the intestines of salmonids are far from clear and require further exploration. Nonetheless, demographic modelling of microbial communities suggests colonisation of salmonid guts by these microorganisms as *non-neutral*, *i.e.* the rate at which these bacteria colonise the gut indicates a significant degree of specific adaptation to the host environment (Cheaib et al., 2020; Heys et al., 2020). Interestingly, Mycoplasma sp. are also prevalent in different extraintestinal organs (gills, liver, spleen, kidney, reproductive organs, serous membrane from the peritoneum and the swim bladder) of different fish species (EI-Jakee Ei-Jakee, 2020; Sellyei et al., 2021). Even after 40 years form their discovery, the role of Mycoplasma sp. in fisheries and aquaculture production is not clear. In particular, questions around Mycoplasma pathogenicity remains unresolved.

Mycoplasmas, as well as related taxa included in the class Mollicutes (*Spiroplasmas*, *Ureaplasma* and *Acholeplasmas*), are recognized as the

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smallest and simplest free-living and self-replicating forms of life (Bové, 1993; Trachtenberg, 2005). Mycoplasmas lack a peptidoglycan cell wall and are bounded by a simple cell membrane (Miyata and Ogaki, 2006). In addition to being physically small, mycoplasmas have the smallest genomes of any free-living organism (Razin et al., 1998). *Mycoplasma genitalium*, in particular, has a genome size of 580 kilobases comprising of only 482 protein-coding genes(Citti and Blanchard, 2013), whilst *Mycoplasma mycoides*, typically has 473 protein-coding genes, of which 149 still have no known function (Citti and Blanchard, 2013).

The simplicity of mycoplasmas, their small genome sizes, as well as their close association with metazoan hosts has led them to be considered as a target species to explore theories around genome erosion or reductive evolution (Fadiel et al., 2007; Rocha and Blanchard, 2002). Dependence on host organisms can theoretically lead to mutual interdependence of metabolic processes. This results in its relaxed selection among the pool of bacterial genomes, with the main process being the accumulation of loss-of-function mutations in coding genes, and the eventual loss of genetic material from the bacterial genome (Boscaro et al., 2017). Genetic drift can also play a significant role as host-associated microbes have relatively fewer opportunities to exchange genetic material with the wider microbial community (Moran, 1996). Isolation from microbial congeners and host dependence may be further enhanced in mycoplasmas that exploit an intracellular niche, which several species have been shown to do within the literature (Razin et al., 1998; Yavlovich et al., 2004). Mycoplasmas, likely owing to their dependence on their hosts, have fastidious requirements for *in vitro* culture. Culture-free approaches for microbial identification, especially, with the advent of DNA sequencing approaches, have markedly increased in recent years to identify new *Mycoplasma* strains (Aceves et al., 2018; Costello et al., 2013; Martin et al., 2013).

In the current study, we aimed to explore the characteristics of *Mycoplasma* in salmonids, including a potentially intracellular niche, taxonomic affiliations, genome structure and gene content. We also examined genetic features and metabolic functions that reveal the role of reductive evolution in shaping its genome. Finally, we discuss what role *Mycoplasma* may have in impacting host fitness, especially in the context of aquaculture, by reviewing genomic features it possesses that are consistent with parasitic or symbiotic lifestyles.

2. Materials and methods

2.1. Sample collection

Farmed *Atlantic salmon* (*Salmo salar*) subadults (individuals from 3 to 5 kg) fed on a commercial diet were sampled from 3 marine cages at an aquaculture facility at Corran Ferry, near Fort William, Scotland, in Autumn 2017 in collaborations with MOWI Ltd. *Salmo salar* freshwater parr (30-50 g) and ova were sampled at the Institute of Biodiversity, Animal Health and Comparative Medicine aquarium facility, University of Glasgow. The fishes were euthanized by blunt cranial trauma under a Schedule 1 procedure and gut compartments (stomach, pyloric caecum, and midgut) samples were dissected under aseptic conditions before being fixed in formalin for subsequent microscopy, or flash frozen in liquid nitrogen prior to DNA analysis.

2.2. Fluorescence in-situ hybridisation (FISH)

Previous work has established the dominance of *Mycoplasma* in marine *Salmo salar* GI (gastrointestinal tract) (Heys et al., 2020). To explore their physical distribution in different gut compartments and life cycle stages, FISH was undertaken on stomach, pyloric caecum and hind gut from marine farmed adults (three individuals, MOWI, Scotland) and juvenile parr reared in aquaria at the University of Glasgow (three individuals). Samples were fixed in a freshly made sterile-filtered solution of 4% paraformaldehyde in PBS (pH 7.4) for 16–24 h and maintained at room temperature for 16–48 h. Fixed samples were then washed with sterile-filtered PBS (pH 7.4) three times before being stored in 70% ethanol. Samples were then gradually dehydrated in a series of ethanol-xylene-paraffin treatment steps (Copper et al., 2018). Before sectioning, samples were embedded in paraffin and stored at 4 °C. At least four 3–4 μm sections were taken from each embedded tissue block, rehydrated in sterile ddH_20, and mounted on slides for pepsin treatment and straining. Pepsin treatment was undertaken in a 0.05% pepsin solution and 0.01 M HCL. Samples were DAPI stained to target cell nuclei of host cells, and FISH probes were hybridized at 55 °C to the 16S rDNA small subunit of bacterial cells. Multiple FISH probes labelled with Cy3 and Cy5 dyes were deployed to distinguish *Mycoplasma* strains from other microbes present in samples (Table 1). To improve the visualization of non-mycoplasma bacteria, multiple probes were deployed using the same dye. A *Mycoplasma* probe (Myc1–1) (Table 1) probe was designed based on Illumina amplicon sequences based upon the most abundant operational taxonomic (OTU) sequence identified in Adult Salmon that we identified in previous work (Heys et al., 2020). To establish respective specificity of probes, positive controls of for both universal probes (*E. coli*) and *Mycoplasma* probes (*Mycoplasma muris)* were used. Attempts culture Mycoplasma from salmon were not successful. All samples were visualised at 20-30× magnification on a DeltaVision-Core microscope (Applied Precision, GE), equipped with a CoolSNAP HQ camera (Photometrics) and operated with SoftWoRx software (Applied Precision, GE).

2.3. DNA extraction, library annotation and sequencing

DNA was extracted from a section of pyloric caecum derived from a single individual on which FISH analyses had identified the presence of *Mycoplasma* organisms, based on their labelling with a targeted 16S probe. The sample homogenised *via* bead beating and DNA extracted using a Qiagen DNAeasy Stool Kit. A sequencing library for Illumina Next-Seq WGS (whole genome shotgun) was prepared using a sonication protocol and a TruSeq library protocol and adaptors. Sequencing was undertaken at the University of Glasgow Polyomics facility.

2.4. Data preprocessing, assembly, binning and annotations

The short paired-end NextSeq Illumina reads (2 × 63 million reads) were preprocessed for quality filtering using sickle V1.2 (https://github.com/najoshi/sickle). Decontamination of good quality reads was performed by mapping reads against the *Salmo salar* genome (available at NCBI sequence archive with the accession number GCF_000233375.1) using Deconseq V 0.4.3 (Schmieder and Edwards, 2011) based on BWA mapper V 0.5.9 (Li, 2013). The decontaminated paired-end reads (~18 millions of bacterial reads) were assembled using the Megahit V1.1 software (Li et al., 2015). The assembled contigs (~93,400) were processed for binning using MetaBAT V2.12.1 (Kang et al., 2015). Quality assessment for completeness and contamination of sequence was performed using CheckM V1.0.18 software (Parks et al., 2015). Annotation of gene content was performed using the pipeline ATLAS-metagenome (Kieser, 2019), which involves the prediction of open reading frames (ORFs) using Prodigal (Hyatt et al., 2010). Translated gene products were clustered using LinClust (Steinegger and Söding, 2018) to generate non-redundant gene and protein catalogues, which were mapped to the eggNOG catalog (Huerta-Cepas et al., 2019) using DIAMOND (Buchfink et al., 2015).

2.5. Phylogenetic analyses

Two approaches were undertaken to construct phylogenetic trees: a) MLST-based (Multi Locus Sequence Typing); and b) 16S gene markers (recovered from the genome) of the *mycoplasma* MAG (metagenome-assembled genome) from this study as well as what is previously available in the literature. Using CheckM software, the MLST-based strategy focused on a concatenation of 21 conserved housekeeping genes

Table 1
Fish probes and sequences deployed in this study.

Probes	Target group	Sequence (5′-3′)	Reference
Myc1–1	*Mycoplasma*	GCGGTAATACATAGGTYGCAAGCG	This study
Gam-1	Gammaproteobacteria	GCCTTCCCACATCGTTT	Manz *et al.*, 1992
FIR-1	Firmicutes	GGAAGATTCCCTACTGCTG	Hallberg *et al.*, 2006
EUB338	All bacteria	GCTGCCTCCCGTAGGAGT	Amann *et al.*, 1990
EUB338 II	*Planctomycetes*	GCAGCCACCCGTAGGTGT	Daims *et al.*, 1999
EUB338 III	*Verrucomicrobia*	GCTGCCACCCGTAGGTGT	
Non EUB338	None	CGACGGAGGGCATCCTCA	Wallner *et al.*, 1993

This table resumes the probes used for targeting general and specific bacterial groups including the Mycoplasmas and control negative.

annotated in the mycoplasma MAG supplemented with the orthologues available for all the *Mycoplasma* genera, to date. The MLST-based dataset included 55 orthologues of protein sequences of concatenated 21 conserved markers. The 16S rDNA sequence dataset included: one sequence of 16S rDNA gene annotated from the mycoplasma MAG; five Operational Taxonomic Units (OTUs) sequences of mycoplasmas characterised form the same farmed salmon system (Heys et al., 2020); 101 and 17 sequences of 16S rDNA from the *Mycoplasma* sp. and *Sprioplasma* sp. genomes respectively from IMG database; and 11 sequences, from environmental studies, detected in marine species including shrimp, fish and isopods. DNA and protein sequences were aligned using MAFFT version 6.24 (Katoh and Standley, 2013). Phylogenetic inference was performed using PhyML version 3.0 (Guindon and Gascuel, 2003) and MrBayes V.3.2.6 (Huelsenbeck and Ronquist, 2001). The evolutionary model was chosen using MODELTEST(Posada and Crandall, 1998), and parameters were iteratively estimated in PhyML using the GTR + I + G model for the nucleotide sequence of 16 s trees and the LG + I + G model for amino-acid sequences of concatenated markers trees (Le and Gascuel, 2008). Bootstrap values were calculated using 100 replicates (Felsenstein, 1985). With MrBayes, posterior probability values were calculated using an average standard deviation of partition frequencies <0.01 as a convergence diagnostic (Ronquist et al., 2012). MrBayes runs consisted of eight simultaneous Markov chains, each with 1,000,000 generations, a subsampling frequency of 1000, and a burn-in fraction of 0.15. Trees were then visualised and adapted for presentation in FigTree version1.4.3 as a graphical viewer of phylogenetic trees (http://tree.bio.ed.ac.uk).

2.6. Metabolic pathways comparison and genome reduction analysis

All Pfam V.32 (comprehensive and accurate collection of protein domains and families) annotations were predicted with Prodigal and analysed in terms of function categories and metabolic content (focusing on Enzyme EC numbers). The 570 genes identified were associated with 746 Pfam functions. The Pfam functions led to the recovery of Gene Ontology (GO) terms and were then mapped to the KEGG database. Simultaneously, the alternate approach involving the MetaCyc database was employed to elucidate metabolic pathways from all domains of life (Caspi et al., 2018). The EC numbers of the coding sequence regions in *Mycoplasma penetrans* were extracted from the KEGG database and were then compared with those annotated within the mycoplasma MAG from *Salmo salar* in this study. The mapping of metabolic pathways from both genomes was visualised using the iPath (Yamada et al., 2011). From the IMG genomic database, all available metadata on sequenced mycoplasma strains were then collected and compared to the mycoplasma MAG for the genome size, GC content, gene content and their preference (*e.g.* intracellular, free-living etc). Annotations for the mycoplasma MAG were submitted to CG view (Grant and Stothard, 2008) for radial visualization of its genomic. Using a core gene approach, the 570 predicted genes were compared at the DNA and protein sequence levels against all the available genes of *Mycoplasma penetrans* using BLAST+ V 2.8.1 (Altschul et al., 1990). The best hits for each query were represented in a radial plot using Circoletto software version V.069–9 (Darzentas, 2010). Complimentary annotations were performed using RAST software which, consisted of subsystem classification of microbial functions available in the curated database, *i.e.* SEED subsystems (Overbeek et al., 2014).

3. Results

3.1. Fluorescence in situ hybridization (FISH) of mycoplasmas in the farmed salmon

The set of probes used in FISH for the identification of bacterial populations is summarized in Table 1. The *Mycoplasma* probe Myc1–1 showed specific hybridization, and its specificity was evaluated against in pure culture *Escherichia coli* and *Mycoplasma muris* (Supplementary Fig. S1). The probe gives a positive signal solely with cultured *Mycoplasma muris*. We made multiple attempts in both solid and liquid culture mycoplasmas from the salmon intestines, but without success. FISH visualization in salmon ova demonstrated a low abundance of bacteria and no signal of *Mycoplasma* sp. (Fig. 1A; Supplementary Fig. S2.1). In *Salmo salar* freshwater parr, *Mycoplasma* sp. aggregates were observed on the stomach lining of all samples (Fig. 1B; Supplementary Fig. S2.2), as well as on the muscular mucosae, and epithelium of the pyloric caecum (Fig. 1C; Supplementary Fig. S2.3). In the midgut of salmon parr (distal to the pyloric caecum) the aggregation of *Mycoplasma* sp. in the epithelium cells was also identified (Supplementary Fig. S2.4). In the stomach (Fig. 1D; Supplementary Fig. S2.5) and pyloric caecum (Fig. 1E–F; Supplementary Fig. S2.6) of adult salmon, *Mycoplasma* sp. signals were clustered in small aggregates in the lumen around the nuclei of epithelial cells. Fig. 1 indicates this intracellular clustering most clearly. In the midgut of adult salmon, *Mycoplasma* sp. showed lower abundance and the signals of *Mycoplasma* sp. showed aggregations near epithelium cell nuclei (Supplementary Fig. S2.7). More comprehensive, 16S -amplicon-seq based surveys of Mycoplsama abundances in farmed sub adults from the same site can be found in Heys et al., 2020.

3.2. Mycoplasma MAG (metagenome-assembled genome) features and orthologs

Using a total of 63,180,207 reads, and after decontamination, 93,397 contigs were assembled using megahit software (see Materials and methods). The assembled contigs were binned, annotated, and assessed for completeness (see materials and methods). The best quality assembled bins corresponded to a nearly complete genome assigned to a *Mycoplasma sp.* (see the genome sequence in Supplementary File 2). The completeness of this metagenome-assembled genome (MAG) was estimated at 92.18% with 0.38% of contamination (Table 2). The metagenome and MAG were deposited into the NCBI database under the Bio project accession number PRJNA714611.

The average size of the assembled genome was estimated to be 0.57 Mb and comprised a set of 570 predicted genes accounting for a total of 694 CDS regions found on the 5′3’ and 3′5’ ORFs. The GC percentage was estimated to be 24.98% (Table 2). Circular representation of the genomic structure of the mycoplasma MAG highlights CDS annotations on the negative (Fig. S3-a) and positive (Fig. S3-b) strands, respectively. To further resolve CDS annotations, a supplementary annotation

framework was applied using the curated SEED database and the RAST server (Glass et al., 2010). The number of curated annotations was reduced to 600 CDS across the negative (275 CDS) and positive strands (325 CDS). Among these CDS regions, 390 had functional annotations, and within these, three annotated CDS regions (> 85% of similarity threshold against SEED) were identified as riboflavin kinase (EC 2.7.1.26/EC 2.7.1.26;1278 bp) along with two Riboflavin/purine transporters of length 1383 bp and 1608 bp, respectively. Other functions required for host-microbiota symbioses, such as ribonucleotide reductase, were annotated with SEED and are reported (Supplementary Table 1).

3.3. Phylogenetic proximity to Mycoplasma penetrans

Robust phylogenetic trees were recovered based on 16S rDNA and MLST data. The 16S rDNA tree includes four OTUs of *Mycoplasma* detected in the digestive tract of farmed salmon previously (Jin et al., 2019), as well as all OTUs recovered from our recent work on the same farm system (Heys et al., 2020). All *Mycoplasma* OTUs, including the MAG we sequenced lie in a clade alongside *M. muris* and *M. penetrans*. (Fig. 2). No evidence of an ecological association between mycoplasmas from similar ecotopes (*e.g.* marine, freshwater, terrestrial) was noted.

To further ascertain the above clustering of 16S rDNA sequences of *Mycoplasma*, and the phylogenetic relatedness, a second analysis based on MLST approach using 21 concatenated housekeeping genes (see PFAM IDs of markers and their functions in Supplementary File 1) increased our confidence in *M. penetrans* being close to the recovered mycoplasma MAG (Fig. 3). These 21 markers are detected in single copies and are conserved in the bacteria and the mycoplasmas lineage. The MLST tree shows high posterior probabilities in support of this topology (post prob. >0.9). Tip labels of the selected *Mycoplasma* sp. were

Table 2
Summary of *Mycoplasma* metagenome assembled genome (MAG).

Genome	*Mycoplasma* assembled genome
Completeness	92.18
Contamination	0.38
Unique markers (of 43)	39
Multi-copy	0
Taxonomy (contained)	Genus: *Mycoplasma*
Taxonomy of sister lineage	*Mycoplasma penetrans*
GC content	24.98
Genome size (mbp)	0.57
Gene count	570
Coding density	0.93
Length	577,903
N50	14,796
Genome completeness	92.18%

The shotgun metagenomics data (63 million reads) were trimmed after a quality control assessment, then assembled using Megahit, binned using Metabat and checked for binning quality using CheckM software. The metagenome was sequenced from the pyloric caecum of one individual subadult farmed Atlantic salmon (*Salmo salar*).

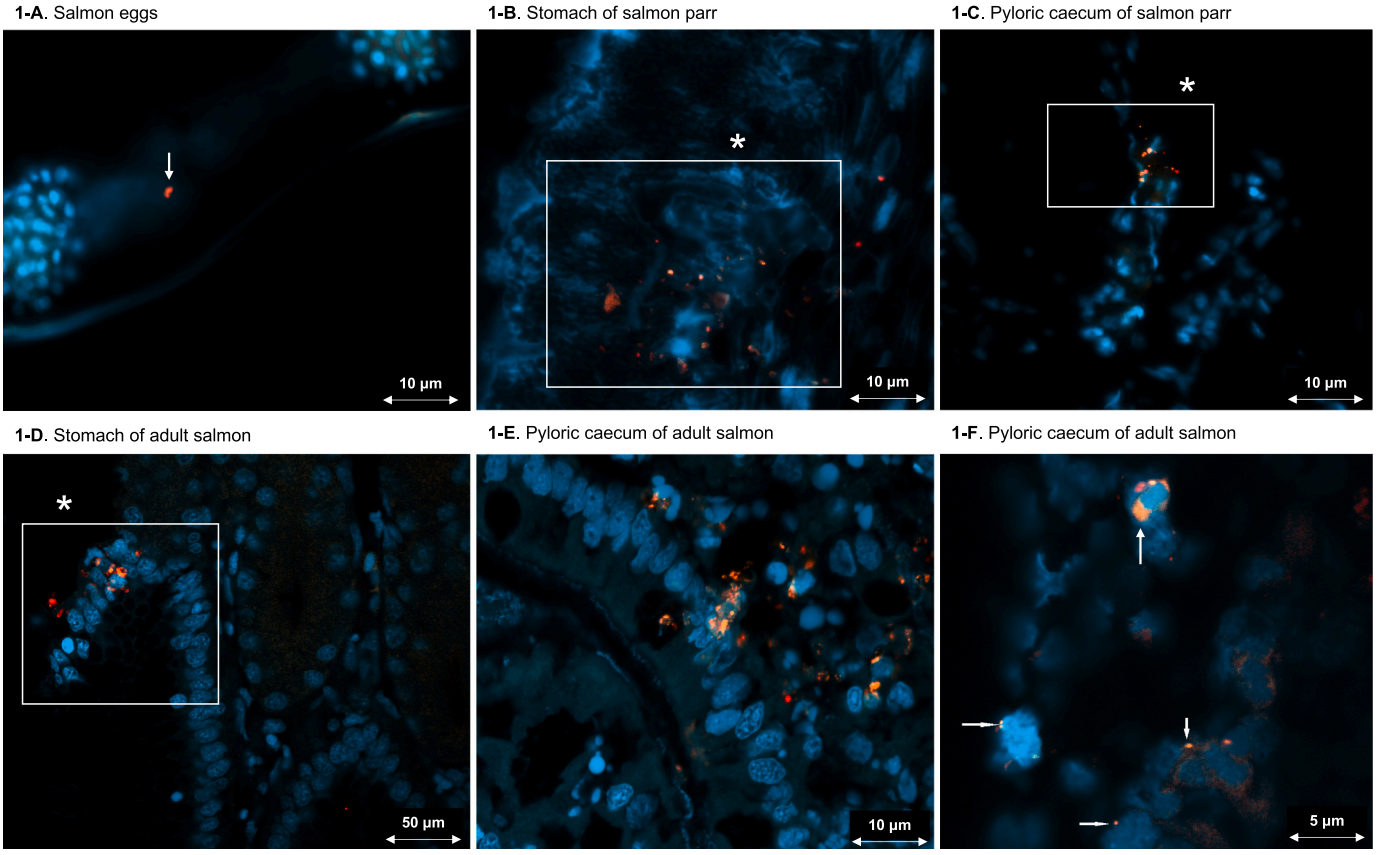

Fig. 1. FISH visualization of Mycoplasma in salmon parr and adults. The images were an overlay of DAPI signals (blue), hybridization signals of Gam-1, FIR-1, EUB338, EUB338 II, EUB338 III probes (Cy5, red) and *Mycoplasma* sp. Specific Myc1–1 probe (Cy3, orange). Scale bars are shown in the bottom left corner of each image (A) *Mycoplasma* sp. are absent from salmon ova, and bacteria scarce. (Scale 10um). (B) Distribution of *Mycoplasma* sp. in the stomach of salmon parr, scaled at 10 µm. Orange signals indicate mycoplasmas were clustered in small groups. (C) Distribution of *Mycoplasma sp.* in the epithelium of pyloric caecum of salmon parr (Scale 10um). (D) Distribution of *Mycoplasma sp.* in the stomach of adult salmon (Scaled 50 µm). (E, F) Distribution of *Mycoplasma sp.* in the pyloric caecum of adult salmon scaled at 10 µm (E) and 5 µm (F) respectively. Mycoplasmas signals were aggregated on the muscularis mucosae, lamina propria (E) and clustered in high abundance around epithelial cell nuclei (white arrows, F). These experiments were performed using at least three technical replicates of each life stage and digestive tract compartment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

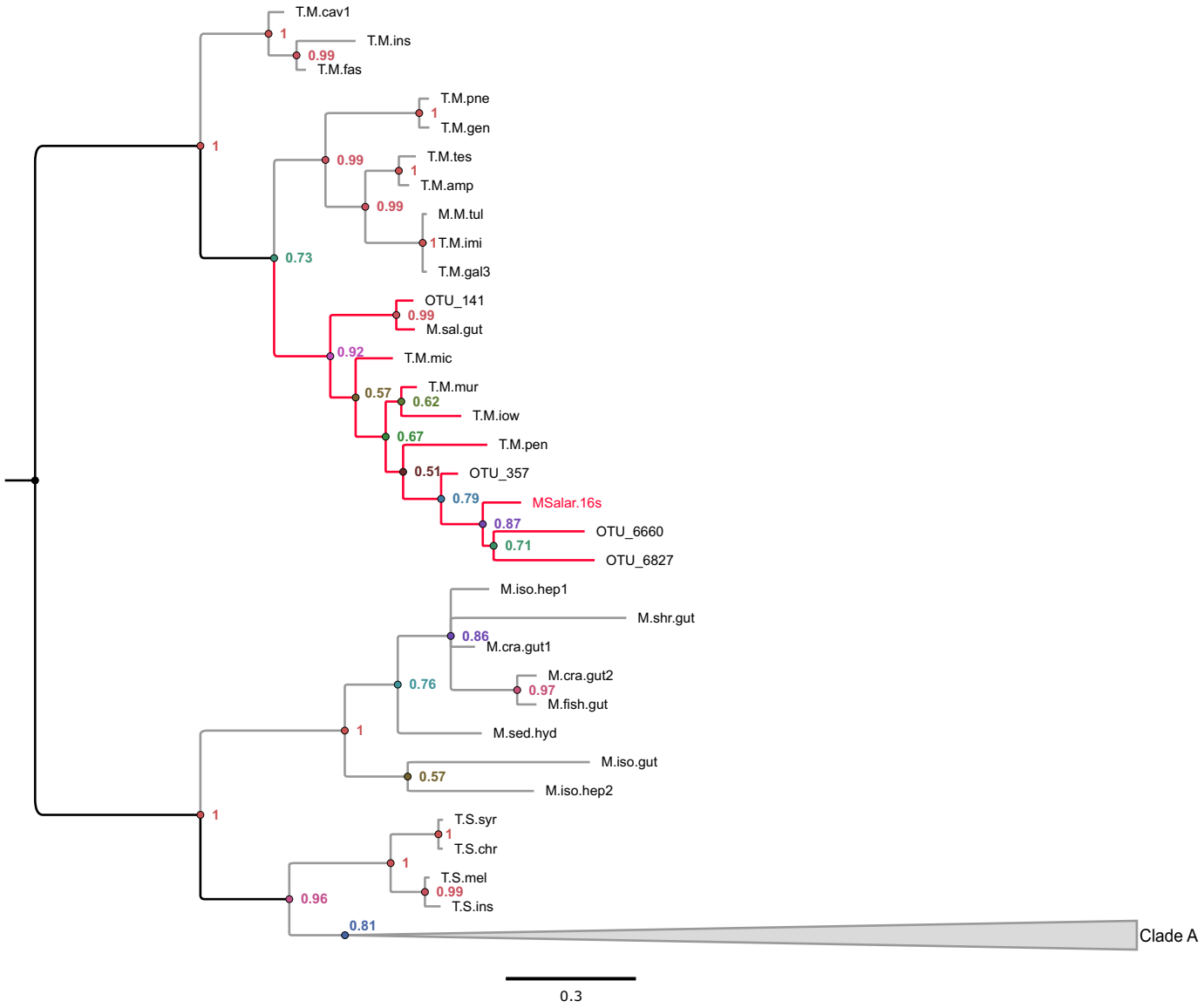

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences of mycoplasmas. Sequence name abbreviation of tree tips labels and clade A (including Spiroplasmas) are reported in Supp. File (sheet 2). The tree as consrtucted using MrBayes software , the nodes labels represent the calculated posterior probability values (see Materials and methods).

further annotated with the information of genome size in Mbp. It should be noted that the genome sizes did not appear to correlate with the phylogenetic distribution of *Mycoplasma* sp. in the tree. The genome size of *M. penetrans* (1.36 Mb) is approximately double to that of the mycoplasma MAG and, is the highest among the *Mycoplasma* sp. genomes.

3.4. Orthology, metabolic pathways and genome reduction analyses

A core genome analysis including the amino acid sequences of predicted CDS and all the available CDS from the closely related *M. penetrans*, available on NCBI repository, were blasted against the COG database. Circular track of the genome including the orthology clearly showed the difference in genome size between the mycoplasma MAG and *M. penetrans*. We observed heterogeneity across in terms of GC content and GC skew (Fig. 4). An orthology analysis based on SEED annotations indicated 14 functions (oxidative stress, periplasmic stress, protein biosynthesis, detoxification, ribonuclease H, cation transporters, ABC transporters) specific to the mycoplasma MAG, 144 functions specific to *M. penetrans*, and 156 functions that are common to mycoplasma MAG and *M. penetrans*. The shared functions between these two genomes belong to nine different general subsystems including those related to commensalism such as riboflavin metabolism; intracellular resistance; and resistance to antibiotics and toxic compounds (RATC) (Supplementary Table 2). We only found two similarity hits associated with RATC. Complimentary analysis pointed out a bifunctional riboflavin kinase/FMN FMN adenylyltransferase among the best reciprocal similarity's hits between the mycoplasma MAG in this study and *Mycoplasma penetrans* (Fig. S5; Supplementary Table 3).

To understand genome reduction in the mycoplasmas lineage, the genome size and genes count were compared across 247 strains (Fig. 5A; Supplementary Table 4) of the mycoplasmas available in the integrated microbial database (IMG). These strains include a wide variety of human and animal sources and comprising both parasitic and commensal. Given the collected data (Supplementary Table 4), also, this study mycoplasma MAG, gene content and genome size are strongly correlated. The average genome size of the 247 available mycoplasmas was 0.87 Mbp ± 0.15, and the average genes count was 790 ± 157 genes; however, this was not the case with all considered genomes. For instance, 8

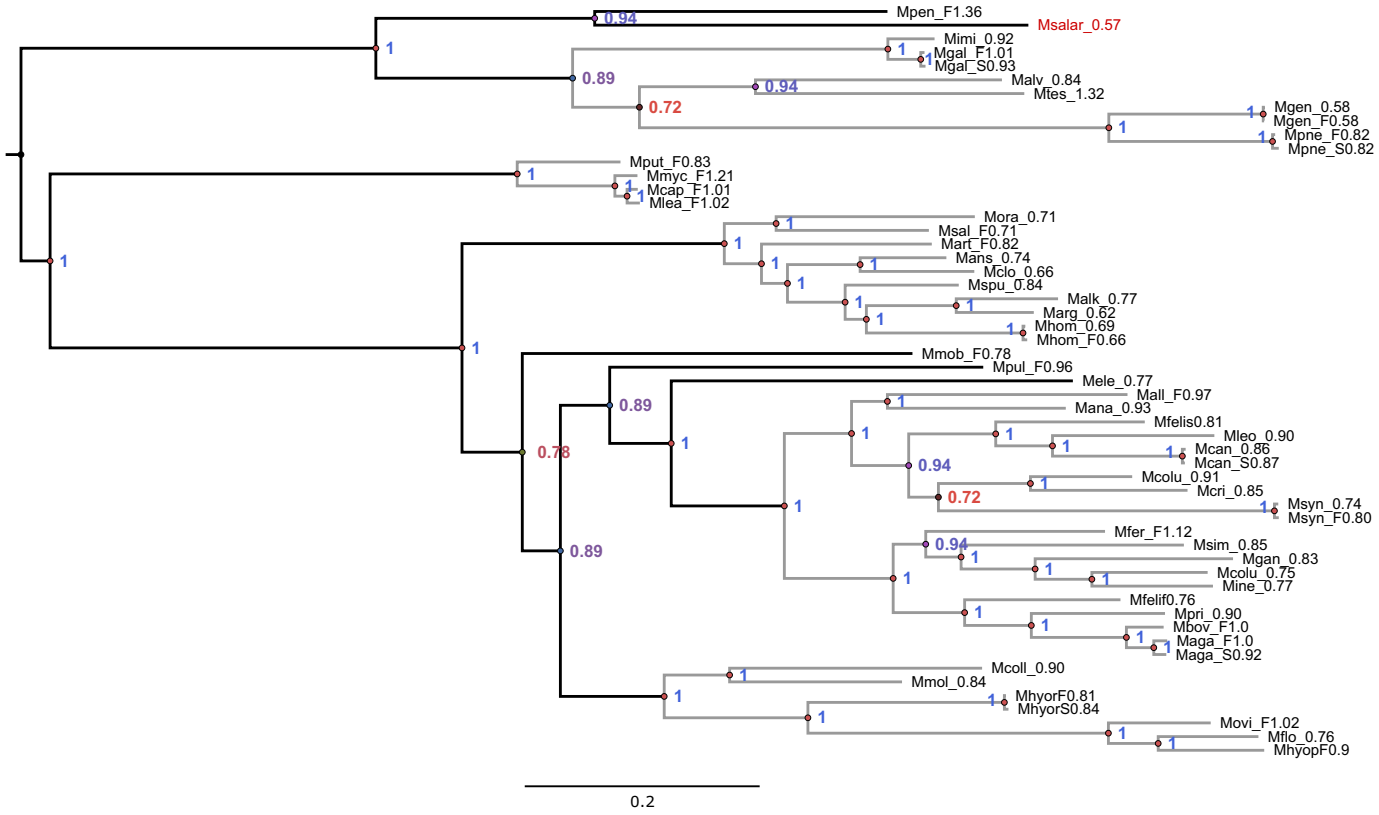

Fig. 3. Phylogenetic tree of mycoplasmas based on 21 MLST markers with the details given in the supplementary data. Sequence name abbreviation of tree tips labels is explained in Supp. File 1 (sheet 3). The tree as consrtucted using MrBayes software , the nodes labels represent the calculated posterior probability values (see Materials and methods).

genomes are lower than 0.8 Mb, accumulating somewhere between 829 and 1036 genes. Further analysis revealed that pseudogenes count had no relationship with genome size whilst both the transmembrane proteins and GC content were correlated with *Mycoplasma* sp. genome sizes (Fig. 5B). Furthermore, the average count of pseudogenes was significantly higher in free-living than within intracellular mycoplasmas (Supplementary Fig. S4), although available databases contain incomplete information with regards to mycoplasmas lifestyles. Finally, enzyme content was analysed in terms of metabolic pathways by comparing the annotated EC numbers of the mycoplasma MAG and *M. penetrans*. Common pathways of both genomes are highlighted in red lines (Fig. 6) including the riboflavin biosynthesis pathway.

4. Discussion

Mycoplasmas are hyper-abundant commensals of salmonid guts. Our study suggests, based on FISH data, that in *Salmo salar*, these organisms grow intracellularly in the epithelial and possibly muscular lining of the fish's GI tract, both in freshwater and during marine lifecycle stages. Mycoplasmas were not visualised on salmon ova, but we cannot rule out vertical transmission between individuals. The *Mycoplasma* sp. sequences recovered from *Salmo salar*, including the mycoplasma MAG reported here, had a strong phylogenetic similarity to *M. penetrans*. Comparative analysis of genome size and content across *Mycoplasma* sp. strains suggest that the genome we recovered in this study is, to the best of our knowledge, among the smallest ever observed. Comparative genomics analyses between the mycoplasma MAG and *M. penetrans* were undertaken and provide insight into the potential host-microbe interaction. Several features of the *Mycoplasma*'s genome organisation and content suggest a strong level of dependence on the salmon host, as well as a potential role for nutrient provisioning relevant to aquaculture.

Mycoplasmas have been widely reported within *Salmo salar* (Holben et al., 2002), and other teleosts (Cheaib et al., 2021; Ei-Jakee, 2020; Sellyei et al., 2021). It is not uncommon to find that communities of gut microorganisms are dominated by mycoplasmas (Dehler et al., 2017). The modelling approaches comparing environmental and intestinal frequency distributions of these organisms undertaken in this study have previously suggested that salmon mycoplasmas are well adapted to the colonisation of their hosts (Heys et al., 2020). Culture-based approaches have had been less successful in isolating these organisms (Llewellyn et al., 2014) and despite numerous attempts, we failed to obtain pure cultures of *Mycoplasma* sp. from the adult salmon used in this study (data not shown). This may be attributed to a potential source of bias arising from cell wall deficiency (Razin, 1995) in mycoplasmas which decreases their growth in presence of inhibitors such as nucleoside and nucleobase as demonstrated in *Mycoplasma pneumoniae* (Sun and Wang, 2013) *and others mycoplasmas* (Wehelie et al., 2004). FISH data from the current study, however, indicate that many mycoplasmas could be sequestered within the basal the epithelial cells, suggesting potential unknown parameters in symbiosis with *Salmo salar* which were missed from the culture media trials and reduce their cultivability. Although only a qualitative assessment is possible by employing FISH, consistent with recent 16S amplicon-seq data from the same farm site (Heys et al., 2020), our data suggest that *Mycoplasma* comprised the majority of the resident microbes (Fig. 2).

A high level of adaptation to, and dependence on the host organism, is a key feature of many *Mycoplasma* species (Faucher et al., 2019). The exploitation of an intracellular niche, dependence on the host, and relative isolation from the other microorganisms and mobile genetic elements are thought to have contributed to genome decay in mycoplasmas (Sirand-Pugnet et al., 2007). One result of this decay is a reduction in genome size and the number of genes, and the mycoplasma

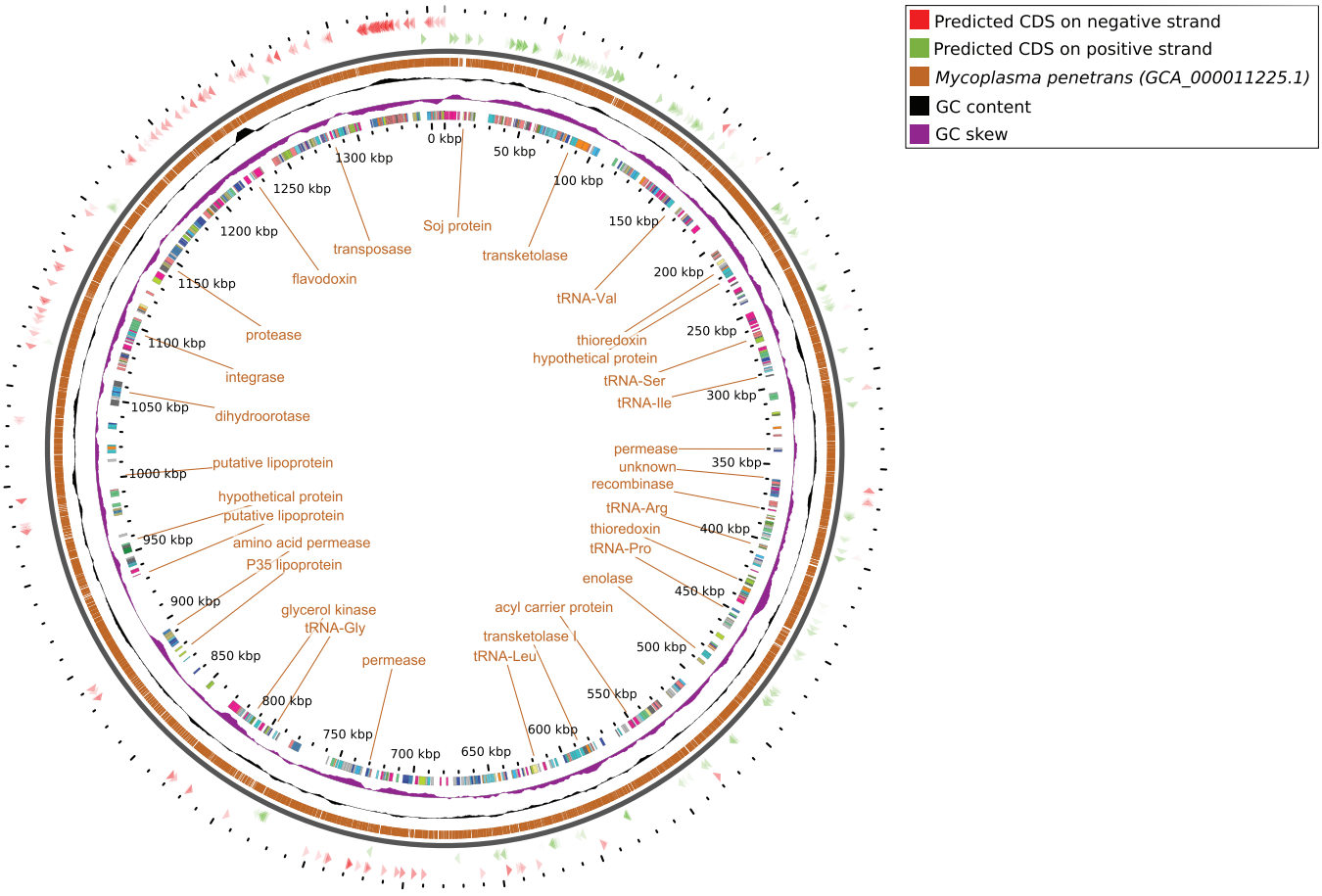

Fig. 4. Circular track of the core genes. This figure highlights the orthologs genes shared between the mycoplasma MAG from this study and related *Mycoplasma penetrans* species.

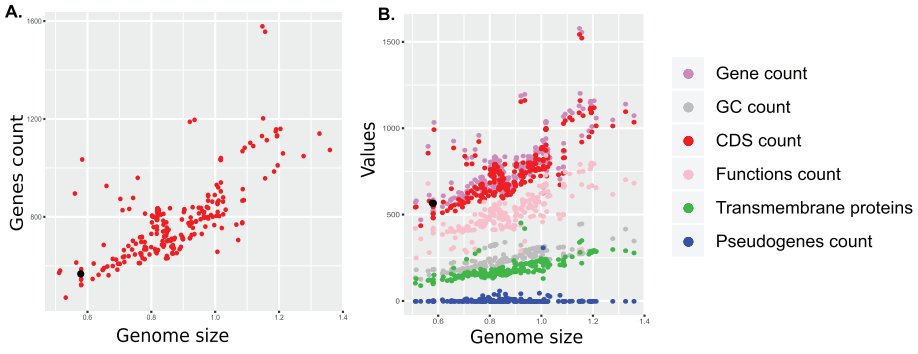

Fig. 5. Genomic features of Mycoplasmas. (A) The plot of genome size and genes count in the Myoplsamas lineage. (B) The plot of functions and GC contents against genome size in the Mycoplasmas lineage.

MAG in this study appears to have been potentially affected by such processes in comparison to the other mycoplasmas (Fig. S5). According to the phylogenetic tree, we did not observe any specific relationship between the tree topology and the genome sizes of mycoplasmas (Fig. 2). Indeed, the closely related *M. penetrans* was over three times larger than the size of the mycoplasma MAG in this study. Despite sharing a recent ancestor with the human pathogen *M. penetrans*, a long and close evolutionary association of this *Mycoplasma* and salmonids is possible given the similarity of another mycoplasma MAG sourced from the Norwegian sea salmon and identified to *M. penetrans* (Jin et al., 2019). We were also able to identify *Mycoplasma* sp. in freshwater parr *via* the FISH method in this study. One potential route for vertical transmission of the *Mycoplasma* sp. among salmon could be observed during oviposition. We were not able to identify microbes colonising eggs in this study, although our sample size was limited. Further development on specific *Mycoplasma* sp. strain markers could potentially reveal their abundance as well as their epidemiology, and potential routes of intergenerational transmission.

Some insight for the potential role of these mycoplasma on salmon health in an aquaculture setting may be possible. Many well-characterised mycoplasmas are pathogens (Meseguer et al., 2003; Rosengarten et al., 2000; Sasaki et al., 2002), with several *Mycoplasma* sp. being responsible for human, animal and plant diseases; however, some species are considered to be commensal organisms (Razin et al.,

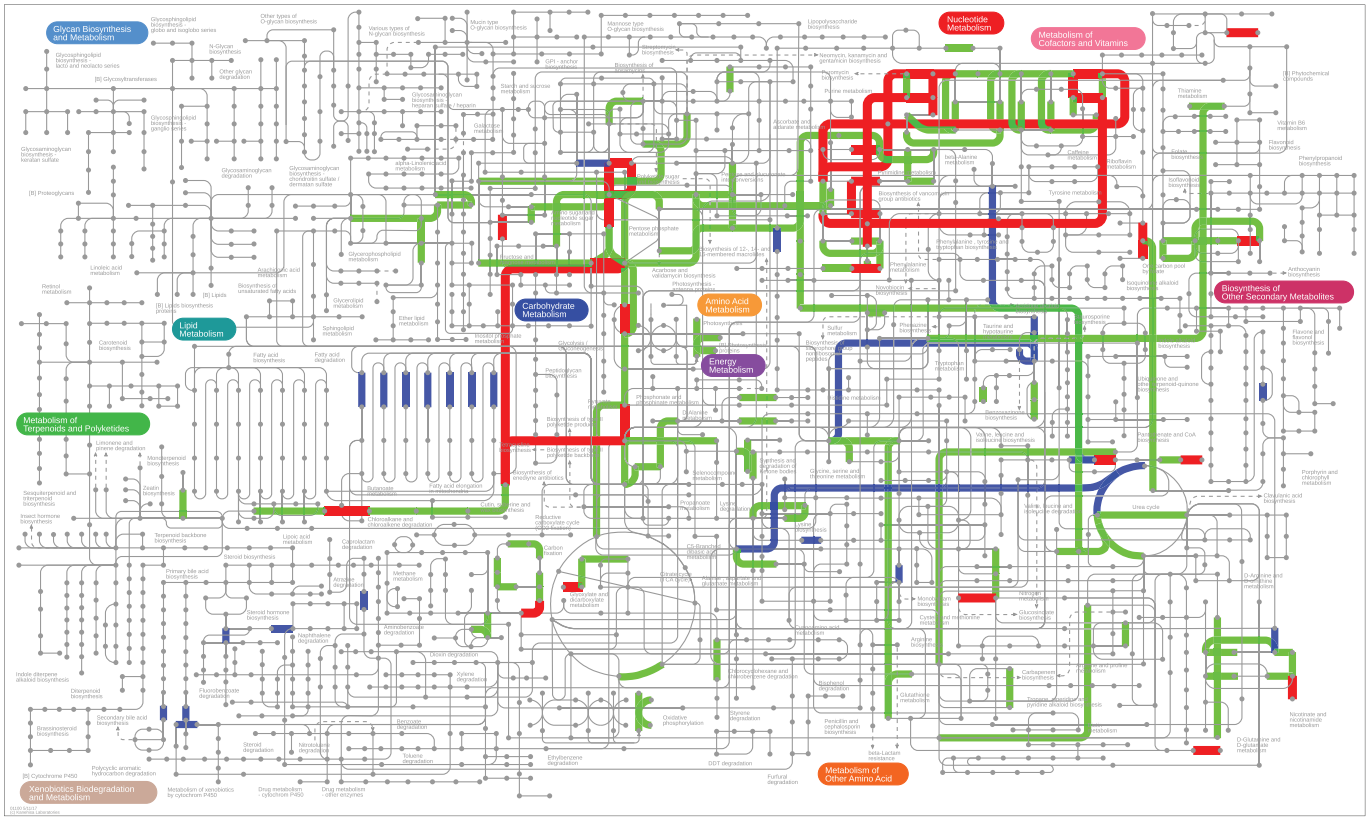

Fig. 6. Pairwise Metabolic pathways comparison of mycoplasma MAG from this study and Mycoplasma penetrans. Red colour represents shared and conserved pathways between the two genomes, whereas blue colour represents the metabolic pathways of the mycoplasma MAG from this study and the green colour represent the metabolic pathways of *Mycoplasma penetrans*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1998; Siqueira et al., 2013). The role of *Mycoplasma* in the context of *Salmo salar* is not well established. Koch's postulates were not applied in this study (Falkow, 2004). Given the challenges encountered in culturing these microorganisms, it seems quite likely that they may never be applied. Furthermore, the apparent abundance of *Mycoplasma* in the healthy salmonids (Heys et al., 2020; Holben et al., 2002; Llewellyn et al., 2016), and lack of any clear associated pathology in gut tissues, implies that there is not a significant impact on the host health or fitness. Commensal exploitation of the host intracellular niche is potentially the most parsimonious description of the host-microbe interaction in this case. The ultimate metabolic adaptation to an intracellular lifestyle (i.e *Buchnera*, *Wigglesworthia* and *Blochmannia)* appears to be solely regulated by the metabolic activity of the host cells to which the bacteria may actively contribute to, by delivering essential metabolites that are limited in their habitats and are not produced by the hosts (Fuchs et al., 2012). In this context, the presence of genes encoding riboflavin pathway could potentially indicate benefit from the salmon host perspective of *Mycoplasma* sp. colonisation. Riboflavin, known as the precursor for the cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide, is an essential metabolite in organisms (Fuchs et al., 2012; Gutiérrez-Preciado et al., 2015), although vertebrates cannot synthesize it on their own (Vitreschak et al., 2002). The *Mycoplasma* may play a role in riboflavin supplementation in salmon, as has been suggested in several deep-sea snailfish (Lian et al., 2020), although it must be noted that riboflavin biosynthetic pathways also occur in pathogenic mycoplasmas (Gutiérrez-Preciado et al., 2015). Riboflavin supplementation is not limited to the mycoplasmas; in the bedbug *Cimex lectularius,* the gram-negative Wolbachia can synthesize biotin and riboflavin which, are crucial for the host growth and reproduction (Kubiak et al., 2018; Moriyama et al., 2015). Riboflavin biosynthesis is common for symbiotic associations and therefore occurs even in small and optimized genomes size like *Wolbachia* (~ 1.48 Mb) *and mycoplasmas* (0.51–1,38 Mb).

Also, it is reported that many *Mycoplasma* species can modify their surface antigenic molecules with high frequency (Horino et al., 2003; Rosengarten et al., 2000) which may likely play a key role in outmanoeuvring the host immune system. This ability may generate phenotypic heterogeneity in colonising Mycoplasma populations and provide fitness benefits such as evasion of host immune responses and to the adaptation to the environmental changes (Halbedel et al., 2007; Horino et al., 2003). The majority of the variable surface antigenic molecules of mycoplasmas are lipoproteins (Chambaud et al., 1999; Halbedel et al., 2007; Wise, 1993), which, depending on the species, are encoded by single or multiple genes (Rosengarten et al., 2000; Rosengarten and Wise, 1990). The expression of these lipoproteins, due to extensive antigenic variation, is thought to be a major factor for immune evasion, for example, the P35 lipoprotein and its paralogs, which are distributed across the surface of *M. penetrans* cells, are immunodominant (Distelhorst et al., 2017; Neyrolles et al., 1999; Wang et al., 1992). Two lipoprotein encoding genes were found only in *Mycoplasma penetrans* but not in the mycoplasma MAG of this study, and the lack of such virulence factors or mobile genes could again support a non-pathogenic lifestyle (Supplementary Table 2).

Our work demonstrates a potentially important ecological and functional association between *Mycoplasma* sp. and *Salmo salar* that merits further investigation in the context of aquaculture disease and, potentially, nutrition. Targeted meta-transcriptomics and strain-specific screening for this organism could improve our understanding of its biology, function, and its role in the host homeostasis. Furthermore, targeted studies involving genome reduction and their association with

the host dynamics are also necessary to fully understand the evolution of *Mycoplasma* sp. symbiosis in *Salmo salar*. Furthermore, bespoke infections experiments, informed by the findings of this study, may lead to the development of practices that can improve the aquaculture industry, especially in the context of the probiotic potential of mycoplasmas in salmonids.

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ML and BC conceived and designed the study. PY, RK, MDN, TD, CH, PS, and EL collected the samples, performed FISH experiments, and analysed image data. BC performed the bioinformatics and interpreted the results. BC and ML wrote the original draft of the manuscript. All authors contributed to revisions of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736772.

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